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Inhibition of Nitric Oxide Synthesis Enhances Teratogenic Effects Induced by Valproic Acid

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Abstract. *Background/aim: The mechanism of valproic acid (VPA)-induced teratogenicity is poorly known. This study was carried out to probe into the potential consequences of nitric oxide (NO) deprivation on VPA teratogenicity. Materials and Methods: On gestation day 8, mice were injected with a non-teratogenic dose (20 mg/kg) of the nitric oxide synthase (NOS) inhibitor N(G)-nitro-L-arginine methyl esther (L-NAME). Thirty minutes later, animals received a teratogenic dose of VPA (400 or 500 mg/kg). Developmental end-points were evaluated near the end of gestation. Results: After treatment with VPA at 400 mg/kg, 35.2% of fetuses exhibited skeletal teratogenesis. The rate of skeletally affected fetuses significantly increased to 53.7% after L-NAME co-administration. In the group treated with VPA at 500 mg/kg group, L-NAME pre-treatment increased the incidence of exencephaly from 5.4% to 22.2%. Conclusion: Inhibition of NO synthesis can result in an enhancement of VPA-induced teratogenesis.*

Valproic acid (VPA) is a short-chained fatty acid used in the treatment of epilepsy, migraine and bipolar disorders. It may also have therapeutic potential in neurodegenerative and psychiatric disorders (1), and is currently being tested as an anticancer agent (2). It is well-established that VPA is teratogenic in humans and in all tested animal species (3). Exposure to VPA during pregnancy has been associated with approximately a 1-2% of neural tube defects, which is 10- to 20-times the rate in the general population (3). VPA exposure in pregnant women has also been related to the presence of cardiac, craniofacial, skeletal and limb defects, and a possible set of dysmorphic features, the valproate syndrome (3). Experimentally, administration of VPA to pregnant mice during neurulation resulted in the induction of malformations primarily consisting of exencephaly (a closure defect of the anterior neural tube) and axial skeletal defects (4-6). Experiments with mice have also shown that VPA itself and not one of its metabolites is responsible for the teratogenic effects (7). The molecular events behind VPA teratogenicity remain undefined. Several mechanisms, including disturbances in folic acid metabolism, increased oxidative stress and, inhibition of histone deacetylases (HDAC) have been advocated, but none has been conclusively shown to be the culprit for abnormal development (3).

As an intriguing finding, VPA was recently reported to inhibit nitric oxide synthase (NOS) expression in cell cultures (8, 9). This is of particular interest considering that NO is known to play essential roles in development and its imbalance has been associated with abnormal development (10, 11). We, thus, considered the possible impact of NO imbalance on VPA-induced teratogenesis worthy of investigation. To begin investigating this issue, in the present study, we tested the impact of a NOS inhibitor on the teratogenic response induced by VPA in the mouse.

Materials and Methods

Sexually mature ICR (CD-1) mice from our breeding colony at the University "G. d'Annunzio" of Chieti-Pescara were used. Animal care was in accordance with institution guidelines. Animal husbandry and breeding procedures were performed as previously described (12). All the chemicals used were purchased by Sigma (Milan, Italy). Testing compounds were dissolved in sterile saline solution shortly before injection. A dosage volume of 10 ml/kg was used. On gestation day 8 (plug day=gestation day 0) animals received a single intraperitoneal dose of 20 mg/kg of N(*G*)-nitro-L-arginine methyl esther (L-NAME). Control animals were injected with an appropriate dose of vehicle. The dose of L-NAME was selected because it is known to be several times below the teratogenic threshold (12). Thirty minutes after L-NAME administration, mice received a single intraperitoneal injection of VPA at 400 or 500 mg/kg.

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Table I. *Maternal and litter parameters observed in ICR (CD-1) mice after combined treatment with N(G)-nitro-L-arginine methyl esther (L-NAME)† and valproic acid (VPA)‡.*

	Control	L-NAME	VPA (400 mg/kg)	L-NAME + VPA (400 mg/kg)	VPA (500 mg/kg)	L-NAME + VPA (500 mg/kg)
Number of pregnant animals treated	11	11	12	13	17	16
Maternal body weight (g±SEM) at term gestation	58.49±1.70	56.97±1.72	56.79±2.84	57.58±2.05	52.94±1.99	57.39±2.00
Maternal absolute weight (g±SEM)#	39.24±0.86	38.72±0.94	38.28 ±1.02	39.27±0.95	37.82 ±1.02	39.46±0.86
Uterine weight (g±SEM)	18.91±1.73	18.10 ±1.08	16.87±2.45	17.85 ±1.50	15.48 ±1.05	17.78 ±1.47
No. of animals at term gestation with live fetuses	11	11	12	13	16	16
No. of implantations per litter (mean±SEM)	12.42±0.73	12.00±0.96	12.83±0.77	12.38±0.92	12.12±0.55	12.75±0.92
Post-implantation loss§	11/141 (11.34%)	19/132 (14.39%)	25/154 (16.23%)	22/161 (13.66%)	59/206 (28.64%)*	42/202 (20.79%)**
No. of fetuses at necropsy	130	113	129	139	147	162
Mean fetal body weight per litter (g±SEM)	1.32±0.04	1.29±0.05	1.29±0.04	1.25±0.02	1.17±0.08	1.19±0.03

†Injected intraperitoneally on gestation day 8 at 20 mg/kg; ‡injected intraperitoneally 30 min after L-NAME; #maternal body weight at term minus gravid uterine weight; §including early resorptions, late resorptions and dead fetuses; *statistically significant ($p<0.05$) *vs*. control and L-NAME groups; **statistically significant ($p<0.05$) *vs*. control group.

Pregnancies were terminated near term, on gestation day 18, and the selected end-points, including maternal weight, pregnant uterus weight, maternal absolute weight (maternal weight–pregnant uterine weight), number of living and dead fetuses, fetal gender, fetal weight and gross malformations were recorded. Half of the fetuses from each litter were prepared for double-staining skeletal examination as previously described (12). The remaining fetuses were fixed in Bouin's solution and subsequently examined for visceral anomalies, using the free-hand slicing method of Wilson (13). All morphological evaluations were carried out under a stereo microscope.

For statistical analysis, continuous data were compared using Student's *t*-test or ANOVA and *post hoc* Student–Newman–Keuls test for multiple comparisons. Binomial data were compared using the chi-square test. Differences were considered statistically significant when $p<0.05$.

Results

Table I, summarizes maternal and litter parameters observed in control and treated animals. VPA at the dosages used was not lethal to any pregnant animal. No treatment-related effects on maternal weight parameters after VPA, alone or in combination with L-NAME, were detected. Regarding post-implantation loss, a frequency of 11.3% was recorded in the control (vehicle) group, and no significant increases were noted after L-NAME alone, or after VPA at 400 mg/kg (alone or in combination with L-NAME). A statistically significant increase of post-implantation loss, in comparison to the control group, was recorded when VPA was injected at 500 mg/kg, with 28.6% of embryos dying before birth. This percentage was unaffected by L-NAME pre-treatment. There was a trend towards a reduction of the mean fetal weight with VPA dose increment, but differences were not statistically significant.

Type and frequencies of external malformations observed in fetuses from the various experimental groups are shown in Table II. Frequencies and description of skeletal defects detected in fetuses double-stained with Alcian blue and alizarin red are given in Table III. No malformations were observed in the control group and only one malformed fetus, exhibiting exencephaly and skeletal anomalies, was observed in the L-NAME-treated group. As a main teratological effect, VPA induced axial skeletal defects, affecting 35.2% (31/88) and 67.0% (67/100) of fetuses exposed to 400 mg/kg or 500 mg/kg, respectively. The spectrum of vertebral anomalies included fused, asymmetric, and cleaved vertebrae, and vertebrae with asymmetric, cleaved, and dumbbell-shaped

Table II. *Congenital defects observed in ICR (CD-1) mice after combined treatment with N(G)-nitro-L-arginine methyl esther (L-NAME)† and valproic acid (VPA)‡.*

	Control	L-NAME	VPA (400 mg/kg)	L-NAME + VPA (400 mg/kg)	VPA (500 mg/kg)	L-NAME + VPA (500 mg/kg)
No. of fetuses examined	110	113	129	139	147	162
No. of fetuses with neural tube defects	0	1 (0.9%)	3 (2.3%)	8 (5.7%)*	6 (4.1%)	36 (22.2%)**
No. of fetuses with facial clefting	0	0	0	0	0	4 (2.5%)
No. of fetuses with cleft palate	0	0	2 (1.5%)	1 (0.7%)	1 (0.7%)	0
No. of fetuses with anus imperforatus	0	0	1 (0.7%)	0	0	2 (1.2%)

†Injected intraperitoneally on gestation day 8 at 20 mg/kg; ‡injected intraperitoneally on gestation day 8 (30 min after L-NAME); *statistically significant ($p<0.05$) *vs*. control group; **statistically significant ($p<0.05$) *vs*. VPA at 500 mg/kg.

centrum. Abnormal vertebral phenotypes were found in thoracic, lumbar, sacral, and caudal vertebrae. Rib malformations consisted mainly of rib fusion. Regarding neural tube defects, exencephaly was observed in 2.3% (3/129) and in 4.1% (6/147) after VPA at 400 mg/kg or 500 mg/kg, respectively. Malformations of the internal viscera were virtually absent.

Pre-treatment with L-NAME enhanced VPA teratogenicity. A significant increase in the rate of fetuses displaying axial skeletal defects was observed when L-NAME was co-administered to VPA at 400 mg/kg, yielding this treatment regimen a 53.7% of skeletally affected fetuses. This 50% increase in comparison to rate observed after VPA 400 mg/kg alone (35.2%) was statistically significant. There was a trend toward increase in the rate of skeletally affected fetuses when L-NAME was co-administered to VPA at 500 mg/kg group, from 67% (VPA at 500 mg/kg) to 76.1% (L-NAME plus VPA 500 mg/kg), but there were no statistically significant differences between these values among the groups.

Concerning exencephaly, the low incidence of affected fetuses observed after VPA 400 mg/kg alone was not significantly altered by L-NAME. On the other hand, when L-NAME was administered with VPA at 500 mg/kg a significant enhancement in the rate of exencephalic fetuses resulted. Indeed, only 4.1% of fetuses exposed to VPA at 500 mg/kg were exencephalic, while the percentage of affected fetuses increased to 22.2% after pre-treatment with L-NAME.

Discussion

This study represents the first report showing that inhibition of NO production can lead to an enhancement of VPA teratogenicity. NO is generated from L-arginine by three NOS isozymes: neuronal NOS, inducible NOS, and endothelial NOS. All these isoforms can be directly inhibited by the L-arginine analogue L-NAME. This inhibitor has been shown to be able to cross the placenta and to reach the embryonic/fetal compartment (14), making it feasible that inhibition of embryonic NOS was a determinant of the teratological interaction observed in the present study. Administration of a non-teratogenic dose of L-NAME imposed a two-fold increase in the per fetus incidence of skeletal malformations caused by VPA at 400 mg/kg, and a three-fold time increase of the incidence of exencephaly induced by VPA at 500 mg/kg. VPA pharmacokinetics has been fairly well-established (15). Following a single intraperitoneal dose, peak plasma levels are reached in the mouse in about 30 minutes, and the half-time of drug clearance from serum is about 1 h. It is known that a teratogenic interaction can reflect changes in pharmacokinetic parameters resulting in increased exposure to the toxic agent. For instance, potentiation of VPA-induced teratogenesis by ethanol was related to decreased VPA elimination (16). Determinations of drug levels were not carried out in the present study, but a pharmacokinetic basis for the teratological interaction seen seems unlikely, considering that in a previous study in the mouse, L-NAME was found to be unable to alter VPA plasma levels (17).

NO is a short-lived mediator molecule serving essential roles in reproduction and development, and proper endogenous levels of NO are required for normal embryonic development (10, 11). Treatment of pregnant rodents with NOS inhibitors has resulted in abnormal phenotypes. When pregnant mice received L-NAME early in organogenesis (day 8 or 9) at doses equal or exceeding 150 mg/kg axial skeletal defects resulted (12). Neural tube defects and underdevelopment of the hyoid arch and optic cup were induced when N(*G*)-monomethyl-L-arginine was injected directly into the amniotic fluid of cultured rat embryos (18). Treatment of pregnant of rats (19, 20) and mice (21) with NOS inhibitors during mid-gestation caused limb disruptions secondary to tissue breakdown of normally developed structures. Neural tube defects and axial skeletal malformations were also seen after *in utero* exposure to the soluble guanylate cyclase inhibitor methylene blue (22). The phosphodiesterase inhibitor zaprinast, which will prolong the

Table III. *Skeletal abnormalities observed in ICR (CD-1) mice after combined treatment with with N(G)-nitro-L-arginine methyl esther (L-NAME)† and valproic acid (VPA)‡.*

	Control	L-NAME	VPA (400 mg/kg)	L-NAME + VPA (400 mg/kg)	VPA (500 mg/kg)	L-NAME + VPA (500 mg/kg)
No. of fetuses with axial skeletal defects (%)	0/64	1/76 (1.3%)	31/88 (35.2%)#	51/95 (53.7%)**	67/100 (67.0%)*	83/109 (76.1%)*
Cervical vertebrae						
Fused vertebrae	0	0	2/88	5/95	9/100	12/109
Hemivertebra	0	0	1/88	4/95	11/100	7/109
Misshapen vertebra	0	0	1/88	2/95	6/100	5/109
Thoracic vertebrae						
Misshapen vertebra	0	0	15/88	23/95	23/100	17/109
Vertebral hypoplasia	0	0	2/88	4/95	1/100	3/109
Hemivertebra	0	0	2/88	14/95	12/100	18/109
Bipartite vertebra	0	0	2/88	3/95	6/100	12/109
Fused vertebrae	0	1/76	2/88	9/95	8/100	13/109
Fused centrum	0	0	1/88	2/95	3/100	2/109
Misshapen ossification of centrum	0	1/76	11/88	16/95	14/100	23/109
Bipartite ossification of centrum	0	0	4/88	5/95	10/100	14/109
Dumbbell ossification of centrum	0	0	2/88	8/95	8/100	8/109
Lumbar vertebrae						
Vertebral agenesis	0	0	1/88	2/95	12/100	7/109
Misshapen vertebra	0	0	1/88	5/95	1/100	0
Hemivertebra	0	0	2/88	4/95	1/100	1/109
Fused vertebrae	0	0	0	1/95	0	0
Misshapen ossification of centrum	0	0	0	3/95	0	0
Bipartite ossification of centrum	0	0	1/88	1/95	0	0
Dumbbell ossification of centrum	0	0	0	2/95	1/100	1/109
Sacralization of the 6th lumbar vertebra§	0	0	2/88	2/95	0	2/109
Ribs						
Wavy	0	0	0	0	2/100	0
Fused	0	1/76	19/88	37/95	40/100	80/109
Branched	0	0	3/88	5/95	9/100	21/109
Bifurcated	0	0	1/88	3/95	4/100	5/109
Lumbar rib (14) §	4/64	12/76	40/88	47/95	44/100	33/109
Supernumerary thoracolumbar short (13+1)§	3/64	2/76	4/88	1/95	2/100	1/109
Caudal vertebra						
Fused vertebrae	0	0	0	1/95	0	0
Short tail	0	0	0	1/95	0	0

†Injected intraperitoneally on gestation day 8 at 20 mg/kg; ‡injected intraperitoneally on gestation day 8 (30 min after L-NAME); *statistically significant ($p<0.05$) *vs*. control group; **statistically significant ($p<0.05$) *vs*. VPA 400 mg/kg group and control group; §variation.

lifetime of cGMP, alleviated methylene blue-mediated teratogenesis, supporting the etiological role of NO signaling disruption. With specific reference to the role of NO in neural tube development, studies with the chick embryo have shown NO is detectable in the neural tube at time of neurulation, and is important in the morphogenesis and differentiation of the neural tube system (23-26). Moreover, NO was found to control the balance between mitosis and programmed cell death in the developing neural tube (27).

The present study was stimulated by the evidence that VPA has the capacity to interfere with the NO pathway. VPA inhibited enodothelial NOS expression in cell cultures (8), and this capacity was also shared by other HDAC inhibitors, including trichostatin A, butyric acid, and MS-275 (28). Decreased expression of endothelial NOS accounted for the antiangiogenic effects mediated by VPA in human umbilical vein endothelial cells, as the antiangiogenic response was fully normalized in endothelial cells by co-exposure with an

NO donor or a cGMP analog (8). Rossig *et al*. (28) proposed that HDACs exert effects on NOS through the induction of an endothelial NOS mRNA- destabilizing protein which causes a decrease of endothelial *NOS* mRNA, thereby leading to the decline in its protein. From a teratological perspective, it is relevant that HDACs share the capacity to inhibit enodothelial NOS expression, given that increased histone acetylation/deacetylation has been linked to VPA-induced teratogenesis (29, 30), and that analogs of VPA displaying HDACs are teratogenic, whereas VPA analogs that do not have HDAC activity are devoid of teratogenic effects (30, 31). It is noteworthy that the inhibitory effects elicited by VPA are not limited to the endothelial NOS, but also extend to inducible NOS. Guo *et al*., reported VPA to significantly reduce nitrite, inducible NOS protein and mRNA of inducible NOS in RAW 264.7 murine macrophages (9).

Mechanisms and etiological factors behind VPA teratogenicity have been extensively investigated, but are still undefined (3). The potential role of NO deprivation in VPA-initiated teratogenic effects has not been investigated. The need for exploring this line of research is suggested by the following facts: VPA, like other HDACs, has the capacity for inhibiting NOS expression *in vitro* (8, 9); proper levels of NO are required for normal development, and NOS inhibitors have been shown to be able to induce teratogenic effects in various experimental models (10, 11); when administered during early organogenesis, a NOS inhibitor causes a teratogenic effect similar to that induced by VPA (12); as firstly-reported in the present study, the teratogenic effects of VPA are enhanced by pre-treatment with a non-teratogenic dose of the NOS inhibitor L-NAME.

Conflicts of Interest

None declared.

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