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Inhibition of Thalidomide Teratogenicity by Acetylsalicylic Acid: Evidence for Prostaglandin H Synthase-Catalyzed Bioactivation of Thalidomide to a Teratogenic Reactive Intermediate[1]

RICHARD R. ARLEN and PETER G. WELLS

Faculty of Pharmacy (R.R.A., P.G.W.) and Department of Pharmacology (P.G.W.), University of Toronto, Toronto, Canada

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ABSTRACT

Thalidomide is a teratogenic sedative-hypnotic drug that is structurally similar to phenytoin, which is thought to be bioactivated by prostaglandin H synthase (PHS) and other peroxidases to a teratogenic reactive intermediate. The relevance of this mechanism to thalidomide teratogenicity was evaluated in pregnant New Zealand White rabbits treated with thalidomide at 11:00 A.M. on gestational days 8 to 11, with day 0 indicating the time when sperm were observed in the vaginal fluid. Thalidomide (7.5 mg/kg i.v.) produced mainly fetal limb anomalies analogous to those observed in humans. Thalidomide (25–200 mg/kg i.p.), produced a dose-related increase in a spectrum of fetal anomalies, and in postpartum lethality, but did not produce a reliable incidence of limb anomalies. In subsequent studies, pregnant does received the irreversible PHS inhibitor acetylsalicylic acid (ASA), 75 mg/kg i.p., or its vehicle, followed 2 hr later by thalidomide, 7.5 mg/kg i.v., or its vehicle. ASA pretreatment was remarkably embryoprotective, resulting in respective 61.2 and 61.4% decreases in thalidomide-initiated fetal limb anomalies ($P = .002$) and postpartum fetal lethality ($P < .02$), and a small but significant reduction in thalidomide-initiated fetal weight loss. ASA alone did not produce significant embryopathy. These results show that ASA can protect the embryo from thalidomide teratogenicity, suggesting that thalidomide may be bioactivated by PHS to a teratogenic reactive intermediate.

Thalidomide, [(±)-α-phthalimidoglutarimide], a sedative-hypnotic drug with inherent anti-inflammatory properties, was independently identified as a human teratogen by McBride (1961) in Australia and Lenz (1961, 1962) in Germany. These observations followed an alarming increase in the incidence of rare, severe limb anomalies in infants exposed to the drug *in utero* between the 23rd and 38th days of pregnancy (Lenz and Knapp, 1962). The predominant malformations characteristic of the human syndrome were amelia and phocomelia of varying severity, although a host of internal defects and deficits have been documented (Smithells, 1973; Ruffing, 1977). Because of its potent toxicity to the developing embryo, in the absence of maternal toxicity, thalidomide has become known as the prototypical human teratogen.

Subsequent studies have confirmed the teratogenicity of thalidomide in a number of animal species (Woolam, 1962; King and Kendrick, 1962; Somers, 1962; Ehmann, 1963; Delahunt *et al.*, 1965), although susceptibility and the nature of anomalies are highly species-dependent. Mice and rats are relatively resistant, exhibiting primarily increased embryolethality along with some minor internal deficits. The rabbit, next to primates, is now commonly accepted as being the most sensitive laboratory model of thalidomide teratogenicity, exhibiting fetal anomalies more similar to the human syndrome in terms of type and frequency of malformation. Rather than amelia and phocomelia observed in humans, offspring born to thalidomide-treated rabbit does exhibit contractures and clubbing of the fore and hind limbs (Sawin *et al.*, 1965; Drobeck *et al.*, 1965), as well as anomalies of the alimentary tract, kidney and heart (Larsen and Bredahl, 1966; Vickers, 1966; Jonsson, 1972a).

Although numerous hypotheses have been proposed to explain the teratogenic effects of thalidomide, the biochemical mechanism underlying its embryotoxic response in humans and laboratory animals remains obscure and controversial (McBride, 1979; Helm *et al.*, 1981; Stephens, 1988). The negligible solubility and inherent instability of thalidomide in biological media further complicate the interpretation of mechanisms in both *in vitro* and *in vivo* studies (Schumacher *et al.*, 1965). Current hypotheses include the involvement of

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ABBREVIATIONS: PHS, prostaglandin H synthase; PG, prostaglandin; GSH, glutathione; LPO, lipoxygenase; ASA, acetylsalicylic acid (aspirin); DMSO, dimethylsulfoxide; Pg, propylene glycol.

an enzymatically generated, toxic reactive intermediary metabolite of thalidomide or one of its hydrolysis products. Interspecies differences in the elimination or bioactivation of thalidomide or its hydrolysis products, or the detoxification of a reactive intermediate, might explain differences in species sensitivity to thalidomide teratogenicity. Gordon *et al.* (1981) proposed cytochromes P450-catalyzed oxidation of thalidomide to an electrophilic arene oxide intermediate that covalently binds to embryonic macromolecules, thereby disrupting embryonic growth and differentiation. Arene oxides have been implicated as the proximate teratogens for other embryotoxic drugs such as phenytoin (Martz *et al.*, 1977; Harbison *et al.*, 1977). *In vitro* studies have reported the cytochromes P450-catalyzed formation of a thalidomide metabolite that inhibited cellular attachment to concanavalin A-coated disks (Braun and Weinreb, 1984, 1985; Braun *et al.*, 1986). Cellular adhesion is considered to be an essential process for normal embryogenesis. The toxic intermediate presumably was generated from parent thalidomide, because hydrolysis products had no effect in their assay system (Braun and Weinreb, 1985). Intercalation of thalidomide between embryonic DNA base pairs with subsequent depurination of nucleic acids (Jonsson, 1972b), or a combined arene oxide-intercalation hypothesis (Koch and Czejka, 1986), also have been proposed. These studies provide evidence for the interaction of thalidomide, or thalidomide-generated reactive intermediates, with embryonic DNA, RNA or proteins, which would be expected to have teratological consequences. Moreover, generation of a thalidomide reactive intermediate *in vitro* seems to require the addition of an exogenous drug-metabolizing enzyme system (Gordon *et al.*, 1981; Braun and Weinreb, 1984) or prior induction of an endogenous enzyme (Neubert and Krowke, 1983). The exogenous enzyme source may be either microsomes (Gordon *et al.*, 1981; Braun and Weinreb, 1984) or 9000 $\times$ *g* supernatant (S-9) fraction (Shepard and Shiota, 1983) from the livers of rats pretreated with cytochromes P450 inducers.

The basis for a reactive intermediate hypothesis is the disruption of normal embryogenesis by interaction (covalent binding and/or intercalation) of the intermediate with macromolecules essential for embryonic replication and differentiation. *In vivo* experiments with tritiated thalidomide have shown that radioactive products do become bound to embryonic cellular macromolecules (Schumacher *et al.*, 1968b). More radioactive thalidomide products are bound to the plasma proteins and liver of rabbits (a teratologically sensitive species) than rats (insensitive species) (Schumacher *et al.*, 1968b). Bakay and Nyhan (1968) demonstrated that thalidomide metabolites bind specifically to acidic proteins in the rat fetus, implying a role for involvement of reactive species in thalidomide embryopathy. Covalent binding of [^{14}C]thalidomide to protein in homogenates of day 11 murine embryos occurs only after pretreatment of the dams with the cytochromes P-450 inducer rifampin (Neubert and Krowke, 1983). Intercalative binding of thalidomide into DNA, but not RNA, that may require concomitant metabolic activation also has been reported (Koch and Czejka, 1986).

An alternative bioactivating system that may have a role in the teratogenicity of thalidomide is the PHS pathway, which is known to oxidize a wide range of drugs to toxic free radicals (Marnett, 1990). PHS is a bifunctional enzyme with both cyclooxygenase and hydroperoxidase activities, which catalyze respectively the formation of the hydroperoxy endoperoxide PGG_2 from arachidonic acid, and the subsequent reduction of PGG_2 to the hydroxy endoperoxide PGH_2. PGH_2 in turn is a precursor of other biologically important PGs, thromboxanes and prostacyclin (Samuelsson *et al.*, 1978). It is the hydroperoxidase component of the enzyme that catalyses the oxidation of xenobiotics. For the conversion of PGG_2 to PGH_2, PG hydroperoxidase has a requirement for reducing equivalents, with the physiological reducing cofactor most likely being GSH (Eling *et al.*, 1986). Drugs and/or xenobiotics may also act as reducing equivalents for the hydroperoxidase during PGG_2 reduction, thereby undergoing oxidation to highly reactive and potentially toxic free radical intermediates (Marnett, 1990). Substrate-derived free radical or electrophilic reactive intermediates generated in this fashion may initiate oxidative stress evidenced by the oxidation of DNA, protein and lipid, and/or bind covalently to such molecular targets, leading to altered function or premature cellular death, with subsequent teratologic consequences (Winn and Wells, 1995a; Wells and Winn, 1996; Wells *et al.*, 1996).

A hypothesis involving PHS as a teratologically relevant bioactivating system is especially appealing in terms of *in vivo* chemical embryotoxicity, because rodent embryos have low or negligible activities of most cytochromes P450 (Juchau, 1981; Juchau *et al.*, 1992), but high content and activity of PHS (Mitchell *et al.*, 1985; Wells and Winn, 1996). Furthermore, if a reactive intermediate is involved in thalidomide teratogenesis, then embryonic rather than maternal bioactivation may be the critical teratological determinant. Injection of thalidomide directly into the developing embryo produced malformations (Jonsson, 1972b), and thalidomide inhibits chondrogenic differentiation in tissue culture experiments with human embryonic mesonephrons (Lash and Saxen, 1971). There is *in vivo* and *in vitro* evidence supporting embryonic chemical bioactivation by PHS, LPOs and other peroxidases to free radical reactive intermediates, and reactive oxygen species-dependent oxidation of DNA, protein and lipid, as a teratologic mechanism for anticonvulsant drugs such as the hydantoin anticonvulsants phenytoin, mephenytoin and nirvanol; the structurally related oxazolidinedione anticonvulsant trimethadione and its active, N-demethylated metabolite dimethadione (fig. 1); and the environmental polycyclic aromatic hydrocarbon benzo[*a*]pyrene (reviewed in: Liu and Wells, 1994, 1995a,b; Winn and Wells, 1995a,b; Yu and Wells, 1995; Wells and Winn, 1996; Kim and Wells, 1996).

Thalidomide shares structural features common to the teratogenic hydantoin and oxazolidinedione anticonvulsants (fig. 1), thereby making the oxidation of thalidomide and/or its metabolites by PHS hydroperoxidase to a teratogenic-free radical intermediate an appealing hypothesis (fig 2). The glutarimide nitrogen and adjacent carbonyl groups of the thalidomide moiety are identical to that found on the hydantoin and oxazolidinedione rings and, like mephenytoin and trimethadione, the thalidomide analogue N-methylthalidomide may be N-demethylated *in vivo* to give the penultimate teratogenic precursor, thalidomide (fig. 1). In support of this hypothesis, thalidomide: 1) is bioactivated by purified PHS to a free radical intermediate (Parman *et al.*, 1996); 2) initiates horseradish peroxidase-dependent DNA oxidation *in vitro* (Liu and Wells, 1995b); and 3) initiates GSH oxidation *in vivo* (Arlen and

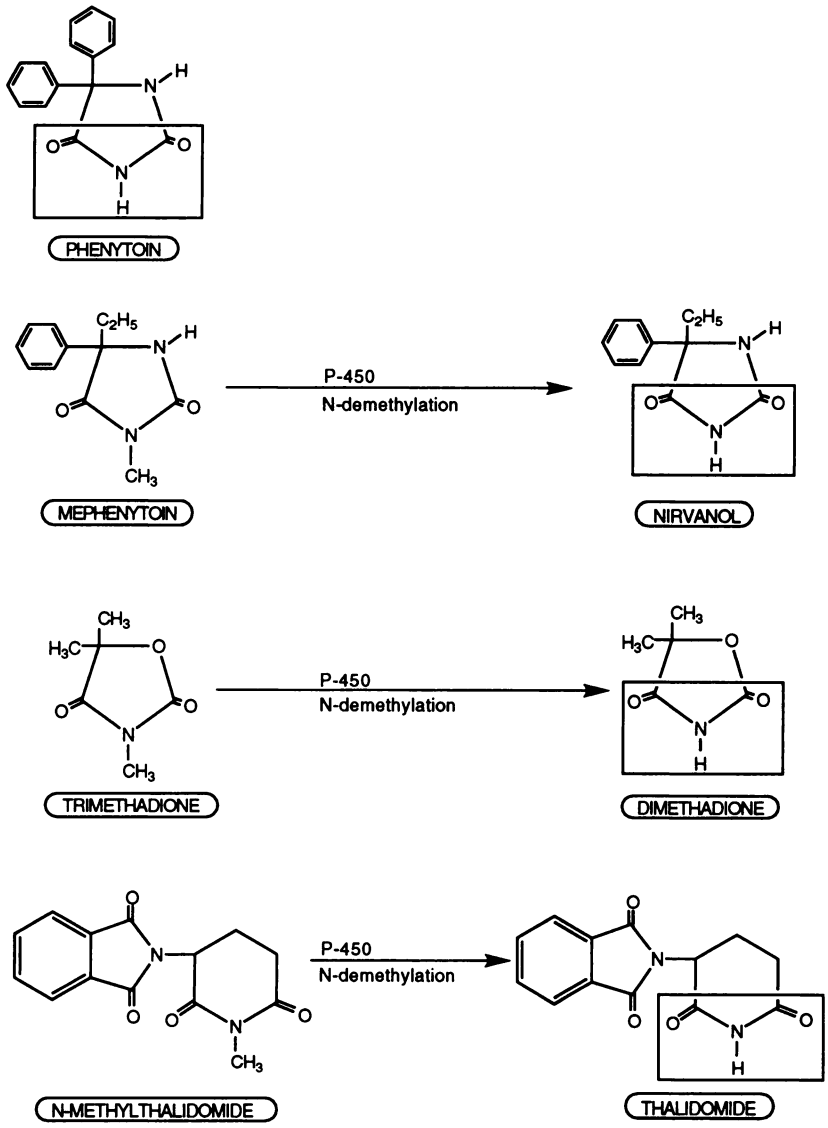

Fig. 1. Thalidomide and structurally related xenobiotics. The relative teratologic properties of these xenobiotics in mice is known (Liu and Wells, 1995b), and the methylated parent compounds are less embryopathic than their N-demethylated metabolites.

Wells, 1990). If thalidomide does act as a cofactor for PG hydroperoxidase-mediated reduction, and is concomitantly bioactivated to an embryotoxic reactive intermediate involved in its teratologic mechanism, then inhibition of this pathway *in vivo* would be expected to decrease the incidence and/or severity of thalidomide-induced limb defects in offspring from thalidomide-treated pregnant rabbit does. ASA is an irreversible inhibitor of the cyclooxygenase component of PHS. The mode of its inhibition is likely due to the irreversible acetylation of serine residues at the catalytic site of the enzyme (Roth and Siok, 1978). Hence, new enzyme is required for the restoration of base-line levels of PG synthesis, a process that may take several days (Flower *et al.*, 1985). ASA and/or eicosatetraynoic acid, a dual PHS/LPO inhibitor, have been shown in mice or with purified PHS to inhibit the formation of xenobiotic free radical intermediates, reactive oxygen species, molecular target oxidation and embryopathy initiated by phenytoin and related compounds *in vitro*, in embryo culture and/or *in vivo* (Winn and Wells, 1995a; Wells and Winn, 1996; Kim and Wells, 1996; Parman *et al.*, 1996). Therefore, if PHS-catalyzed bioactivation of thalidomide is involved in its teratogenic mechanism *in vivo*, ASA pretreatment should reduce thalidomide-induced anomalies and embryotoxicity.

Traditionally, teratologic studies of thalidomide have used p.o. therapy in an attempt to duplicate human exposure. This route has demonstrated experimental difficulties, such as substantial interindividual variability in drug bioavailability and toxicity, when undertaking toxicologic or pharmacokinetic studies of a mechanistic nature (de Morais and Wells, 1988, 1989). Due to limited data on alternative methods of administration of thalidomide to pregnant rabbit does, dose-response studies were conducted using the i.p. route of administration to determine the optimal conditions for *in vivo* mechanistic studies of thalidomide embryotoxicity. Based upon these studies, ASA was evaluated for a possible embryoprotective effect in reducing the embryotoxicity of thalidomide in the pregnant New Zealand White rabbit. These results provide the first *in vivo* evidence that the embryopathic effects of thalidomide may be due at least in part to its bioactivation by PHS to a toxic reactive intermediate.

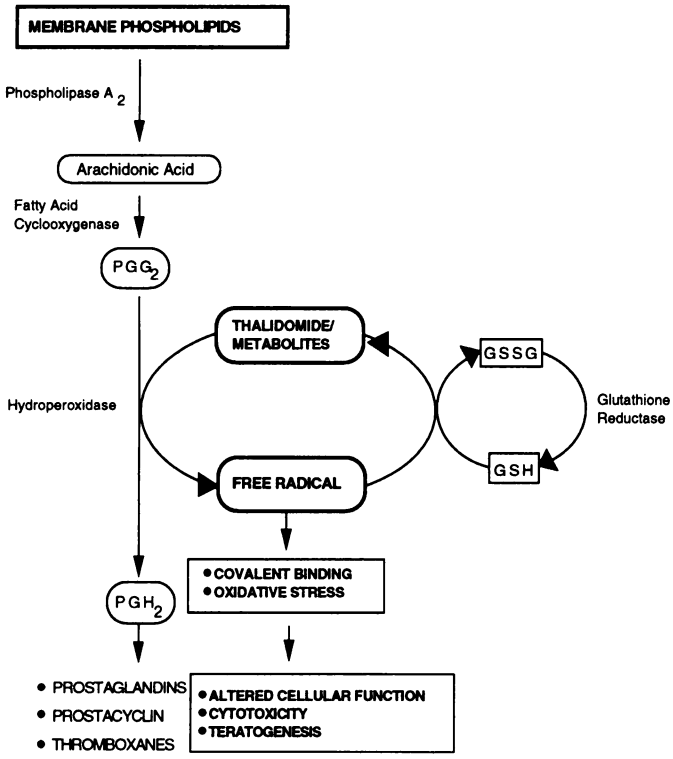

Fig. 2. Putative embryonic peroxidase-catalyzed bioactivation of thalidomide to a teratogenic reactive intermediate. Cyclooxygenase and hydroperoxidase are the two components of PHS. GSSG, oxidized GSH.

Methods

Animal breeding. Virgin female New Zealand White rabbit does, approximately 20 weeks of age and 4 kg, and sexually mature males (Maple Lane Farms, Clifford, Ontario, Canada) were housed individually in wire-bottomed stainless-steel cages with food (Rabbit Chow, Ralston Purina, St. Louis, MO) and tap water available *ad libitum*. Animals were kept in a temperature-controlled room with a 12-hr day/night rhythm automatically maintained. In preliminary studies, after a minimum 1 week accommodation period, between 9:00 to 11:00 A.M., each doe was taken to the cage of a male, allowed to copulate and removed immediately thereafter. Successful pregnancies were ascertained by the presence of sperm in vaginal fluids, and this day was designated as day 0 of pregnancy. Due to limited success with breeding in our animal facility, timed-pregnant does subsequently were purchased from the supplier who was instructed to breed according to the protocol laid out above. Pregnant does were shipped on day 3 of gestation, corresponding to approximately 2 to 3 days before implantation (Hartman, 1974). No significant differences in mean fetal body weight, postpartum death, implantation rate or resorption incidence were detected between the two procedures for obtaining pregnant rabbits.

Drug treatments. Pregnant does received all treatments with thalidomide [(±)-α-phthalimidoglutarimide] (Chemie Grunenthal and Professor D. Neubert, Berlin, Germany) or its vehicle at 11:00 A.M. on gestational days 8 to 11 inclusive. This period in gestation corresponds to heart formation, anterior neuropore formation and closure, appearance of the branchial arches, as well as formation and division of the fore and hind limb (Hartman, 1974). For the i.p. dose-response studies, racemic thalidomide (25–200 mg/kg) was administered as a fine suspension in a corn oil vehicle (3 ml/kg). Thalidomide for i.v. therapy was dissolved in a DMSO/Pg vehicle (50/50 v/v) and was administered at a known teratogenic dose of 7.5 mg/kg (Schumacher *et al.*, 1968a). ASA (Sigma Chemical Co., St. Louis, MO) was dissolved in a 0.24 M sodium bicarbonate buffer to give a final pH of 7.0. ASA and i.v. thalidomide were dissolved in vehicular volumes of 20 and 7.5 mg/ml, respectively, each solution being prepared immediately before use. For the i.v. studies involving pretreatment with the cyclooxygenase inhibitor ASA, pregnant does received a two-dose regimen on days 8 to 11 inclusive, consisting of ASA (75 mg/kg) or ASA vehicle (pH adjusted to 7.0–7.2) i.p. 2 hr before i.v. infusion of thalidomide or its vehicle. The dose of ASA was chosen as the highest dose that was likely to have minimal embryopathic effects in rabbits (McColl *et al.*, 1967). Thalidomide solutions were administered *via* an i.v. catheter (Angiocath, 24-gauge 3/4", Canlab, Mississauga, Ontario, Canada) inserted in an ear vein. Solutions were infused at a flow rate of 0.4 ml/min with the use of an i.v. syringe pump (model 351, Sage Instruments, White, Plaines, NY) and subsequently rinsed with 1 volume of normal saline to inhibit DMSO-induced fibrinogen precipitation (Rubin, 1983). Adverse affects of DMSO administration were minimized by alternating selection of ear veins over the 4-day treatment period.

Toxicological assessment. In all studies, does were sacrificed by cervical dislocation on gestational day 29, corresponding to 1 to 4 days before spontaneous delivery (Hartman, 1974). After laparotomy, the uterus was exteriorized and the number and location of live and dead fetuses and resorptions (*in utero* deaths) were noted. For the purposes of statistical evaluation, no attempt was made to distinguish between *in utero* resorptions and those fetuses delivered dead. Live fetuses were evaluated for 2-hr postpartum survival as a determinant of fetal viability, euthanized by i.p. injection of 0.2 ml of T-61 solution (Hoechst, Montreal, Canada), weighed and fixed in Carnoy's solution for at least 7 days before teratological assessment. Fetuses were examined for characteristic thalidomide-induced limb anomalies (dysmelia or arthyrogryposis), digital anomalies (polydactyly or syndactyly), cleft lip, spina bifida, microcephaly and umbilical herniation. Internal examination involved assessment for cleft palate and renal defects (unilateral/bilateral renal agenesis, fused and ectopic kidneys and absent or shortened ureters). To facilitate internal examination, a transverse incision was first made between the upper and lower jaw of each fetus, and the palate was examined for complete rotation and fusion of the palatal shelves. A further incision was made cephalad to the umbilicus to just above the adrenal glands. The liver and intestines were removed and discarded and a final cut was made through the pelvises of both kidneys, at which time the kidneys were evaluated for the presence or absence of renal anomalies (Manson *et al.*, 1982). Other anomalies not specifically named in the protocol were recorded and included in the statistical analyses. The incidence of resorptions was calculated as the total number of *in utero* deaths divided by the total number of implantations.

Statistical analysis. In all studies, comparisons of differences between treatment groups was undertaken using a statistical software program for microcomputers (SAS Institute, Cary, NC). Statistical significance of differences between treatment groups for continuous data was determined using a one-way analysis of variance procedure followed by Tukey's range test as a *post hoc* test for determination of the location of differences. Chi-square analysis was used to determine differences for binomial data, whereas the Kruskal-Wallis analysis of ranks was utilized to determine whether differences existed between groups of ranked data. Relationships between two dependent variables were analyzed by calculation of Pearson's correlation coefficient. A probability of $P < .05$ was chosen as the minimal level of significance for all statistical tests.

Results

Toxicological assessment. Pregnant does receiving i.v. thalidomide immediately exhibited symptoms of sedation regardless of the pretreatment. This effect was not as pronounced with the does receiving i.p. thalidomide, likely reflecting delayed absorption of the drug. Red cell hemolysis

and resulting hemoglobinuria was observed in all does receiving i.v. treatments. These observations are consistent with DMSO-induced injury to erythrocytes and the underlying vascular epithelium at concentrations of 50% or greater (Rubin, 1983), as was used in these studies. DMSO-induced vascular injury was extensive enough in two does that they were excluded from the study after development of ear infections. Litters from both of these does were resorbed completely after treatment with topical antibiotics (chloramphenicol, neomycin and chlorhexidine) and an anti-inflammatory agent (hydrocortisone).

Studies using i.p. administration of thalidomide to pregnant does revealed a dose-dependant increase in pooled anomalies in the offspring of does treated with thalidomide (fig. 3). At the highest dose (200 mg/kg), a large increase in postpartum death was observed, indicating thalidomide-initiated embryopathy that was not detectable by structural evaluation. Thalidomide at any dose had no significant effect on *in utero* resorption incidence. Fetal body weight was not reduced by increasing doses of thalidomide in comparison to the lowest dose, although weights with the mid-range doses were higher. However, although i.p. thalidomide produced a dose-related increase in total malformations (fig. 3), the incidence of limb anomalies particularly, the sine qua non of thalidomide embryopathy, was insufficient (fig. 4) for definitive mechanistic studies. Therefore, subsequent studies used the i.v. route of administration, which has been characterized in the literature (Schumacher *et al.*, 1968a), and in our hands resulted in a reproducibly significant incidence of limb anomalies (fig. 4).

A comparison of the two routes of administration revealed that i.v. thalidomide is a potent teratogen in terms of limb anomalies ($P < .0001$) (fig. 5), whereas only 3 fetuses of 66 exhibited some type of renal anomaly (table 1). Conversely, i.p. thalidomide exposure gave rise to litters with a larger spectrum of defects and deficits; primarily renal anomalies and deficits in tissues and organs other than the limbs (table 1).

ASA pretreatment of does receiving i.v. thalidomide therapy resulted in a 61.2% decrease in thalidomide-induced anomalies when calculated as the percentage of fetuses affected ($P = .002$) (fig. 5). Interestingly, when the number of thalidomide-initiated anomalies for each fetus was determined, ASA also produced a 78% decrease in this parameter ($P = .0001$) (data not shown). In either case, the decrease was

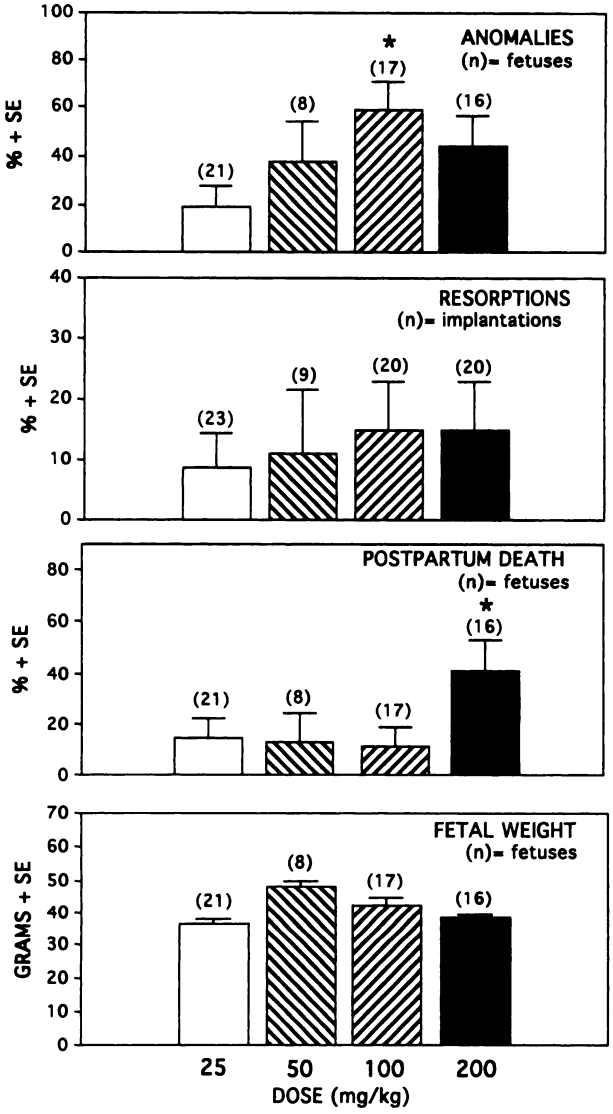

Fig. 3. Effect of dose upon the teratogenicity of i.p. administered thalidomide. Thalidomide, 25 to 200 mg/kg ip, was given at 11:00 A.M. on gestational days 8 to 11. The number (*n*) of fetuses or implantations is given in parentheses. *A difference from the lowest dose group ($P < .05$).

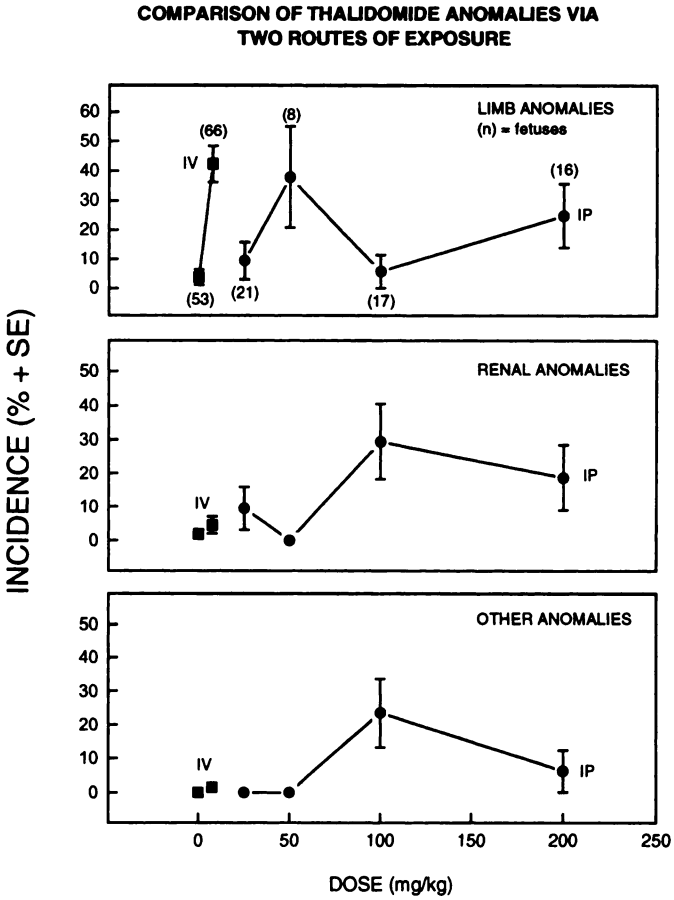

Fig. 4. Comparison of thalidomide teratogenicity *via* i.p. and i.v. administration. Thalidomide, 25 to 200 mg/kg i.p. or 0 to 7.5 mg/kg i.v., was given at 11:00 A.M. on gestational days 8 to 11.

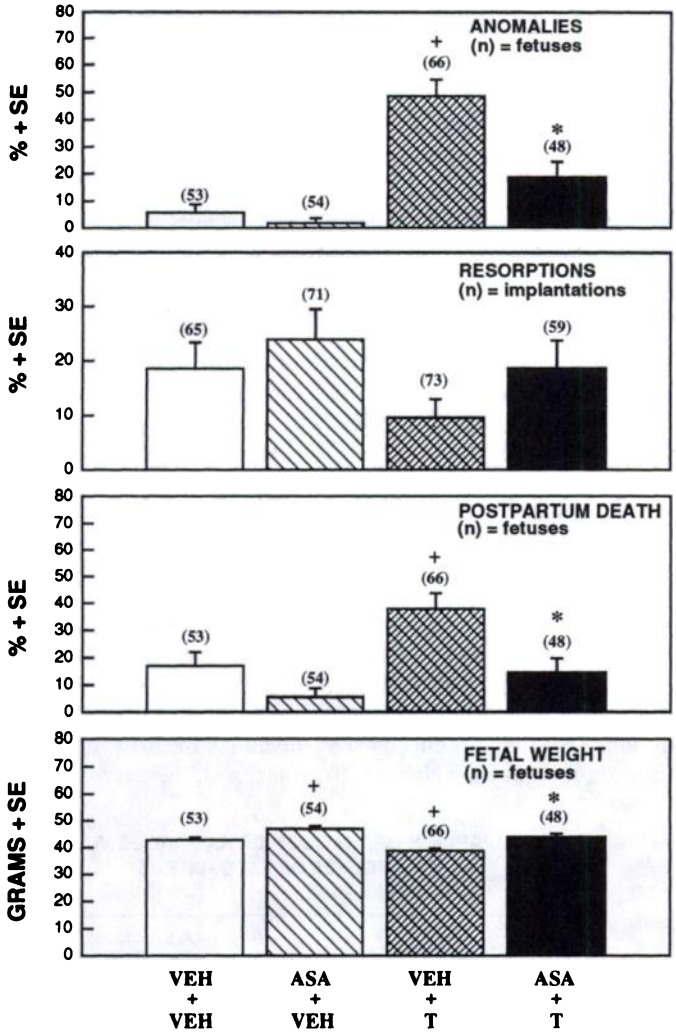

Fig. 5. Effect of pretreatment with ASA upon thalidomide (T) teratogenicity. ASA, 200 mg/kg i.p., was given at 9:00 A.M. on gestational days 8 to 11, 2 hr before T, 7.5 mg/kg i.v. The number (*n*) of fetuses or implantations is given in parentheses. *Differences from groups treated with T; + differences from vehicle (VEH) controls ($P < .05$).

generally manifested in a reduction in limb anomalies (characterized by contractures of the fore and hind limbs), although one digital defect and three renal anomalies occurred in the group receiving only thalidomide that were not observed in the thalidomide group pretreated with ASA (table 1). Although fetal defects did occur in the two groups receiving ASA, ASA alone did not cause limb anomalies. One case of umbilical herniation was observed. One fetus with a cleft palate and one with microcephaly were observed in the group receiving both ASA and thalidomide. Three fetuses receiving the vehicles only for both thalidomide and ASA demonstrated anomalies (table 1). One of these fetuses exhibited unilateral renal agenesis, whereas the other two, both dead fetuses and from the same litter, had mild contractures of the forepaws (table 1).

ASA pretreatment also produced a significant 61.4% decrease in thalidomide-initiated postpartum fetal death ($P < .02$) when compared to the group receiving thalidomide alone (fig. 5). There were no significant differences between the ASA-pretreated thalidomide group and the controls. Compared to vehicular controls, ASA alone produced a small but significant enhancement in fetal body weight, and appeared to reduce postpartum lethality by 67%, although the latter effect was not statistically significant ($P = .06$).

Thalidomide produced a small but significant decrease in fetal body weight, and this effect was prevented by pretreatment with ASA (fig. 5). There was no difference in fetal body weight between the ASA-pretreated thalidomide group and the group receiving both vehicles. The fetal weight of pups from does receiving ASA alone was significantly higher than those of vehicle-treated does. This again implies a possible ASA protective effect on vehicular toxicity. No significant differences in resorption incidence existed among the four treatment groups (fig. 5). The apparent but nonsignificant elevation in *in utero* deaths in the group receiving ASA alone was due to one doe in which all 10 implants were resorbed.

Discussion

The embryopathic effects of thalidomide were inhibited by pretreatment of pregnant does with the cyclooxygenase inhibitor ASA, as evidenced by significant decreases in thalidomide-initiated limb anomalies, postpartum lethality and fetal body weight loss, when compared with animals treated with thalidomide alone. Thalidomide treatment at the 7.5-mg/kg dose did not cause resorptions, contrary to one previous study (Schumacher *et al.*, 1968a). The inhibition of the teratologic and embryopathic effects of thalidomide by ASA is the first *in vivo* evidence of a possible role for PHS-catalyzed bioactivation of thalidomide. A similar embryoprotection by peroxidase inhibitors such as ASA and eicosatetraynoic acid has been reported previously for the structurally related teratogens phenytoin (Wells *et al.*, 1989a; Miranda *et al.*, 1994; Yu and Wells, 1995) and trimethadione and dimethadione (Wells *et al.*, 1989b). According to this hypothesis, irreversible inhibition of PHS by ASA inhibited the hydroperoxidase-catalyzed cooxidation of thalidomide to a reactive-free radical intermediate, thereby protecting the integrity of essential embryonic macromolecules from toxic effects of oxidative stress and/or covalent binding. This hypothesis is consistent with the observed thalidomide-initiated oxidation of glutathione *in vivo* (Arlen and Wells, 1990) and DNA *in vitro*, the latter of which involved a peroxidase-dependent bioactivating system (Liu and Wells, 1995b). Furthermore, GSH oxidation was greater in a species that was sensitive (rabbit) to thalidomide embryopathy compared to a resistant species (rat) (Arlen and Wells, 1990). Finally, *in vitro* PHS-catalyzed bioactivation of thalidomide and formation of a free-radical intermediate has been demonstrated (Parman *et al.*, 1996), as discussed below. More broadly, putative PHS-catalyzed thalidomide bioactivation and reactive oxygen species-dependent teratogenic initiation is consistent with evidence of a similar molecular mechanism underlying the teratogenicity of phenytoin and related xenobiotics. *In vivo*, in embryo culture and/or in *in vitro* preparations of purified enzymes, some or all of these xenobiotics have been found to be bioactivated by embryonic peroxidases (Kubow and Wells, 1989; Miranda *et al.*, 1994; Yu and Wells, 1995) to reactive intermediates that covalently bind to protein (Kubow and Wells, 1989; Yu and Wells, 1995) and DNA (Liu and Wells, 1994b), or initiate the formation of reactive oxygen species (Winn and Wells, 1995b; Kim and Wells, 1996). We have found further evidence of xenobiotic-initiated, reactive oxy-

TABLE 1

Effect of route of administration on the teratogenicity of thalidomide

Thalidomide was administered i.v. or i.p. to pregnant New Zealand White rabbits on gestational days 8 to 11. Abbreviations: RF, right forelimb; LF, left forelimb; RH, right hindlimb; LH, left hindlimb; DIG, digital anomalies; CP, cleft palate; CL, cleft lip; SB, spina bifida; M, microcephaly; UH, umbilical hernia. Vehicle refers to thalidomide vehicle DMSO/Pg.

Thalidomide Treatment	Implants (*n*)	Fetuses (*n*)	Anomalies *n* (%)									
			Limbs				Others					
			RF	LF	RH	LH	DIG	Renal	CP/CL	SB	M	UH
i.p.												
25 mg/kg	23	21	1 (4.8)	1 (4.8)	0	0	0	2 (9.5)	0	0	0	0
50 mg/kg	9	8	0	1 (13)	2 (25)	1 (13)	0	0	0	0	0	0
100 mg/kg	20	17	0	0	1 (5.9)	0	1 (5.9)	5 (29)	3 (18)	0	0	0
200 mg/kg	20	16	3 (19)	2 (13)	1 (6.3)	0	0	3 (19)	0	1 (6.3)	0	0
i.v.[a]												
Vehicle	65	53	1 (1.9)	1 (1.9)	0	0	0	1 (1.9)	0	0	0	0
ASA plus vehicle	71	54	0	0	0	0	0	0	0	0	0	1 (1.9)
Thalidomide	73	66	16 (24)	15 (23)	12 (18)	8 (12)	1 (1.5)	3 (4.6)	0	0	0	0
ASA plus thalidomide	59	48	4 (8.3)	0	1 (2.1)	2 (4.2)	0	0	1 (2.1)	0	1 (2.1)	0

[a] ASA, 75 mg/kg i.p.; thalidomide, 7.5 mg/kg i.p.

gen species-dependent oxidation of protein (Liu and Wells, 1994a, 1995a; Wells *et al.*, 1995), DNA (Winn and Wells, 1994, 1995b; Liu and Wells, 1995b) and lipid (Liu and Wells, 1994a, 1995a), one or more of which may constitute critical molecular targets mediating teratogenic initiation. DNA is a particularly likely teratologically relevant target, because transgenic knock-out mice deficient in the p53 tumor suppressor gene, which facilitates DNA repair, are more susceptible to the teratogenicity of the DNA-damaging xenobiotics benzo[*a*]pyrene (Nicol *et al.*, 1995) and phenytoin (Laposa and Wells, 1995; Laposa *et al.*, 1996).

As detailed under the Introductory section, there is substantial evidence of the bioactivation of teratogenic compounds by PHS and related enzyme systems with hydroperoxidase activity. These compounds (fig. 1) share structural features with thalidomide. The glutarimide portion of the thalidomide structure is similar to the hydantoin nucleus of the anticonvulsants phenytoin and nirvanol, and the oxazolidinedione ring of the structurally similar anticonvulsant dimethadione, the teratogenic potential of which may be due at least in part to their oxidation or bioactivation by PHS. Formation of the free radical may be facilitated by an unhindered nitrogen on the hydantoin and oxazolidinedione rings, given the reduced or negligible embryopathy observed with the methylated phenytoin analogs mephenytoin (Wells *et al.*, 1982) and trimethadione (Wells *et al.*, 1989b) (fig. 1) compared to their respective demethylated metabolites nirvanol and dimethadione. Inasmuch as N-methylthalidomide also is less embryopathic than its demethylated product thalidomide (Jonsson, 1972b), this requirement may extend to thalidomide analogs, involving the unhindered imido nitrogen on the glutarimide ring. By using electron spin resonance spectrometry, a secondary free radical has been demonstrated by spin trapping with α-phenyl-N-*t*-butylnitrone after *in vitro* bioactivation of thalidomide by purified PHS (Parman *et al.*, 1996). Because the N-methoxy and N-hydroxy analogs of thalidomide are not teratogenic (Wuest *et al.*, 1968), it has been hypothesized that, in the case of the N-methylated analog of thalidomide (Wuest *et al.*, 1968), as well as for mephenytoin and trimethadione (Wells *et al.*, 1982, 1989b), N-demethylation *in vivo* may be required to produce thalidomide, nirvanol and dimethadione, respectively (fig. 1), which subsequently may be bioactivated to the ultimate teratogenic reactive intermediate (Arlen and Wells, 1990) (fig. 2).

Substantial evidence exists for the involvement of a toxic electrophilic intermediate in the teratogenicity of thalidomide. Early evidence suggested thalidomide covalent binding to both maternal and fetal tissues (Schumacher *et al.*, 1968b; Bakay and Nyhan, 1968). Subsequently, liver microsomes from maternal rabbits (a sensitive species), but not rats (insensitive species), were shown to generate a cytochrome P450-dependent metabolite, postulated to be an arene oxide reactive intermediate, that was toxic to human lymphocytes (Gordon *et al.*, 1981). Although supported by evidence for the presence of phenolic metabolites in the urine of treated rabbits (Schumacher *et al.*, 1965), other investigators have demonstrated that thalidomide analogs with nitro or amino substitution of the phthalimide aromatic ring, which would be expected to block formation of an arene oxide, are still teratogenic (Fabro *et al.*, 1964). Furthermore, the 3- and 4-hydroxy metabolites of thalidomide, when administered to chick embryos, produced malformations similar to that of thalidomide itself (Boylen *et al.*, 1963, 1964). Braun and Weinreb (1984) have demonstrated that thalidomide metabolites generated by canine liver microsomes inhibited attachment of mouse ovarian ascites tumor cells to concanavalin A-coated surfaces. Their metabolite was unaffected by either 1,2-epoxy-3,3,3-trichloropropene or epoxide hydrolase, and therefore probably not an arene oxide. Both sensitive species (rabbits or rhesus monkeys) as well as insensitive species are able to generate a metabolite that inhibits cellular adhesion (Braun and Weinreb, 1984). The results from these two *in vitro* assays suggest that the potential exists for the generation of a reactive intermediate from thalidomide, but the nature of this metabolite, as well as its mechanism of pathogenesis, remains unclear. The inhibitory effects of ASA on thalidomide teratogenicity *in vivo* in our studies suggest that one possible teratologic mechanism could involve PHS-catalyzed oxidation of thalidomide to a toxic free radical intermediate.

ASA alone, as well as its metabolite salicylic acid, may be

marginally teratogenic at the 75-mg/kg dose used in these studies (McColl *et al.*, 1967). The spectrum of defects that have been attributed to ASA may account for the single cases of microcephaly, cleft palate and umbilical hernia observed in groups receiving ASA pretreatment, and in any event, such defects are not commonly associated with thalidomide. ASA alone does not cause the limb anomalies characteristic of the rabbit thalidomide embryopathy. ASA may reduce the toxicity of the DMSO/Pg vehicle, as evidenced by an increase in fetal body weight and apparent reduction in postpartum lethality. Intravenous administration of DMSO is reported to generate methyl radicals (Rubin, 1983), which could initiate oxidative stress in embryonic tissues leading to cell death and subsequent reductions in fetal growth and viability. The mechanism of the apparent protective effect of ASA against vehicular toxicity is speculative, but salicylic acid can trap free radicals *in vivo* (Grootveld and Halliwell, 1986). Indeed, this free radical trapping effect of salicylate may constitute a secondary mechanism of embryoprotection against thalidomide and phenytoin (Kim and Wells, 1996).

Whereas the majority of *in vivo* studies have used the p.o. route of administration for thalidomide, parenteral administration has substantial advantages. It allows, especially in the case of unstable compounds such as thalidomide, for instantaneously and reliably high plasma concentrations of parent compound. Thalidomide undergoes spontaneous hydrolysis to greater than 12 products at physiological temperature and pH (Williams *et al.*, 1965), with a half-life for thalidomide in the order of 2 to 3 hr (Schumacher *et al.*, 1968b). The majority of thalidomide metabolites are strong acids that are highly ionized at physiological pH, and therefore are not readily absorbed through the gastrointestinal tract or through cellular membranes. Therefore, appreciable amounts of thalidomide may be hydrolyzed before absorption after p.o. administration, leading to unpredictable bioavailability. Inasmuch as i.v. administration of thalidomide in a DMSO vehicle can produce red cell hemolysis and fibrinogen precipitation (Rubin, 1983), the i.p. route also was investigated, given that thalidomide is readily absorbed from the peritoneal cavity (Bakay and Nyhan, 1968). In our studies, differences in the spectrum of malformations attributed to thalidomide after i.p. administration as compared to the i.v. route were apparent. Fetuses of does receiving thalidomide i.v. were more prone to limb anomalies than the corresponding treatment group receiving thalidomide i.p. Conversely, renal and other anomalies were more apparent in the i.p. group. Prolonged absorption of the drug in the corn oil vehicle may have resulted in embryonic exposure over a broader range of sensitive periods of organogenesis. Furthermore, the environment of the peritoneal cavity may alter the spectrum of spontaneously formed hydrolysis products. Because it is not known whether thalidomide alone and/or one or more of its hydrolysis products is the penultimate or ultimate teratogen *in vivo*, this observation may be of some pharmacological importance.

Any hypothesis for the biochemical mechanism of thalidomide teratogenicity must take into account three unique aspects of its biological action. These properties are its severe embryotoxicity, while remaining virtually nontoxic to the adult, the stringent relationships between chemical structure and biologic action of its structural analogs and the dramatic differences in species sensitivity to its teratogenic action. Bioactivation of thalidomide by PHS may satisfy these requirements. First, high levels of PHS in the embryo and fetus, with inversely low levels of related detoxifying and cytoprotective enzymes relative to the adult (Winn and Wells, 1995a; Wells and Winn, 1996), may explain the high embrotoxicity and low maternal toxicity of thalidomide. The postulation of a thalidomide-derived free radical formed within the glutarimide ring and requiring an unhindered nitrogen in that ring, rather than the aromatic ring of the phthalimide group, avoids interpretive contradictions associated with the cytochrome P450-arene oxide hypothesis in terms of structure-activity relationships. Initial studies of detoxifying and cytoprotective biochemical pathways, such as glutathione homeostasis, which are essential for providing cellular resistance to potentially harmful reactive intermediates, indicate that the balance between bioactivation and detoxification/cytoprotection is more unfavorable in sensitive species (rabbit) than insensitive species (rat) (Arlen and Wells, 1990). Similarly, these and other cytoprotective pathways providing direct or indirect defense against reactive oxygen species (Winn and Wells, 1995a; Wells and Winn, 1996) may prove to be critical in determining species-dependent and interindividual teratological susceptibility.

In summary, the inhibition of thalidomide teratogenicity by the cyclooxygenase inhibitor ASA provides the first *in vivo* evidence for PHS-catalyzed bioactivation of thalidomide to a teratogenic reactive intermediate. This reactive intermediate may be a free radical that directly or indirectly oxidizes and/or covalently binds to embryonic DNA, protein or lipid, initiating *in utero* death, growth retardation or teratogenicity.

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Send reprint requests to: Dr. Peter G. Wells, Faculty of Pharmacy, University of Toronto, 19 Russell St., Toronto, Ontario, Canada M5S 2S2.