The Effect of Hypoxia and 2,4 Dinitrophenol on Human Placental Glycogen Metabolism[1] (36540)

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We have recently demonstrated that hypoxia may cause marked alterations in human placental glycogen metabolism. Important adaptive mechanisms involving the enzymes of glycogen metabolism, glycogen synthetase (UDPG α-1,4-glucan α-4-glucosyltransferase, EC 2.4.1.11) and glycogen phosphorylase (EC 2.4.1.1) are responsible for these changes. Glycogen synthetase normally decreases during gestation while phosphorylase is elevated in term placental tissue.

Six hours of oxygen deprivation may cause significant depletion of available placental glycogen stores. Activation of the phosphorylase enzyme occurs and results in the rapid degradation of placental glycogen. If these responses to hypoxic conditions were the immediate result of a decline in intracellular ATP, experimental conditions which are known to cause a decrease in ATP concentration within the cell should evoke similar changes.

The investigations presented in this report compare the effects of 2,4 dinitrophenol, a known inhibitor of oxidative phosphorylation, and "severe hypoxia" (1% oxygen) on placental glycogen metabolism in immature and term placentas.

Materials and Methods. Organ culture. Human placental tissue from six term deliveries and five therapeutic interruptions of pregnancy (11–20 weeks) were studied in an organ culture system. This culture system has been described in detail previously (1). The fresh placental explants were placed on stainless-steel platform grids contained within shallow petri dishes to which the culture medium was added (TC 199, Difco Laboratories, Detroit, Michigan). The petri dishes were then placed in metal canister gas chambers and gassed for 10 min before being sealed. Gas mixtures were renewed at 6-hr intervals and the canisters kept in a 37° incubator. In this study, the gas phase of the cultures was either room air with 5% CO_2 or 1% O_2–5% CO_2–94% N_2 ("severe hypoxia"). The presence of hypoxia and metabolic acidosis under the conditions of reduced oxygen tension was confirmed by measurements of lactic and pyruvic acid and the pH of the culture medium.

The 2.4 dinitrophenol experiments were performed by adding DNP to the culture medium at concentrations ranging from 5×10^{-5} *M* to 1×10^{-4} *M*.

After timed exposure to the individual experimental conditions, the explants were removed from the culture grid, weighed, and immediately frozen at — 60°. The tissue was then assayed for glycogen content, glycogen synthetase, and glycogen phosphorylase activities.

Biochemical Studies. Glycogen content. Tissue glycogen content was determined using a modification of the method described by Montgomery (2). The placental explants were placed in boiling 30% (w/v) KOH containing 0.5% Na_2SO_3, digested, and the glycogen precipitated with ethanol. The glycogen concentration was quantitated colorimetrically with appropriate standards using a phenol–sulfuric acid mixture. Results are expressed as milligrams of glycogen/100 g wet weight of tissue.

Glycogen synthetase activity (I and D

[1] Supported by NIH HD 03736 and The Ford Foundation.

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TABLE I. The Effects of Added 2,4 Dinitrophenol on the Relative Amounts of Synthetase I and Phosphorylase *a* to Glycogen in 16-Week Placental Villi in Organ Culture.

Experimental conditions		Percentage of glycogen synthetase I	Percentage of glycogen phosphorylase *a*	Glycogen
Control fresh tissue[a]		12.9 ± 2.1	41.4 ± 1.4	232 ± 11
Control 6 hr room air	(3)	13.1 ± 0.8	42.0 ± 1.7	213 ± 8
2,4 DNP ($5 \times 10^{-5}M$) 6 hr room air	(3)	23.6 ± 0.7[b]	49.4 ± 0.6[b]	133 ± 6[b]

[a] Control fresh tissue reflects those values observed at the start of the culture period. The percentage of Synthetase I was calculated as enzyme activity in absence of cofactor G-6-p compared to activity in the presence of this compound. Phosphorylase *a* percentage was calculated as that enzyme activity in the absence of the cofactor 5′ AMP.

[b] Represents significant difference ($p < .001$) between Control and DNP-treated groups. Values are shown as Mean ± SE. Numbers in parentheses represent number of culture grids for each placenta at the experimental condition. Three 16-week placentas were studied. Enzyme values are mean of four determinations for each culture grid and are expressed as μmoles glucose incorporated into glycogen/gram wet tissue/hour for both phosphorylase and synthetase. Glycogen concentrations represent mean of two determinations for each placenta and are expressed as mg/100 g wet tissue.

forms). Synthetase activity was determined by a modification (3) of the method described by Villar-Palasi and Larner (4). This assay is based on the incorporation of ^{14}C from UDP-^{14}C glucose into glycogen in the presence of the synthetase enzyme. A 5% (w/v) tissue homogenate (100 μl) prepared in 0.25 *M* sucrose containing 1.7 m*M* EDTA and 0.01 *M* NaF was added to the following reaction mixture: 40 m*M* Tris–maleate buffer, pH 7.6, 1.7 m*M* EDTA, 0.48% glycogen, 32 m*M* glucose-6-phosphate (when required), 5 m*M* uridine 5′ diphosphoglucose (UDPG), 0.06 μCi uridine-diphospho [U-^{14}C] glucose (147 mCi/mmole; New England Nuclear Corp., Boston, MA) in a total volume of 0.3 ml. Incubation was carried out at 37° for 15 min and the reaction stopped with 1 ml 30% (w/v) KOH containing 1 mg glycogen as carrier. The sample was boiled at 100° for 5 min and the labeled glycogen precipitated with 2 ml 95% (w/v) ethanol, washed once with distilled water, reprecipitated, and suspended in 0.3 ml water. A unit of enzyme activity was defined as 1 μmole glucose incorporated/g wet tissue/hr. The synthetase enzyme was determined in the presence (dependent) and absence (independent) of the cofactor glucose-6-phosphate.

Glycogen phosphorylase activity. Phosphorylase activity was assayed according to Demers (3) by measuring the incorporation of ^{14}C glucose-1-phosphate into glycogen. Enzyme activity was determined in the presence and absence of adenosine 5′-phosphate using the following reaction mixture: 100 μl of a 5% (w/v) placental homogenate in 0.25 *M* sucrose containing 1.7 m*M* EDTA and 0.01 *M* NaF added to a solution of 0.01 *M* NaF, 26 m*M* glucose-1-phosphate, 0.42% glycogen, 9.0 m*M* 5′ AMP (when required), 0.035 *M*-β-glycerophosphate, pH 6.1, 0.008 *M* reduced glutathione, and 0.05 μCi [U-^{14}C] glucose-1-phosphate (20 mCi/mmole; New England Nuclear Corp.) in 0.3 ml total volume. Incubation was carried out for 10 min at 37° and the reaction was stopped with 1 ml 30% (w/v) KOH with 1 mg glycogen/ml as carrier. The isolation of radioactive glycogen was performed as described for the synthetase enzyme assay. A unit of enzyme activity was expressed as 1 μmole glucose

TABLE II. Effects of 2,4 Dinitrophenol on Glycogen Metabolism of 13-Week Placenta.[a]

Experimental conditions	Glycogen synthetase			Glycogen phosphorylase			
	+G-6-p (D)	−G-6-p (I)	I/D (%)	+ AMP (*t*)	− AMP (*a*)	*a*/*t* (%)	Glycogen
Control 6-hr culture (3) room air	52.7 ± 1.7	3.3 ± 0.4	7	430 ± 14	149 ± 1.9	34	260 ± 16
DNP $1 \times 10^{-4}M$ (3) room air	27.2 ± 0.2[b]	4.9 ± 0.2[b]	22[a]	302 ± 10[a]	176 ± .6[a]	58[a]	84 ± 6[a]
12-hr DNP $1 \times 10^{-4}M$ (3) room air	13.7 ± .2[b]	2.6 ± .15	23[a]	67.5 ± 2.5[a]	28.9 ± .75[a]	43	46 ± 3[a]

[a] Results expressed as Mean ± SE. Control represents those values obtained after a culture period of 6 hr in a room air and 5% CO_2 environment. Enzyme values are mean of four determinations for each culture grid and are expressed as μmoles glucose incorporated into glycogen/gram wet tissue/hour for both synthetase and phosphorylase. Glycogen concentrations represent mean of two determinations for each placenta and are expressed as mg/100 g wet tissue. Two 13-week placentas were studied. Numbers in parentheses represent number of culture grids for each placenta at the experimental condition.

[b] Indicates significant difference between 2,4 dinitrophenol-treated groups and room air groups ($p < 001$).

incorporated/g wet tissue/hr. By subtracting the 5′ AMP independent activity of phosphorylase *a* from the total activity (*t*), one may calculate the activity of the dependent phosphorylase *b* form. Activities were also calculated on a per milligram protein basis and did not differ statistically from the activity patterns observed using wet weight. Total protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (5) with bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard.

Results. The effects of 2,4 dinitrophenol (DNP) and a 1% oxygen environment on immature and term human placental tissue in organ culture were determined. Six hours after the addition of DNP (5×10^{-5} *M*) to the culture medium of 16-week placental explants, a 2-fold increase in the ratio of glycogen synthetase I to synthetase D (I/D) was observed.

A modest but significant increase (20%) in the phosphorylase *a*/*t* ratio was also noted (Table I). That these enzyme changes represent a metabolic state favoring glycogenolysis, is confirmed by a marked reduction in placental glycogen content.

Hypoxia or DNP treatment of 14-week placental villi produced similar changes in glycogen metabolism (Table II). Increasing the DNP concentration of the tissue culture medium to 1×10^{-4} *M* caused a pronounced shift of synthetase D to the I form, a concomitant elevation in the phosphorylase *a*/*t* ratio, and a decline in the placental glycogen concentration. It was observed that, although the acute enzyme form interconversions were similar at this higher concentration of DNP, total enzyme values were appreciably depressed. The glucose-6-P dependent form of glycogen synthetase exhibited the most pronounced reduction in enzyme activity. Within 6 hr, more than half of the synthetase D activity had been lost. It would appear that ATP is required for the maintenance of this enzyme form. All enzyme activity decreased further between 6 and 12 hr of DNP treatment (Table II).

Explants from term placentas treated with dinitrophenol or cultured in a hypoxic environment (1% oxygen) for 6 hr demonstrated similar responses (Table III). Significant elevations of the I/D and *a*/*t* enzyme form ratios occurred under both conditions. Accompanying these enzyme changes was a pronounced decrease in glycogen concentration. The decline in dependent synthetase activity was not as pronounced in term placental ex-

TABLE III. Effect of Severe Hypoxia and 2,4 Dinitrophenol on Glycogen Metabolism in Term Human Placenta.[a]

Experimental conditions		Glycogen synthetase			Glycogen phosphorylase			
		+G-6-p (D)	−G-6-p (I)	I/D	+ AMP (*t*)	− AMP (*a*)	*a*/*t*	Glycogen
Control 6 hr room air	(3)	39.6 ± 1.8	3.1 ± 0.3	.11	328 ± 28	115 ± 8	.35	189 ± 11
6 hr 1% oxygen	(3)	21.4 ± 0.4[b]	5.2 ± 0.3[b]	.32[b]	281 ± 4[b]	115 ± 5[b]	.55[b]	64 ± 4[b]
6 hr DNP $1 \times 10^{-4}M$ room air	(3)	22.2 ± 0.6[b]	4.7 ± 0.4[b]	.27[b]	236 ± 5[b]	148 ± 5[b]	.63[b]	59 ± 5[b]

[a] Results expressed as Mean ± SE. Enzyme values of both synthetase and phosphorylase are the mean of four determinations for each culture grid and are expressed as μmoles glucose incorporated into glycogen/gram wet tissue/hour. Glycogen content values are the mean of two determinations for each placenta and are expressed as mg/100 g wet tissue. Numbers in parentheses represent number of culture grids for each placenta at the experimental condition. Six term placentas were studied.

[b] Indicates significant difference (Student's *t* test) ($p < .001$) between hypoxia and DNP-treated groups as compared to control cultures in room air 5% CO_2.

plants as in immature villi. As noted previously, synthetase D and I activities are higher in immature placental tissue.

Discussion. Treatment of placental tissue with 2,4 dinitrophenol may reduce intracellular ATP levels (6), impair amino acid transport (7), and alter placental fatty acid oxidation (8). These studies suggest that altering ATP levels by uncoupling oxidative phosphorylation or by oxygen deprivation may also affect placental glycogen metabolism. Both treatments in immature and term human placenta result in an acute glucose-6-P dependent (D) to independent (I) form interconversion. This change accompanied by an elevation of the active/inactive phosphorylase enzyme ratio causes glycogenolysis and a reduction in placental glycogen.

In human placental tissue, glycogen synthetase activity exists predominantly in the glucose-6-P dependent (D) or phosphorylated form. It is well known that ATP is necessary for maintenance of this enzyme form in many mammalian tissues (19). We have observed a marked decline in synthetase D activity in response to hypoxia, or 2,4 dinitrophenol, conditions known to lower intracellular ATP levels. This study suggests, therefore, that an equilibrium exists between the two synthetase enzymes and that ATP is essential to maintain the cofactor dependent form.

The glycogen content of the placenta may also play a role in the observed change in the synthetase enzyme. Danforth (10) among others, has shown an inverse relationship between the tissue glycogen content and the fraction of synthetase in the I form. Synthetase phosphatase, the enzyme which dephosphorylates synthetase D may be directly inhibited by glycogen. Acutely lowering the intracellular glycogen concentration releases this inhibition, allows the interconversion of synthetase D to I, and thus increases the I/D ratio.

It seems likely that in our study, both a decrease in available ATP and a reduction in glycogen concentration could be responsible for elevated placental synthetase I activity. Søvik *et al.* (11) working with heart and diaphragm muscle, used 2-deoxyglucose to reduce intracellular ATP without decreasing tissue glycogen. They observed that, despite normal glycogen levels, a rapid elevation in synthetase I did occur.

Alterations in the glycogen phosphorylase enzyme system produced by hypoxia or 2,4 dinitrophenol treatment are believed to be a

direct consequence of the altered 5′ AMP/ATP ratio in the cell. Elevated AMP levels are known to stimulate phosphorylase *b*. Under conditions of low concentrations of inorganic phosphate or glycogen, AMP may also stimulate phosphorylase *a* and increase the rate of glycogenolysis (12). The consistently elevated *a/t* ratio observed after hypoxia or DNP treatment suggests a similar mechanism in human placental tissue. These findings of an acute mobilization of glycogen under conditions believed to alter intracellular ATP suggest that the homeostasis of placental metabolism during gestation is dependent on an adequate store of glycogen as an available energy reserve.

Summary. The effects of 2,4 dinitrophenol and severe hypoxia (1% oxygen) on human placental glycogen metabolism were studied in immature and term human placental villi isolated in an organ culture system.

It was observed that both treatments evoked similar changes in human placental glycogen turnover in terms of an acute dependent to independent form synthetase enzyme shift, an activation of the glycogen phosphorylase enzyme system, and a rapid breakdown in placental glycogen.

These enzyme changes are consistent with an alteration in intracellular ATP, a known effect of these treatments, and suggest that human placental glycogen may be important to the metabolic energy balance of placental tissue during the course of gestation.

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Received Feb. 14, 1972. P.S.E.B.M., 1972, Vol. 140.