Decreased Ornithine Decarboxylase in the Fetal Hydantoin Syndrome*

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ABSTRACT

The anticonvulsant diphenylhydantoin (DPH) causes embryonic folate antagonism in the animal model of the fetal hydantoin syndrome. Thus, comparisons were made between the metabolic effects of the teratogens DPH and 9-methyl pteroylglutamic acid (9-methyl PGA), a folate antagonist. The DPH inhibited ornithine decarboxylase (ODC), the rate-limiting enzyme in putrescine biosynthesis, and caused reduced levels of this precursor diamine as well as the resultant polyamines, spermidine and spermine. In contrast, embryos from rats treated with 9-methyl PGA had ODC activity similar to controls and increased levels of putrescine, spermidine, and spermine. Because ODC is an enzyme of major importance for embryogenesis, any alterations in ODC activity may indicate abnormal development.

Introduction

Ornithine decarboxylase (ODC), the rate-limiting enzyme of putrescine and polyamine biosynthesis, has higher levels of activity in developing animals, including humans, than in adults. Mammalian embryonic ODC activity and polyamine concentrations typically reach a peak early in embryonic development and decline during the latter portion of gestation and after birth. The high concentrations of polyamines in embryonic development have been theorized to relate to the ability of polyamines to stabilize nucleic acids and ribosomal subunits and to stimulate nucleic acid and protein synthesis.

Because of the importance of ornithine decarboxylase to normal embryonic development, the effects of certain teratogenic agents on ODC and polyamine levels in fetal rodents were examined by the present authors. Buehler and Smith suggested that the anti-convulsant diphenylhydantoin (DPH) may inhibit ODC. Exposure to diphenylhydantoin during treatment for epilepsy has been associated with megaloblastic anemia and reduced folic acid levels in adults and...
with the fetal hydantoin syndrome which includes cleft lip, cleft palate, congenital heart disease, and various central nervous system malformations. It has been suggested that epilepsy, rather than DPH, is the cause of such birth defects. However, Finnell has demonstrated that abnormalities in offspring of inbred mice with seizure disorders are correlated with maternal serum diphenylhydantoin levels rather than being caused by the seizure disorder.

There are resemblances between the side effects of diphenylhydantoin and those of folic acid antagonists. Not only do folic acid antagonists cause adult megaloblastic anemia, but they are also teratogenic in humans. In addition, previous studies have demonstrated similarities between the malformations in offspring of mice treated with DPH and those of rats treated with the folic acid antagonist, 9-methyl pteroylglutamic acid (9-methyl PGA). Embryos from rodent mothers treated with either DPH or 9-methyl PGA also share certain metabolic characteristics.

The two chemicals cause similar gross malformations in rodent embryos. Whether or not alterations in ODC activity and polyamine levels are also common to the two teratogens was tested by comparison of embryonic rodent polyamines, ODC activities, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein levels.

Methods

TREATMENT WITH DIPHENYLHYDANTOIN

Virgin ICR mice were caged overnight with males of the same strain, using one male for four to six females. Females were weighed the next morning, which was considered day one of pregnancy and again on day ten. A gain of 2 g was considered evidence of pregnancy. On day ten of pregnancy, experimental females were injected intraperitoneally with Dilantin with a dose of 88 mg per kg of body weight. Uninjected mice were used as controls, because previous reports indicate that vehicle-injected and uninjected control mice have the same resorption rate and negligible frequency of abnormalities. Treated animals were housed in hanging cages; controls remained caged with non-pregnant mice and consumed the same diet as did treated mice of Wayne Lab blocks and water ad libitum. Mice were sacrificed on day 12 and embryos obtained by laparotomy.

| TABLE I |
| Mean Protein Contents (mg ± S.E.) of Embryos of DPH-treated Mice and 9-methyl PGA-treated Rats Compared to Control Embryos |

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experimental</th>
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<tbody>
<tr>
<td>DPH</td>
<td>2.67 ± 0.09</td>
<td>2.38 ± 0.10*</td>
</tr>
<tr>
<td>Sample size</td>
<td>87</td>
<td>63</td>
</tr>
<tr>
<td>9-methyl PGA</td>
<td>3.09 ± 0.07</td>
<td>2.68 ± 0.09†</td>
</tr>
<tr>
<td>Sample size</td>
<td>93</td>
<td>80</td>
</tr>
</tbody>
</table>

*p < 0.05
†p < 0.01

DPH = Diphenylhydantoin
PGA = Pteroylglutamic acid

\* Harlan Sprague-Dawley Company.
† Allied Feed Company

![Figure 1](image-url)
TREATMENT WITH 9-METHYL PTEROYLGLUTAMIC ACID

Virgin proestrous Long-Evans black hooded rats § were caged overnight with males of the same strain. The presence of a sperm plug or sperm in a vaginal smear the following morning indicated day 1 of pregnancy. On day 11, experimental rats were intubed with one mg of 9-methyl pteroylglutamic acid (9-methyl PGA) in one ml of saline and subsequently fed a folate-deficient diet including 10 mg of 9-methyl PGA per 100 g diet. The rats were housed in hanging cages and consumed at least 10 g of diet daily. Control rats were fed Wayne Lab Blocks † ad libitum. Water ad libitum was available to both groups. Treated and control rats were sacrificed on day 14 and embryos obtained by laparotomy.

Embryos were dissected free of embryonic membranes and frozen in glass tubes in a dry ice-acetone mixture at ~80°C.

ASSAY FOR ORNITHINE DECARBOXYLASE

Aliquots (50 to 100 µl volumes) of homogenate were assayed for ODC activity using the double-chamber assay system of Moskal and Basu. The final concentrations of reagents* in the incubation mixture were those described by O'Brien and Diamond: 50 mM sodium phosphate, pH 7.2; 5 mM dithiothreitol; 1 mM EDTA; 1 mM l-ornithine; 200 µM pyridoxal-5-phosphate. The reaction was initiated by adding 0.5 µCi L-(1-14C)-ornithine † to the homogenate and reagent mixtures, capping the tubes with sleeve-type rubber stoppers, and placing them in a waterbath at 37°C. The 14CO2 evolved by the reaction was trapped in 25 µl of hyamine hydroxide on a strip of filter paper. After incubation for 60 minutes, the reaction was terminated by injecting 200 µl of 2M citrate through the rubber stopper and replacing tubes in the water bath for an additional four hours. The filter paper was then placed in 10 ml of toluene scintillation fluid in a plastic scintillation vial and the amount of radioactivity determined using a Beckman LS-230 scintillation counter.

* All chemicals were obtained from Sigma Chemical Company.
† Amersham.
NUCLEIC ACID DETERMINATIONS

After aliquots were taken for ODC assay, the remaining tissue was used for DNA/RNA determinations. Thawed tissue was sonicated for three seconds with a sonifier at lowest power (output control setting 1). The RNA and DNA were determined from 0.1 ml samples on a spectrophotometer using the method of Seiler and Gleinewinkel, with the exception that DNA samples were stored overnight in a water bath at 37°C. Cuvettes with optical path length of 10 mm were used and calf liver RNA and calf thymus DNA were used as standards.

POLYAMINE DETERMINATIONS

One-tenth ml aliquots were diluted 1:1 with 0.2N perchloric acid in preparation for polyamine determination. Putrescine, spermidine and spermine were quantitated on an amino acid analyzer as described by Rennert et al. Protein contents of embryos were determined by the method of Lowry et al. Statistical comparisons were performed using the Student t-test as described by Dunn and Clark. All statistics are expressed as means ± standard errors.

Results

Treatment with either DPH or 9-methyl PGA reduced the protein contents of embryos (P < 0.05, table I). Diphenylhydantoin reduced the embryonic levels of putrescine (3.7 ± 0.2 versus 5.1 ± 0.2 nmoles per mg of embryonic protein), spermidine (9.9 ± 0.4 versus 12.6 ± 0.5 nmoles per mg of embryonic protein), and spermine (4.8 ± 0.2 versus 6.0 ± 0.2 nmoles per mg of embryonic protein) in embryos of treated mothers as shown in figure 1. In contrast (figure 2), 9-methyl PGA-treated pregnancies resulted in higher levels of putrescine (11.4 ± 1.2 versus 8.2 ± 0.8 nmoles per mg of embryonic protein), spermidine (25.4 ± 1.7 versus 20.2 ± 1.5 nmoles per mg of embryonic protein), and spermine (16.9 ± 2.1 versus 10.0 ± 1.1 nmoles per mg of embryonic protein, P < 0.05).

Maternal treatment with DPH was associated with decreased embryonic ODC activity (treated versus control values were 6.4 ± 0.4 versus 8.7 ± 0.6 pmoles 14CO2 evolved per mg embryonic protein, figure 3). However, treatment with 9-methyl PGA did not decrease embryonic ODC activity (figure 4). In fact, ODC activity appeared increased in embryos of 9-methyl PGA-treated mothers, although the change was not statistically significant (6.5 ± 0.6 versus 6.0 ± 0.5 pmoles 14CO2 evolved per mg of embryonic protein).

The nucleic acid contents of embryos from either DPH- or 9-methyl PGA-treated mothers were reduced (tables II and III). Embryonic DNA concentration was not affected by either treatment (table II). The DPH had no effect on embryonic RNA concentration, but 9-methyl PGA

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† Branson model W140C.
‡ Zeiss PM QII.
§ Sigma Chemical Company.
** Durrum D-500, Durrum Instrument Corp., Sunnyvale, CA.
TABLE II
Mean DNA Contents (µg DNA/embryo ± S.E.) and Concentrations (µg DNA/mg embryonic protein ± S.E.) in Embryos of DPH-treated Mice and 9-methyl PGA-treated Rats Compared to Control Embryos

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contents</td>
<td>202.6 ± 10.4</td>
<td>165.1 ± 6.8*</td>
</tr>
<tr>
<td>Concentrations</td>
<td>84.4 ± 4.8</td>
<td>93.7 ± 10.1</td>
</tr>
<tr>
<td>Sample size</td>
<td>69</td>
<td>74</td>
</tr>
<tr>
<td>9-methyl PGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contents</td>
<td>179.1 ± 6.0</td>
<td>153.0 ± 4.0*</td>
</tr>
<tr>
<td>Concentrations</td>
<td>58.3 ± 2.6</td>
<td>58.8 ± 2.5</td>
</tr>
<tr>
<td>Sample size</td>
<td>68</td>
<td>68</td>
</tr>
</tbody>
</table>

*p < 0.01
DNA = Deoxyribonucleic acid
DPH = Diphenylhydantion
PGA = Pteroylglutamic acid

decreased the RNA concentration in embryos of treated mothers (P < 0.01, table III).

Discussion

The levels of control rodent embryonic ornithine decarboxylase activities obtained in this study are similar to those reported by Sturman and Gaull for human fetuses. 33 Embryos of mice treated with diphenylhydantoin had reduced ODC activity and correspondingly lower putrescine, spermidine, and spermine concentrations than controls. Maternal treatment with 9-methyl pteroylglutamic acid, however, resulted in elevated embryonic polyamine levels although the increase in ODC activity was not statistically significant. Increases in ODC are usually transient 15 and ODC elevations induced on day 11 by 9-methyl PGA-treatment may have returned to normal levels by day 14 when the embryos were collected.

Nucleic acid contents of embryos of treated mothers are lower than those of controls. Because protein contents were also reduced by both treatments, the nucleic acid concentrations (µg of nucleic acid per mg of embryonic protein) are not significantly affected, except for the RNA concentrations of embryos of 9-methyl PGA-treated rats. There is previous evidence that 9-methyl PGA has severe effects on the amount of cytoplasmic RNA and ribosomes in embryos of treated rats. 16

Nucleic acid concentrations have been observed to increase in parallel with polyamine concentrations during normal development and after various stimulatory treatments. 14,15,29,31,36 Parallel changes in nucleic acids and polyamines were not observed in our study after DPH or 9-methyl PGA treatment. Experiments by Poso and Janne 28 and Fillingame and Morris 8 indicate that inhibition of polyamine biosynthesis is not always associated with a decrease of nucleic acid concentrations.

The ODC has an essential role in embryogenesis. Inhibition of ODC is associated with diminished growth in cultured cells, regenerating liver, and certain neoplasms. 15,31 Embryos are particularly sensitive to ODC interference. Treatment with DL-α-difluoromethylornithine (α-DFMO), an irreversible and specific inhibitor of ornithine decarboxylase, causes complete resorption of rodent, rabbit, and cat embryos. 8 Decreases in heart ODC activity are correlated with lower weights and reduced growth in hyperthyroid neonatal rats. 19 The DPH treatment at doses comparable to those used in our study re-
roduces fetal weight and shortens fetal long bones as well as reducing the activity of ODC.\textsuperscript{13} Injections of 150 mg DPH per kg of maternal weight produce ectrodactyly, cleft lip, cleft palate, hydronephrosis, internal hydrocephalus and skeletal defects.\textsuperscript{13}

Not only is inhibition of ODC harmful to the embryo, but elevations of ODC activity may also indicate abnormal development. Maternal morphine administration is associated with elevated neonatal brain ODC activity and with central nervous system abnormalities.\textsuperscript{2} Higher than normal levels of brain ODC shortly after birth occur in conjunction with reserpine- or methadone-induced deficits of brain tyrosine hydroxylase.\textsuperscript{34} Elevations of ODC and subsequently of polyamines may reflect a change in growth rates or an attempt to compensate for some other injury. For example, maternal treatment with teratogenic doses of 9-methyl PGA in our study produced elevated polyamine levels. Because folic acid antagonists inhibit dihydrofolate reductase but polyamines stimulate the enzyme,\textsuperscript{35} it is possible that the elevation of polyamines in offspring of 9-methyl PGA-treated rats is an attempt to re-establish appropriate levels of dihydrofolate reductase, an enzyme crucial for DNA synthesis.

High values of ODC and polyamines usually occur during situations of rapid growth, cell proliferation and/or cell death. Neoplastic tissue \textit{in vitro} and \textit{in vivo} exhibits increased ODC activity and polyamine levels.\textsuperscript{15,27,31} Increased plasma levels of polyamines are observed in patients with polycythemia vera.\textsuperscript{31} Urinary polyamines are elevated in patients with acute and chronic lymphocytic leukemia and acute myelogenous leukemia.\textsuperscript{4} The polyamine elevations are not believed a causative factor, but rather may reflect the high turnover and active proliferation of cells in these diseases.

It appears that any alterations of ODC and polyamines can be indicative of developmental disorder. The ODC assays require tissue, but polyamines can be measured in urine, plasma, erythrocytes and amniotic fluid. Giles et al\textsuperscript{3} report significant elevations of spermidine, cadaverine, and other polyamines in amniotic fluid of embryos experiencing intrauterine growth retardation as well as exceedingly high values of cadaverine in amniotic fluid of patients with intrauterine fetal death. Unfortunately, this test is not sufficiently sensitive; other patients with serious pregnancy complications did not have elevated polyamine levels in amniotic fluid. The importance of appropriate controls for such comparisons cannot be over-estimated. Not only do normal placental polyamine levels vary with gestational age,\textsuperscript{11} but sex, hormone levels, and health can affect adult urinary polyamine levels\textsuperscript{31} and may well influence polyamine contents in amniotic fluid. Although much basic research would be required before the limitations and uses of comparative polyamine determinations become well-defined, it is possible that alterations of fetal polyamine levels could be a valuable, though non-specific, predictor of abnormal development.

\textit{Acknowledgments}

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