Total parenteral nutrition induces sustained hypomethylation of DNA in newborn guinea pigs

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BACKGROUND: Neonatal total parenteral nutrition (TPN) is associated with animals with low glucose tolerance, body weight, and physical activity at adulthood. The early life origin of adult metabolic perturbations suggests a reprogramming of metabolism following epigenetic modifications induced by a change in the pattern of DNA expression. We hypothesized that peroxides contaminating TPN inhibit the activity of DNA methyltransferase (DNMT), leading to a modified DNA methylation state.

METHODS: Three groups of 3-d-old guinea pigs with catheters in their jugular veins were compared: (i) control: enterally fed with regular chow; (ii) TPN: fed exclusively with TPN (dextrose, amino acids, lipids, multivitamins, contaminated with $350 \pm 29 \mu\text{mol/l}$ peroxides); (iii) H_2O_2 : control + $350 \mu\text{mol/l}$ H_2O_2 intravenously. After 4 d, infusions were stopped and animals enterally fed. Half the animals were killed immediately after treatments and half were killed 8 wk later ($n = 4\text{--}6$ per group) for hepatic determination of DNMT activities and of 5'-methyl-2'-deoxycytidine (5MedCyd) levels, a marker of DNA methylation.

RESULTS: At 1 wk, DNMT and 5MedCyd were lower in the TPN and H_2O_2 groups as compared with controls. At 9 wk, DNMT remained lower in the TPN group, whereas 5MedCyd was lower in the TPN and H_2O_2 groups.

CONCLUSION: Administration of TPN or H_2O_2 early in life in guinea pigs induces a sustained hypomethylation of DNA following inhibition of DNMT activity.

Total parenteral nutrition (TPN) may be required in neonates with impaired oral feeding capability following intestinal surgery or in premature newborns with immature gastrointestinal tract. Even if this mode of nutrition enables sustained growth and development, its contamination by oxidant molecules such as peroxides (1) is associated with oxidative stress (2). The neonatal impacts of this stress have been demonstrated in an animal model of TPN (3) as well as in premature neonates (2). An oxidative stress early in life is also suspected to modulate the health status into adulthood (4,5).

Administration of TPN or H_2O_2 during the first week of life in guinea pig pups has been shown to induce, 3 mo later, a modification in energy metabolism that was associated with a

phenotype of energy deficiency (6). As compared with orally fed controls, animals receiving TPN had a lower body weight, weaker spontaneous physical activity, and poorer glucose tolerance. These characteristics are also reported in young adults 20 y of age who were born very low birth weight (7–9) and in whom the use of TPN was a frequent occurrence. This early life origin of adult metabolic perturbations suggests a reprogramming of metabolism following epigenetic modifications induced by a change in the pattern of DNA expression. We suspect that this might take place through a permanent effect of TPN on DNA methylation.

Metabolic activities are dependent of the expression of genes. Transcription of genes is dependent on their availability to interact with transcription factors and other components required to induce the biochemical processes leading to production of transcripts. The availability of genes is mainly regulated by epigenetic phenomena such as methylation/acetylation of histones as well as methylation of DNA (10–15). Three enzymes are implicated in the methylation of DNA: DNA methyltransferase (DNMT)1, DNMT3a, and DNMT3b. DNMT3a and 3b are implicated in *de novo* methylation of DNA (16–18), whereas DNMT1 is responsible for maintenance of DNA methylation between cell replications (19,20). The activity of DNMT1 is reported to be sensitive to the redox status of its cysteine residues (21,22). We hypothesized that the peroxide content of TPN favors DNA hypomethylation through inhibition of DNMT activity caused by oxidation of its sensitive cysteine residues. Therefore, the current study aims to evaluate in guinea pigs (i) the impact of TPN infused early in life on hepatic DNMT activity as well as on DNA methylation level immediately after treatment and 2 mo later and (ii) whether the effects of TPN are associated with its H_2O_2 content.

RESULTS

At the end of the treatment, at 1 wk of age, the DNMT activity (Figure 1a) was lower in the TPN and H_2O_2 groups, with no difference between the TPN and H_2O_2 groups. At 9 wk of age, 2 mo after cessation of treatment, the activity of the enzyme (Figure 1b) remained lower only in the TPN group.

The methylation of DNA in the liver, expressed as the concentration of 5'-methyl-2'-deoxycytidine (5MedCyd) in whole

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Figure 1. Hepatic activity of DNA methyltransferase (DNMT) as a function of treatments. Control: animals fed orally with regular chow ($n = 6$ in each panel); TPN: animals fed exclusively with TPN between days 3 and 7 of age, followed by regular chow ($n = 4$ in (a); $n = 6$ in (b)); H₂O₂: animals fed regular chow and infused with 350 $\mu\text{mol/l}$ H₂O₂ between days 3 and 7 of age ($n = 5$ in (a); $n = 6$ in (b)). (a) After treatments, at 1 wk of age, the activity of DNMT was lower in the TPN and H₂O₂ groups as compared with the control group. There was no difference between the TPN and the H₂O₂ groups. (b) Eight weeks after cessation of treatments, the activity of DNMT remained lower in the TPN group. Means $\pm$ SEM; * $P < 0.05$; ** $P < 0.01$. TPN, total parenteral nutrition.

DNA, was lower in the TPN and H₂O₂ groups than in controls, both at the end of the treatments (Figure 2a) and 2 mo later (Figure 2b). The concentrations of 5MedCyd did not differ between the TPN and H₂O₂ groups. The mean value for the control group in Figure 2b excludes two data points (2.7 and 3.3 nmol/ μg DNA) that are outside the 99.9% confidence interval (0–2.3 nmol/ μg DNA) of the mean.

DISCUSSION

The main finding of the present study was that administration of TPN early in the life of the guinea pig induces a sustained DNA hypomethylation following inhibition of DNMT activity. The effects of TPN are reproduced by an infusion of H₂O₂. To our knowledge, this is the first time that a link has been reported between a short-term exposure (4 d) to TPN and a modification of the pattern of DNA methylation, a modification that appears to be sustained into adulthood.

DNA methylation occurs on cytosine, preferentially in the CpG islets, by the action of DNMT. Methylation in the promoter area of the gene is recognized to modulate the level of its transcription. Hypermethylation is associated with a silencing of genes, whereas hypomethylation favors gene transcription. Hence, the level of methylation of a gene is associated with the number of copies of the protein encoded by this gene. Therefore, a change in methylation pattern of a gene is associated with a modification of the global activity of this protein in the cell.

Pradhan *et al.* (22) showed that the activity of DNMT is dependent on the integrity of sensitive thiol functions (R-SH). Different molecules can react with R-SH. For instance, H₂O₂ induces the formation of R-SOH, whereas 4-hydroxynonenal (HNE), derived from lipid peroxidation, leads to formation of

Figure 2. Hepatic levels of 5'-methyl-2'-deoxycytidine (5MedCyd) in DNA as a function of enteral vs. parenteral nutrition. Control: animals fed orally with regular chow ($n = 6$ in each panel); TPN: animals fed exclusively with TPN between days 3 and 7 of age, followed by regular chow ($n = 5$ in (a); $n = 6$ in (b)); H₂O₂: animals fed regular chow and infused with 350 $\mu\text{mol/l}$ H₂O₂ between days 3 and 7 of age ($n = 5$ in each panel). The levels of 5MedCyd in whole DNA were lower in TPN and H₂O₂ groups than in controls, both at (a) the end of the treatments and (b) 2 mo later. The concentrations did not differ between TPN and H₂O₂ groups. In (b), the mean value of the control group excludes two data (2.7 and 3.3 nmol/ μg DNA) because they are outside the 99.9% confidence interval (0–2.3 nmol/ μg DNA) of the mean. Means $\pm$ SEM; ** $P < 0.01$. TPN, total parenteral nutrition.

R-S-HNE. Both molecules (H₂O₂ and HNE) are generated in TPN solutions (23). The infusion of H₂O₂ will also induce *in vivo* lipid peroxidation (24,25). We can expect that the quality of the antioxidant defenses or/and the capacity to detoxify peroxides and HNE molecules would play a significant role in the process leading to hypomethylation of DNA. Thus, the level of glutathione, which is the essential cofactor in reducing peroxides by glutathione peroxidase and in detoxification of HNE by glutathione *s*-transferase, should be a key player in the process leading to DNA methylation. In human neonates, the glutathione level increases with gestational age (26). Because our results show that early exposure to TPN affects DNA methylation in a term animal model, we would expect to observe a similar effect in newborn infants receiving TPN. Premature infants would be even more at risk of TPN-induced DNA hypomethylation because of their immature antioxidant system and low glutathione levels.

An interesting secondary observation is that the level of methylation in DNA increases as a function of age. It was increased by three to four times between 1 and 9 wk of age, independently of the treatment received during the first week of life of the animals. This suggests that normal development of the organism could require silencing of some genes. If the level of methylation is important for the evolution of some metabolic pathways during life, it should be no surprise that a neonatal administration of TPN would cause metabolic perturbations later in life (6). The persistence of a lower DNMT activity until 9 wk of age in the TPN group relative to the control and H₂O₂ groups is surprising and not well understood. This observation should be investigated in a further study.

In accordance with the finding of epigenetic modifications associated with the administration of intravenous nutrition solutions contaminated with oxidant molecules, the current report underlines the importance of studying in humans the long-term impact of TPN administered early in life and of developing new and improved TPN solutions devoid of undesirable oxidant molecules.

METHODS

The institutional committee for good practice with animals in research of CHU Sainte-Justine, in accordance with the principles of the Canadian Council, approved the present research protocol.

Experimental Design

At 3 d of life, guinea pigs (Charles River Laboratories, St-Constant, Quebec, Canada) (109 ± 4 g) were anesthetized in order to fix a catheter (SAI Infusion, Lake Villa, IL) in a jugular vein (24,27,28). Animals were assigned to receive one of the following treatments for 4 d:

Control. Animals were fed orally with the laboratory food for guinea pigs, with no intravenous solution provided; a knot obstructed the catheter.

TPN. Animals were fed exclusively with TPN (8.7% (wt/vol) dextrose, 2.0% (wt/vol) amino acids (Travasol; Baxter, Mississauga, Ontario, Canada), 1.6% (wt/vol) lipids (Intralipid 20%; Pharmacia Upjohn, Baie d'Urfé, Quebec, Canada), 1 U/ml heparin, electrolytes, and 1% (vol/vol) pediatrics multivitamins (Multi-12 pediatric; Sandoz, Montreal, Quebec, Canada)). This solution was contaminated with 350 ± 29 $\mu\text{mol/l}$ peroxides generated spontaneously (1).

H₂O₂. Animals were infused through the catheter with a solution containing 350 $\mu\text{mol/l}$ H₂O₂, 6% (wt/vol) dextrose, 0.3% (wt/vol) NaCl, and 1 U/ml heparin. Animals had free access to regular laboratory food for guinea pigs.

The studied intravenous solutions were infused continuously through the catheter at the rate of 200 ml/kg body/d. The solutions were changed daily.

After 4 d, at 1 wk of age, half of the animals from each group ($n = 4$ –6 per group) were killed for liver collection. For the other animals, intravenous infusions were stopped and animals had free access to regular chow and water until the end of study, i.e., 8 wk later, when the animals were killed for liver sampling. The collected specimens were divided into aliquots and stored at -80°C until biochemical determinations.

Isolation of Nuclei and Preparation of Nuclear Extract

The liver nuclei were isolated following the protocol of Gorski *et al.* (29) and Rose *et al.* (30) by homogenization in a high-density sucrose buffer and centrifugation at 100,000g for 30 min at 4°C . After dissolution of the pellet in a buffer containing 0.3 mol/l KCl, DNMT protein was isolated following the protocol of Wadzinski *et al.* (31) by centrifugation at 100,000g for 30 min at 4°C . The supernatant was mixed with 0.3 g (NH₄)₂SO₄/ml and centrifuged at 150,000g for 30 min at 4°C . The pellet was recovered in 500 μl buffer (25 mmol/l Na-Hepes, 40 mmol/l KCl, 0.1 mmol/l EDTA, 1 mmol/l DDT, and 10% glycerol) before filtration by centrifugation on Amicon with a cutoff of 100,000 Daltons (Amicon Ultra-0.5 ml, 100,000 molecular weight cutoff, Millipore, Billerica, MA).

DNMT Activity

The DNMT activity from the nuclear extract was determined by a colorimetric assay, using the EpiQuik DNMT Activity/Inhibition Assay Ultra kit (Epigentek, Farmingdale, NY), according to the manufacturer's user guide. This assay measures the global activity of DNMT, including all isoforms of the enzyme.

DNA Extraction

The extraction of DNA from the liver was performed by using the E.Z.N.A HP Tissue DNA Midiprep Kit (Omega Bio-Tek, Norcross,

GA) according to the manufacturer's instructions. Briefly, samples were homogenized in a high-salt buffer containing cetyltrimethyl ammonium bromide followed by proteinase digestion. After addition of 3 ml chloroform:isoamyl alcohol (24:1; vol/vol), the homogenate was separated into aqueous and organic phases by centrifugation at 4,000g for 5 min at room temperature. The aqueous phase was extracted and purified using a DNA affinity column (provided by the DNA Midiprep Kit; Omega Bio-Tek). Extracted DNA was quantified by measuring absorbance at 280 and 260 nm to determine the A260/A280 ratio. Values of this ratio between 1.4 and 1.9 indicate 85–90% purity. The concentration of DNA was determined according to Beckman Coulter notes as follows: concentration = $50 \mu\text{g/ml} \times \text{Absorbance}_{260} \times (\text{dilution factor})$.

DNA Methylation Level

The methylation of DNA occurs on the position 5' of the 2'-deoxycytidine. The level of this compound (5MedCyd) was quantified on 1 μg of hepatic DNA by colorimetric assay using the Global DNA methylation ELISA kit (5'-methyl-2'-deoxycytidine Quantification) from Cell Biolabs (Arjona Drive, San Diego, CA).

Statistical Analysis

Results are expressed as means $\pm$ SEM and compared by ANOVA ((TPN vs. H₂O₂) vs. control) after verification of homoscedasticity by the test of Bartlett's χ^2 . The threshold of significance was set at $P < 0.05$.

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