Lipid and fatty acid profiles in the brain, liver, and stomach contents of neonatal rats: effects of hypoxia

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Bruder, Eric D., Ping C. Lee, and Hershel Raff. Lipid and fatty acid profiles in the brain, liver, and stomach contents of neonatal rats: effects of hypoxia. Am J Physiol Endocrinol Metab 288: E314–E320, 2005. First published October 5, 2004; doi:10.1152/ajpendo.00362.2004.—Neonatal hypoxia leads to clinically significant fatty liver, presumably due to disturbances in lipid metabolism. To fully evaluate lipid metabolism, the present study analyzed the complete lipid profile of the brain, liver, and ingested stomach contents of 7-day-old rats exposed to hypoxia from birth. Hypoxia had negligible direct effects on lipid metabolism in the brain. Conversely, hypoxia exhibited direct effects on hepatic lipid metabolism that could not be fully explained by changes in dietary intake. Triacylglyceride concentration was significantly increased in the hypoxic liver but remained unchanged in the brain and stomach contents. Diacylglyceride concentration was increased in both the brain and liver, and this was associated with increased diacylglyceride in the stomach contents. Most n-3 and n-6 fatty acids were increased in the liver, but not in the brain, of hypoxic pups. These changes did not reflect those measured in the stomach contents. Saturated fatty acid concentrations were increased in both the hypoxic brain and liver, and these changes reflected those in the stomach contents. Hypoxia also increased total phospholipid concentration in the brain and stomach contents. We conclude that neonatal hypoxia directly affects specific lipid and fatty acid concentrations in the brain and liver through alterations in the absorbed stomach contents. Hypoxia also exhibits some direct affects through modulation of metabolic pathways in situ, mostly in the liver. In this respect, the neonatal brain exhibits tighter control on lipid homeostasis than the liver during neonatal hypoxia.

MATERIALS AND METHODS

Animal treatment. All animal protocols were approved by the Institutional Animal Care and Use Committees of the Medical College of Wisconsin and Aurora Health Care. Timed-pregnant Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were obtained at 14 days gestation and maintained on a standard diet with water ad libitum in a controlled environment (0600–1800 lights on). Parturition usually occurred on the afternoon of gestational day 21, during which time rats were kept under observation.

Hypoxia from 0 to 7 days of age. As soon as a litter was completely delivered, the dam and her pups were immediately moved to an environment chamber and exposed to normobaric normoxia (21% O2; room air) or hypoxia (12% O2) as described previously (31, 33). A total of six litters (8–10 pups per litter; mixed sexes) was used. The experimental day was at the end of 7 days of exposure of dams and their litters to either normoxia or hypoxia (32). We have previously shown that this exposure leads to arterial P02 levels in adults of ~50–55 Torr, with sustained respiratory alkalosis and metabolic derangements and alterations in brain lipids, thereby affecting the function of the central nervous system.

To assess the metabolic effects of hypoxia on selected tissues during development, it is critical to generate a highly comprehensive and detailed lipid profile. The purpose of the present study was to use comprehensive lipid profiling to 1) evaluate the effect of neonatal hypoxia on all lipid classes and individual fatty acid metabolites in the brain, liver, and stomach contents; 2) assess tissue specificity; and 3) compare changes in dietary lipids, reflected in the ingested stomach contents, with results for the liver and the brain.

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compensation (33). Chambers were briefly opened on day 4 to clean the cages. At 0800 of day 7, litters from the hypoxic (n = 3) and normoxic (n = 3) groups were decapitated. Whole livers and brains were quickly removed and snap frozen in liquid nitrogen. Recently ingested stomach contents were harvested from the forestomach and frozen in liquid nitrogen. Only full stomachs were analyzed. The use of contents from full stomachs to reflect the lipid and fatty acid composition of ingested milk has been extensively validated (5, 6, 27, 29, 34).

Lipid profiling. Brain, liver, and stomach contents were subjected to lipid profile analyses (Lipomics Technologies, West Sacramento, CA). Briefly, the technique involved extraction of stomach content and tissue lipids with authentic internal standards added by the method of Polch et al. (18) using chloroform-methanol (2:1, vol/vol). Brain (n = 3) or liver (n = 4) tissue (25 mg) and stomach contents (100 mg; n = 3) were used for each analysis. Samples were obtained from pups from three normoxic and three hypoxic litters. Preparative thin-layer chromatography (TLC) was used to separate individual lipid classes within each extract (38). Authentic lipid class standard compounds were run on the two outside lanes of the same TLC plate to enable identification of the sample lipid classes. Individual lipid fractions were scraped from the plate and transerified in 3 N methanolic HCl in a sealed vial under nitrogen at 100°C for 45 min. Fatty acid methyl esters obtained were extracted with hexane containing 0.05% butylated hydroxytoluene and used for gas chromatography after sealing of the hexane extracts under nitrogen. Separation and quantification of fatty acid methyl esters were achieved with capillary gas chromatography, using a gas chromatograph (Hewlett-Packard model 6890, Wilmington, DE) with a 30-m DB-225MS capillary column (J & W Scientific, Folsom, CA) and a flame-ionization detector. The intra-assay coefficients of variation for lipid analytes ranged from 0.4% for triacylglycerides (TG) to 13% for phosphatidylethanolamine (PE) (n = 5). Assay sensitivity was set at 0.1 μmol/g because concentrations below this value increased the analytic variability to >20%.

Statistics. Quantitative data (μmol/g specimen) were analyzed with the Surveyor software system supplied by Lipomics Technologies. A “heat map” graph, based on the percent difference between data for hypoxic and normoxic samples, was generated. Significance of differences between hypoxic and normoxic tissue total lipids and metabolite concentrations was assessed by unpaired Students t-test, with P < 0.05 considered significant. This statistical approach has been used previously to evaluate this type of lipid profile analysis (7, 38). Results are reported as means ± SE or as percent change from normoxic control (heat map).

RESULTS

The total concentrations of diacylglyceride (DG) and TG in the brain, liver, and ingested stomach contents are shown in Fig. 1. Note that the abscissa is on a logarithmic scale. Exposure of rat pups to hypoxia from birth to 7 days of age resulted in an increase in DG in brain (P < 0.02), liver (P < 0.02), and ingested stomach contents (P < 0.03). The concentration of TG in the brain tended to increase during hypoxia, with concentrations at or below the sensitivity of the assay. TG concentrations were significantly increased in the liver (P < 0.0003), but not stomach contents (P > 0.05), from rats exposed to hypoxia from birth. There were no significant changes in individual PL concentrations in the liver and stomach contents (P > 0.05), although total PL concentration was increased in the brain (P < 0.02) and stomach contents (P < 0.05) (data not shown). In the brain, there were increased concentrations of both phosphatidylcholine (PC; P < 0.05) and cardiolipin (CL; P < 0.05) (data not shown).

The sum of DG and TG (DG+TG) and the sum of total PL, each as a percentage of total lipid, are shown in the top and middle panels of Fig. 2. Hypoxia had no effect on the percentage of these two classes of lipids in the brain or recently ingested stomach contents (P > 0.05). However, hypoxia resulted in an increase in the percentage of DG+TG (P < 0.001), with a concomitant decrease in the percentage of PL (P < 0.0005), in the liver. There was no effect of hypoxia on the ratio of free fatty acids (FFA) or cholesterol esters (CE) to total lipids in any of the tissues analyzed (P > 0.05) (data not shown). The bottom panel of Fig. 2 shows the concentrations of total lipids on a logarithmic scale. Exposure of pups to hypoxia from birth significantly increased the concentration of total lipid in the brain (P < 0.0003), liver (P < 0.02), and ingested stomach contents (P < 0.02).

The total concentrations of individual saturated fatty acids in the brain, liver, and ingested stomach contents are shown in Fig. 3. Hypoxia increased the concentration of 16:0 and 18:0 (both P < 0.03), but not 14:0 (P > 0.05), in the brain. The liver from rat pups exposed to hypoxia showed increases in the concentration of 14:0 (P < 0.05) and 16:0 (P < 0.02) but not 18:0 (P > 0.05). Ingested stomach contents from hypoxic pups also showed increases in the concentrations of 14:0 and 16:0 (both P < 0.001). There were no effects of hypoxia on the concentrations of 20:0, 22:0, and 24:0 in any tissue analyzed (P > 0.05) (data not shown).

The total concentrations of individual n-6 and n-3 fatty acids in 7-day-old rats exposed to normoxia or hypoxia are shown in
Fig. 4 (on a logarithmic scale). There were no significant effects of hypoxia on the brain. The hypoxic liver showed increases in 18:2n-6 ($P < 0.0001$), 20:4n-6 ($P = 0.04$), 18:3n-3 ($P < 0.0001$), 20:5n-3 ($P < 0.02$), 22:5n-3 ($P < 0.004$), and 22:6n-3 ($P < 0.03$). The concentrations of 18:3n-3 ($P < 0.03$) and 20:5n-3 ($P < 0.05$) were significantly increased in stomach contents. The concentrations of 22:4n-6 and 22:5n-6 were too low to be reliably detected in all three tissues.

Figure 5 displays the heat map generated to highlight changes in the fatty acid profile of each tissue studied. Each colored square indicates a percent change from the normoxic control, with $P < 0.05$ ($t$-test).

DISCUSSION

The present study used comprehensive lipid profiling to investigate the effects of hypoxia on lipid homeostasis in the brain, liver, and recently ingested stomach contents of neonatal rats. The evaluation of specific lipid classes, and of fatty acid metabolite distribution within each class, provided unique insights into the effect of neonatal hypoxia on metabolic pathways within the liver and brain. These analyses also allowed an estimation of dietary influence on lipid status in the liver and brain. The most striking novel finding was the relative lack of a direct effect of hypoxia on lipid metabolism in the brain and liver.
activity of phosphatidate phosphohydrolase (25). It has been shown that, in the adult rat, hypoxia increases the hepatic TG may also be due to increased de novo synthesis. It further decrease in the assembly of VLDL, causing the observed increase in hepatic TG. Hypoxia-related increases in hepatic TG may also be due to increased de novo synthesis. It has been shown that, in the adult rat, hypoxia increases the activity of phosphatidate phosphohydrolase (25).

We have recently reported an increased serum concentration of TG in hypoxic rat pups as well as a 60% increase in serum PL concentration (7). These findings may be partially explained by the present results. The stomach contents of hypoxic pups had increased concentrations of PL, and this is most likely reflected in the serum. It seems unlikely that the hypoxic liver contributed to this increase in serum PL. There were no changes in hepatic PL concentrations and only a decrease in percent PL, however, is fully explained by a relative increase in hepatic TG and DG concentrations. The hypertriglyceridermia present in hypoxic pups is probably not due to increased release of TG from the liver. Instead, increased serum TG concentration is probably due to impaired lipolysis in the circulation. We have previously shown that the development of hepatic lipase, an enzyme similar to lipoprotein lipase (LPL), is delayed during hypoxia (26). It is possible that hypoxia also affects LPL activity. This could not only help explain increased serum TG concentrations but could also provide an explanation for increased serum PL.

Increased saturated fatty acids (14:0 and 16:0) in the hypoxic liver reflected increased concentrations in dietary intake, as assessed by analysis of recently ingested stomach contents. The liver of hypoxic pups also had significant increases in most n-6 and n-3 acids. Particularly striking was the higher docosahexaenoic acid (22:6n-3) content of the hypoxic liver compared with the stomach contents. Interestingly, the stomach contents of hypoxic pups had increased concentrations of 18:3n-3 and eicosapentaenoic acid (20:5n-3), both precursors of 22:6n-3. Dams fed diets high in 18:3n-3 produce milk high in 18:3n-3, which results in elevated 18:3n-3 and 22:6n-3 in the liver of pups (5). Because the maternal dietary content was not altered in our experiments, it is possible that increased n-3 fatty acid concentrations in the milk were a homeostatic adaptation of the dam to benefit the developing pups.

Again, note that we used recently ingested stomach contents as an index of milk composition. This approach has been extensively validated (5, 6, 27, 29, 34) and takes into account a small potential for lipolysis in the stomach (12, 16, 23). Furthermore, it has been suggested that the absorption of lipids and fatty acids in the stomach is negligible (16). Therefore, we are confident that the stomach contents represent the composition of the dam’s milk and also represent the composition of ingested material presented to the small intestine for absorption.

Retroconversion of 22:5n-6 to arachidonic acid (20:4n-6) has been demonstrated in rat hepatocytes (35). The present results showed a significantly higher level of 20:4n-6 in the liver from hypoxic pups compared with the normoxic liver, despite no difference in the 20:4n-6 levels in the hypoxic and normoxic stomach contents. The hypoxia-induced increase in hepatic 20:4n-6 concentration was probably not due to retroconversion of 22:5n-6, since the concentration of 22:5n-6 was negligible. However, an increase in the conversion of 18:2n-6 to 20:4n-6 may have occurred. Elevated concentrations of 20:4n-6 in the hypoxic liver were likely caused by the large increase in hepatic 18:2n-6 concentration.

Hepatic tumor cells have a characteristically low ratio of 18:0 to 18:1n-9, due to a modulation of fatty acid desaturation enzymes (39). In the present study, there was a significant decrease in the ratio of 18:0 to 18:1n-9 in the liver from
hypoxic pups compared with normoxic control. This suggests a likely modification of fatty acid enzymes during hypoxia, similar to tumorigenesis, possibly due to an alteration in the desaturation of 18:0 (3). Furthermore, changes in the ratio of 18:0 to 18:1n-9 have been related to alterations in membrane fluidity (3). Such changes in the ratio of 18:0 to 18:1n-9 in the hypoxic liver might play a role in the modulation of hepatic membrane function.

The brain from hypoxic pups showed a proportional increase in both PL and non-PL, such that the relative ratios of these lipids to total lipids remained unchanged. This is physiologically significant, since stabilizing the activity of the central nervous system is of paramount importance for survival. Neuronal activity is heavily dependent on lipids, particularly PL (14). It is, therefore, advantageous for the brain to possess inherent mechanisms to maintain PL concentrations relative to non-PL. Our results also showed that the hypoxic brain tended to have higher concentrations of 22:6n-3. In humans, the developing brain accumulates 22:6n-3 at a rapid rate (48% from breast milk) during the first 6 mo of life (17). Alterations in brain 22:6n-3 and other long-chain polyunsaturated fatty acids have been associated with changes in biochemical function, for example, enzyme activity and behaviors such as learning ability (4, 11, 37, 40). In the present study, a significant increase in 22:6n-3 in the hypoxic brain was observed. Whether this increase affects neural function and development is an important issue to be considered.

In contrast, there was no effect of hypoxia on 20:4n-6 in the brain. In both the normoxic and hypoxic brain, the concentration of 20:4n-6 was lower than in the stomach contents, indicating minimal or no conversion of 18:2n-6 to 20:4n-6. The concentration of 14:0 remained unchanged while both 16:0 and 18:0 were increased. Our results suggest a dietary influence on tissue concentrations of saturated fatty acids in the brain, in addition to those in the liver.

In conclusion, neonatal hypoxia differentially affected lipid metabolism in both the liver and brain of rat pups as revealed by lipid profile analyses. Perhaps the most important observation was that the neonatal brain maintained lipid homeostasis to a greater degree than the liver (i.e., hepatic lipid metabolism was directly affected by low oxygen). This suggests that the neonatal brain possesses inherent mechanisms involved in the adaptation to an adverse environment, at least as these mechanisms pertain to lipid metabolism. The results also suggest that changes in dietary lipid concentrations are reflected at the organ/tissue level. The overall effects of neonatal hypoxia on

Fig. 5. Heat map showing individual fatty acid metabolite concentrations, as they appear in each lipid class in the brain, liver, and recently ingested stomach contents of 7-day-old rats exposed to hypoxia from birth (n = 3–4/analyte). Quantitative data were used to calculate the percent increase (green squares) or decrease (red squares) of each hypoxic measurement from the normoxic control. Significance of differences was analyzed by t-test, with colors denoting $P < 0.05$. Differences not meeting $P < 0.05$ are shown in black.
any specific tissue are a combination of dietary changes and alterations in cellular homeostasis. These findings, using the power of comprehensive lipid profiling, provide a starting point into the investigation of the metabolic effects of neonatal hypoxia and highlight the utility of performing comprehensive lipid profiling.

The present results allow for speculation on the metabolic consequences of neonatal hypoxia. It is clear that lipid metabolism in the neonatal brain remains relatively protected in a low-oxygen environment; this is clearly an adaptive advantage in the development of normal central nervous system function under hypoxic conditions. Hepatic lipid metabolism seems to be affected by hypoxia both directly and indirectly. The presence of fatty liver during hypoxia, whether an outcome of increased lipid intake or metabolic effects due to hypoxia, needs to be assessed further in the context of neonatal health; that is, fatty liver may confer a physiological advantage. Likewise, such metabolic derangements occurring during a critical stage of development could play a role in the onset of disease in adult life (e.g., metabolic disorders). Finally, these studies again raise the issue of the fat content of ingested milk in neonates who are hypoxic because of cardiopulmonary disease. It may be that subtle changes in the lipid composition of ingested milk may confer an advantage to the survival of the hypoxic neonate. This is important to consider when assessing neonatal hypoxia in humans, since these infants are normally fed standard lipid emulsions.

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GRANTS

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