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PHENYTOIN-INDUCED STRESS PROTEIN SYNTHESIS IN MOUSE EMBRYONIC TISSUE

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Abstract. Several proteins have been shown to be synthesized in response to various environmental stimuli, including treatment with teratogens. The role of these proteins in the teratogenic process is unknown. Pregnant A/J mice were treated with either a teratogenic or a non-teratogenic dose of the anticonvulsant drug, phenytoin (PHT). Protein synthesis in embryonic craniofacial (target) tissue or forelimb buds (non-target) was determined by incorporation of radiolabeled leucine and analysis by two-dimensional polyacrylamide gel electrophoresis. Synthesis of three proteins in target tissue and one protein in non-target tissue was stimulated by drug treatment. These results suggest that synthesis of specific stress proteins may serve as biomarkers of drug-target tissue interaction.

Introduction. Phenytoin (PHT) is the drug of choice for the treatment of epileptic grand mal seizures; however, it is a suspect human teratogen (1,2). The drug is an animal teratogen and increases the frequencies of resorptions and malformations, especially orofacial anomalies, in mice (3,4). A/J mice are particularly sensitive to the teratogenic effects of the drug (4). The mechanisms underlying the developmental toxicity of the compound are unknown.

The synthesis of a set of proteins has been shown to be induced in prokaryotes and eukaryotes, including humans, by heat as well as by a variety of metals and chemicals including several teratogens (5). These proteins are termed heat-shock or stress proteins and are generally identified by their isoelectric points and molecular weights. It has been suggested that these proteins may be involved in teratogenesis. It was reported that a teratogenic dose of PHT was capable of inducing a subset of stress proteins in embryonic Drosophila cells; synthesis of low molecular weight proteins of 22,000 and 23,000 relative molecular mass units (Mr) was induced in vitro by PHT (5,6). The purpose of the present investigation was to determine if a teratogenic dose of PHT would induce stress protein synthesis in vivo in mouse embryonic tissues, and to determine if such synthesis was target tissue specific.

Materials and Methods. Mice of the A/J strain (The Jackson Laboratory, Bar Harbor, ME) were used in all studies. On the morning of gestation day 10 (plug day = day 0), PHT (Aldrich Chemical Company, Milwaukee, WI) at a concentration of 5 mg/ml was dissolved in distilled water adjusted to approximately pH 11 with 1 N sodium hydroxide and was injected ip at 0, 25, or 75 mg/kg body weight. Twenty-four hr later, 50 uCi of ^{3}H-leucine (80 Ci/mmol, New England Nuclear, Boston, MA) was injected ip. One and one-half hr later, dams were sacrificed by cervical dislocation, and embryos were removed from all extraembryonic membranes and washed in 0.5 M phosphate-buffered saline.

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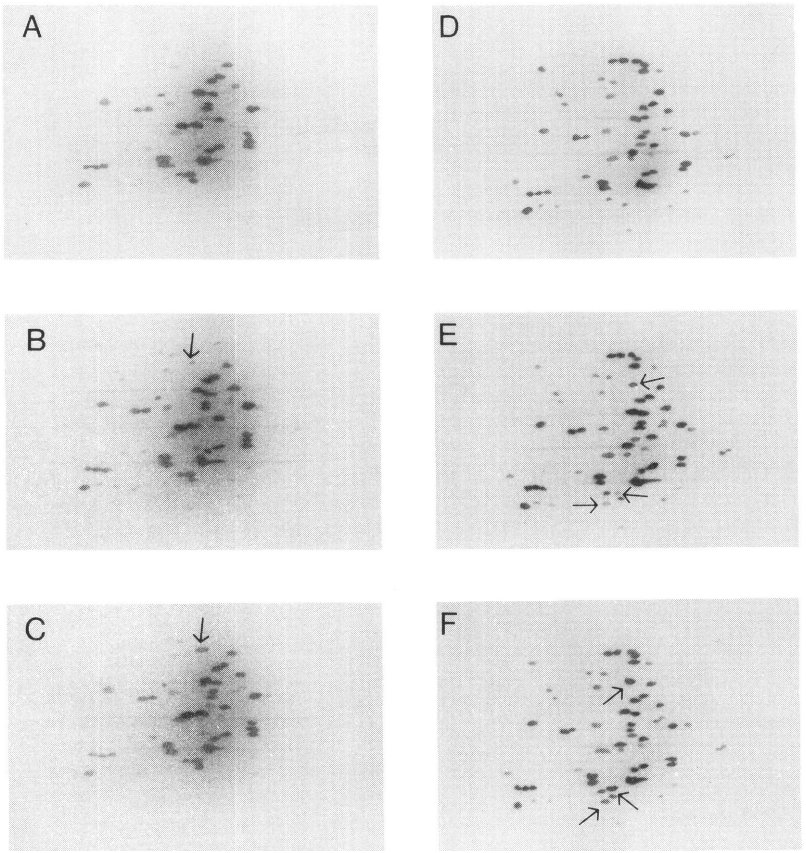

Fig. 1. Two-dimensional polyacrylamide gel electrophoretic pattern of proteins synthesized in embryonic craniofacial tissue. The pH of the first dimension IEF gel was 4 - 9. The acrylamide concentration was 10% in both first dimension and second dimension gels. (A) Unsorted nuclear proteins from control embryos. (B) Unsorted nuclear proteins of embryos from dams exposed to PHT at 25 mg/kg. A protein of Mr = 84,000-90,000 is indicated by the arrow; this protein was not synthesized in control tissue. (C) Unsorted nuclear proteins of embryos from dams exposed to PHT at 75 mg/kg. The protein from (B) is again marked. (D) $G_0 + G_1$ nuclear proteins from control embryos. (E) $G_0 + G_1$ nuclear proteins of embryos from dams exposed to PHT at 25 mg/kg. The Mr = 84,000-90,000 protein is marked by an arrow. In addition, several proteins of Mr = 20,000-25,000 are clearly resolved. Synthesis of two of these proteins (arrows) appears to be induced by PHT since these proteins are not present in control tissue. (F) $G_0 + G_1$ nuclear proteins of embryos from dams exposed to PHT at 75 mg/kg. Synthesis of the Mr = 84,000-90,000 protein and two of the Mr = 20,000-25,000 proteins (arrows) appear to be induced by PHT treatment.

Under dissecting microscopes, forelimb buds and craniofacial tissue were excised and pooled. Nuclei were isolated and stained with propidium iodide according to the method of Anson et al. (7). In some cases, nuclei were sorted from the $G_0 + G_1$ phases of the cell cycle using a fluorescence-activated cell sorter (FACS II; Becton-Dickinson, Sunnyvale, CA). Proteins were extracted from both sorted $G_0 + G_1$ nuclei and from unsorted nuclei using an imidazole extracting solution and dialyzed prior to electrophoresis (7,8). Nuclear protein concentration was determined according to the method of Bramhall et al. (9), and 800 ng of protein (3 ul of sample containing approximately 10,000

cpm) was introduced into the first-dimension gel in the ultra-micro-electrophoresis apparatus (10). Following two-dimensional electrophoresis (7), autoradiography was utilized to visualize labeled proteins (11). The numbers of embryos and litters in each experimental group were as follows: 1) vehicle treated, unsorted sample - 23 embryos from 5 litters; 2) PHT at 25 mg/kg, unsorted sample - 29 embryos from 7 litters; 3) PHT at 75 mg/kg, unsorted sample - 27 embryos from 9 litters; 4) vehicle treated, $G_0 + G_1$ nuclei - 44 embryos from 7 litters; 5) PHT at 25 mg/kg, $G_0 + G_1$ nuclei - 68 embryos from 9 litters; and 6) PHT at 75 mg/kg, $G_0 + G_1$ nuclei - 141 embryos from 21 litters.

Results. When nuclear proteins from embryonic craniofacial tissue were analyzed, synthesis of a high molecular weight protein (Mr = 84,000-90,000) was induced by PHT treatment (Fig. 1B,C,E and F). This protein was present in sorted and unsorted nuclei after treatment of dams with both non-teratogenic (25 mg/kg) and teratogenic (75 mg/kg) doses of the anticonvulsant. The amount of synthesis was not quantitated, but based upon the density of the radiolabeled bands, there appeared to be a dose-related increase in synthesis in sorted and unsorted samples (compare Fig. 1B to 1C or 1E to 1F). This protein was not synthesized in control tissue (Fig. 1A and D). When proteins from sorted nuclei were analyzed, synthesis of two low molecular weight proteins (Mr = 20,000-25,000) became apparent; synthesis appeared to be induced in a dose-dependent manner (compare Fig. 1E to 1F).

The total pattern of protein synthesis in forelimb buds was somewhat more complex than that observed in craniofacial tissue (Fig. 2). The Mr = 84,000-90,000 protein was not synthesized in either control or treated forelimb buds; however, synthesis of a low molecular weight protein (Mr = 20,000-25,000) was induced in forelimb buds by PHT treatment. It has not been determined if this protein is identical to one of the low molecular weight proteins observed in craniofacial tissue.

Discussion. Administration of PHT at 75 mg/kg to pregnant A/J mice on day 10 of gestation increased the incidence of cleft lip and palate to 96%; treatment with 25 mg/kg produced a frequency of this defect of 5.6% which is not significantly higher than the spontaneous incidence of 5% observed in this strain (12). Forelimb defects have been found occasionally in other mouse strains treated with PHT but have not been reported in A/J mice (4). Administration of either dose of the drug induced the synthesis of a Mr = 84,000-90,000 and two or three lower molecular weight, Mr = 20,000-25,000, proteins. Synthesis of these proteins occurred in the $G_0 + G_1$ phases of the cell cycle. Since nuclei were not sorted from other phases, it is not known if synthesis also occurred at other times during the cell cycle. However, previous work has demonstrated that stress protein synthesis occurs predominantly in the $G_0 + G_1$ phases *in vivo* as well as *in vitro* (14,15).

Functions of stress proteins are unknown. Data has indicated that a non-steroid binding phosphoprotein of Mr = 90,000 associated with untransformed glucocorticoid and progesterone receptors is a heat shock protein (15), and glucocorticoid receptors have been found in A/J embryos as early as day 12 of gestation (16). Further work will be needed to determine if the high molecular weight protein synthesized in response to PHT treatment is the same as the heat shock protein associated with the glucocorticoid receptor.

The presence of certain stress proteins only in target tissue would appear to indicate that these proteins may have some role in teratogenesis. At least two interpretations are possible with regard to their synthesis in target tissue at both teratogenic and non-teratogenic doses of PHT. It is plausible that stress proteins are causal in teratogenesis, and the level of synthesis after the non-teratogenic dose was insufficient to disrupt normal development. Alternatively, these proteins could have a protective function that was adequate at the low PHT dose but was overwhelmed by the higher dose. Further experimentation will be required to determine between these possibilities.

To our knowledge, this is the first report to compare stress protein synthesis in teratogenic target and non-

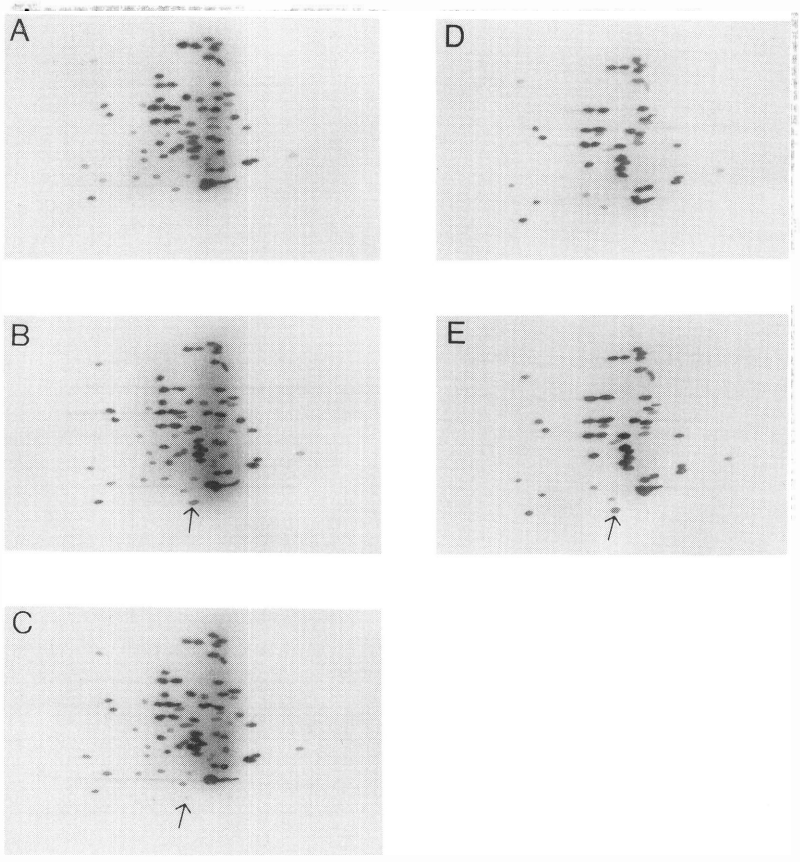

Fig. 2. Two-dimensional polyacrylamide gel electrophoretic pattern of H-leucine labeled proteins synthesized in embryonic forelimb buds. The high molecular weight protein present in craniofacial tissue was not synthesized in forelimb buds in either control or treated embryos. The pH of the first dimension IEF gel was 4 - 9. The acrylamide concentration was 10% in both first dimension and second dimension gels. (A) Unsorted nuclear proteins from control embryos. (B) Unsorted nuclear proteins of embryos from dams exposed to PHT at 75 mg/kg. A protein of Mr = 20,000 - 25,000 is marked by the arrow. (C) Unsorted nuclear proteins of embryos from dams exposed to PHT at 25 mg/kg. A low molecular weight stress protein is again marked by an arrow. (D) $G_0 + G_1$ nuclear proteins from control embryos. (E) $G_0 + G_1$ nuclear proteins of embryos from dams exposed to PHT at 75 mg/kg. Synthesis of a protein of Mr = 20,000 - 25,000 appeared to be induced by PHT (arrow).

target tissue. The role of the three proteins synthesized in craniofacial tissue in PHT-induced teratogenesis remains to be determined. Regardless of their role, these proteins may have potential use as biological markers of drug-target tissue interaction, since at least two of them appear to be synthesized only in target tissue.

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