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Antisense Evidence for Nuclear Factor-κB–Dependent Embryopathies Initiated by Phenytoin-Enhanced Oxidative Stress

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ABSTRACT

Endogenous and xenobiotic-enhanced oxidative stress may initiate embryonic death and birth defects via reactive oxygen species (ROS) signaling pathways involving nuclear transcription factor-κB (NF-κB). Using embryo culture and a transgenic mouse engineered with a NF-κB–dependent β-galactosidase reporter gene, we employed NF-κB antisense oligonucleotide therapy to determine whether NF-κB signaling contributes to the embryopathic effects of the ROS-initiating teratogen phenytoin. Phenytoin selectively increased NF-κB activity in target tissues and caused embryopathies, both of which were blocked by NF-κB antisense oligonucleotides but not by sense and nonsense oligonucleotide controls. NF-κB signaling may therefore contribute to the mechanism of ROS-mediated embryopathies.

Although endogenous biochemical changes during pregnancy as well as exposure to numerous drugs and environmental chemicals may cause embryonic death, birth defects, and postnatal functional deficits, the embryopathic mechanisms and risk factors are poorly understood. Recent studies suggest that both endogenous and xenobiotic-enhanced oxidative stress may contribute to such embryopathies via reactive oxygen species (ROS)-initiated damage to cellular macromolecules (DNA, protein, lipid) (Nicol et al., 2000). However, these studies do not preclude an embryopathic contribution via ROS-mediated alterations in signal transduction, which has been implicated for ROS-initiating teratogens, such as phenytoin (Winn and Wells, 2002) and thalidomide (Hansen et al., 2002).

Phenytoin is a widely used anticonvulsant drug that, when taken by women during pregnancy, can double the incidence of both structural and functional birth defects in their children (Kaneko, 1991; Wells et al., 1997a). However, pregnant women with epilepsy are advised to continue taking phenytoin throughout pregnancy to protect themselves and their fetus from the complications of uncontrolled seizures. Because women of childbearing age also may be similarly or inadvertently exposed to other ROS-enhancing conditions or xenobiotics, there is a need to elucidate the embryopathic mechanisms involved in endogenous and xenobiotic-enhanced oxidative stress, and the associated determinants of risk.

Embryonic tissues have substantially lower levels of ROS-detoxifying enzymes than comparable adult tissues, leaving the embryo highly susceptible to ROS-initiating teratogens (Wells et al., 1997a,b). Evidence from our laboratory suggests that ROS-initiating teratogens, including phenytoin, benzo[*a*]pyrene, and thalidomide, can be bioactivated by embryonic prostaglandin H synthase (PHS) to a free radical intermediate that initiates the formation of ROS (Wells et al., 1997b; Winn and Wells, 1997; Parman et al., 1998; Parman and Wells, 2002). Some teratogens may also enhance ROS formation via the redox cycling of catechol metabolites, reperfusion effects of cardioactive drugs, and receptor-mediated mechanisms. If not detoxified by antioxidants and antioxidative enzymes, these ROS can oxidatively damage embryonic cellular macromolecules, which, if not repaired, can initiate embryopathies (Wells et al., 1997a; Parman et al., 1999; Winn and Wells, 1999; Nicol et al., 2000). Because the pathways of ROS formation, detoxification, and macromolecular repair reside within the embryo, therapeutic concentrations

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ABBREVIATIONS: ROS, reactive oxygen species; PHS, prostaglandin H synthase; NF-κB, nuclear factor-κB; GD, gestational day; MRS, male rat serum; PCR, polymerase chain reaction; PB, phosphate buffer; PBS, phosphate-buffered saline; SOD, superoxide dismutase; BSO, buthionine sulfoximine; TGF, transforming growth factor.

of phenytoin that have no adverse effect on the expectant mother may cause embryopathies in embryos with an unfavorable balance among these pathways.

Although there is evidence for the involvement of macromolecular damage in ROS-initiated birth defects, the role of ROS-mediated alterations in signal transduction remains unclear. Inappropriate activation of ROS-signaling molecules can change the activity of transcription factors and alter cellular function, which under some conditions could have embryopathic consequences. We showed previously that phenytoin increases levels of active embryonic Ras, which could contribute to the embryopathic effects of this ROS-initiating teratogen (Winn and Wells, 2002). Ras is a protein that relays extracellular stimuli and activates intracellular signaling pathways influencing cell growth, differentiation, and apoptosis (Campbell et al., 1998). More directly, phenytoin embryotoxicity was reduced by α-hydroxyfarnesylphosphonic acid, an inhibitor of Ras farnesylation, suggesting that Ras activation plays an important role in the embryopathic mechanism (Winn and Wells, 2002).

The nuclear transcription factor-κB (NF-κB) family regulates expression of many genes, some of which have developmental roles, whereas others play important roles in immune and inflammatory responses (Baeuerle and Baltimore, 1996). NF-κB is activated by numerous stimuli and was the first transcription factor found to be responsive to oxidative stress (Nakamura et al., 1997). Several lines of evidence indicate that NF-κB can by activated by ROS: 1) H_2O_2 can activate NF-κB in some cell lines, 2) many NF-κB activators can also increase intracellular ROS, 3) antioxidants can decrease NF-κB activation in response to different stimuli, 4) overexpression of antioxidants can inhibit NF-κB, and 5) inhibition of antioxidants or introduction of oxidants can potentiate NF-κB activation by some stimuli (Li and Karin, 1999; Bowie and O'Neill, 2000). Excessive NF-κB activation is implicated in the pathogenesis of several diseases, including cancer, and is also required for transduction of oncogenic Ras transformations (Finco et al., 1997; Jo et al., 2000). The potentially critical pathogenic role of NF-κB in cancer was revealed by studies involving inhibition of NF-κB, including the use of antisense oligonucleotides, which decreased the growth rate and tumorigenicity of oncogenic Ras transformed cells (Higgins et al., 1993). In the current study, using a transgenic mouse engineered with a NF-κB–dependent β-galactosidase reporter gene, we employed an embryo culture model and NF-κB antisense oligonucleotide therapy to determine the role of NF-κB signaling in the embryopathic effects of the ROS-initiating teratogen phenytoin.

Materials and Methods

Animals. Homozygous (+/+) κB-lacZ females of the B6SJL strain were genetically engineered in the laboratory of Dr. Alain Israel (Unite de Biologie Moleculaire de l'Expression Genique, Institut Pasteur, Paris, France) (Schmidt-Ullrich et al., 1996) and supplied by Dr. Inder M. Verma (Laboratory of Genetics, The Salk Institute, San Diego, CA). Promoter constructs p105 and $(Igk)_3$, containing multiple NF-κB binding sites, were cloned upstream of the *lacZ* bacterial gene and used to generate different lines of transgenic κB-lacZ mice.

In cell culture experiments, both p105lacZ and $(Igk)_3$conalacZ constructs showed low basal NF-κB activity. Both constructs displayed NF-κB activation in response to classic NF-κB activators and cotransfection with a p65 expression vector. Control constructs containing altered κB-dependent promoters did not display activation upon exposure to the same activators (Schmidt-Ullrich et al., 1996).

Most κB-lacZ transgenic mice lines generated containing either p105lacZ or $(Igk)_3$conalacZ constructs showed nearly identical NF-κB expression patterns, although stronger expression was found in the $(Igk)_3$conalacZ lines. This was taken as evidence that the pattern of expression found was in fact a result of κB-driven sites. Negative controls were generated containing mutated constructs (p105mutlacZ and conalacZ), which showed no β-galactosidase activity in all but two lines derived from p105mutlacZ that showed some ectopic expression. For this study, the $(Igk)_3$conalacZ line 252-1 was employed. These homozygous (+/+) females were mated to B6SJLF1/J wild-type males (The Jackson Laboratory, Bar Harbor, ME) to produce F1 κB-lacZ heterozygous (+/−) offspring.

Virgin female and male CD-1 (Charles River Canada Ltd., St. Constant, Quebec, Canada), heterozygous (+/−) κB-lacZ B6SJL male, and wild-type B6SJLF1/J female mice (The Jackson Laboratory) were housed in plastic cages with ground corncob bedding (Beta Chip; Northeastern Products Corporation, Warrensburg, NY). Food (Laboratory Rodent Chow 5001; PMI Feeds Inc., St. Louis, MO) and tap water were provided ad libitum. The animal facility was maintained in a temperature-controlled room with a 12-h/12-h light/dark cycle. During mating, three females were housed with one male. CD-1 mice were mated from 5:00 PM to 9:00 AM, and B6SJLF1/J mice were mated from 7:00 AM to 10:00 AM. The presence of a vaginal plug in a female mouse was indicative of insemination and designated gestation day (GD) 1. Inseminated females were housed together in groups of four animals or less per cage.

Male rat serum (MRS) is required for embryo culture media, because it contains nutrients and other factors necessary for mouse embryonic survival and growth. MRS was obtained from retired Sprague-Dawley rat breeders (Charles River) as described previously (Winn and Wells, 1995).

Chemicals. Phenytoin, sodium chloride, 100% ethanol, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, *N*,*N*-dimethyl-formamide, glutaraldehyde, EGTA, magnesium chloride, sodium deoxycholate, Nonidet P-40, monobasic sodium phosphate (NaH_2PO_4), dibasic sodium phosphate (Na_2HPO_4), potassium chloride, potassium phosphate (KH_2PO_4), potassium ferrocyanide, and potassium ferricyanide were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide (NaOH) solution (10 N) was purchased from Fischer Scientific (Nepean, ON, Canada). Fetal bovine serum, Hanks' balanced salt solution, Waymouth's MB 752/1, HEPES, agarose, and penicillin-streptomycin were obtained from Invitrogen Canada (Burlington, ON, Canada).

Polymerase chain reaction (PCR) reagents (Amplitaq, $MgCl_2$, and 10× reaction buffer) were obtained from PerkinElmer Life and Analytical Sciences (Woodbridge, ON, Canada), deoxynucleoside triphosphates (dNTPs) from Roche Diagnostics (Indianapolis, IN), bromphenol blue and xylene cyanole from Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada), and Ficoll type 400 from Pharmacia Canada (Mississauga, ON, Canada). PCR primers were synthesized by ACGT Corporation (Toronto, ON, Canada). Antisense and control oligonucleotides were synthesized by the Hospital for Sick Children DNA Synthesis Facility (Toronto, ON, Canada).

Embryo Culture. Pregnant CD-1 and B6SJL dams were sacrificed by cervical dislocation on GDs 9.5 and 9.0, respectively. The uterus (containing the embryos) was immediately removed, rinsed, and maintained in HBSS at 37°C. Using a dissecting microscope (Carl Zeiss, Germany), the individual implantation sites were exposed using five watchmaker's forceps (Fine Science Tools, North Vancouver, BC, Canada). The decidua, trophoblast, parietal endoderm, and Reichert's membrane were removed, leaving the amnion, visceral yolk sac, and ectoplacental cone intact. Explanted embryos were maintained at 37°C in pregassed (5% CO_2 in air, unanalyzed) "holding medium" consisting of 50 ml of "embryo medium" (14 mM $NaHCO_3$, 50 units/ml of penicillin, 50 mg/ml of strep-

tomycin, 2.5 mM HEPES, and 10× Waymouth's MB 752/1 medium) and 17 ml of MRS (Winn and Wells, 1997). Embryos at the same stage of development were identified by somite development (four to six somite pairs), and were pooled and cultured in "embryo culture medium" (50 ml of "embryo medium", 35 ml of MRS, and 15 ml of fetal bovine serum). B6SJL embryos showed comparable somite development (four to six somite pairs) earlier than CD-1 embryos and were therefore explanted earlier on GD 9.0.

To address phenytoin embryopathy, CD-1 and B6SJL embryos were incubated in a therapeutic concentration of phenytoin (20 μg/ml, 80 μM) or its vehicle (0.002 N NaOH) at 37°C for 24 h in autoclaved scintillation vials containing 10 ml "embryo culture media" on a platform rocker (Bellco Biotechnology, Vineland, NJ). At the end of the culture period, embryos were assessed for embryopathy according to developmental (anterior neuropore closure, dorsal-ventral flexure (turning) and somite development) and morphological parameters (yolk sac diameter and crown-rump length) using a dissecting microscope (Carl Zeiss, Germany) (Winn and Wells, 1995).

NF-κB Activity after Phenytoin Exposure. GD 9.0 κB-lacZ embryos were explanted and cultured for 2, 4, 6, 12, or 24 h in either phenytoin (20 μg/ml; 80 μM) or its vehicle (0.002 N NaOH). At the end of the specified culture period, the yolk sacs were removed and stored for genotyping at −20°C, and the embryos were tested for β-galactosidase expression.

κB-lacZ Genotyping. Transgenic embryos carrying the lacZ gene were identified by PCR. DNA was isolated from each yolk sac sample using a standard DNA extraction kit (QIAGEN Inc., Valencia, CA). The DNA concentration was determined spectrophotometrically at A_{260}. PCR analysis was carried out according to the method of Hanley and Merlie (1991).

Whole-Mount β-Galactosidase Staining. Whole-mount β-galactosidase staining was performed as described previously (Schmidt-Ullrich et al., 1996; Li et al., 2000). Embryos were rinsed in phosphate buffer (PB) solution (25 mM NaH_2PO_4 and 75 mM Na_2HPO_4) and then fixed in "fixing solution" for 30 min (0.2% glutaraldehyde solution, 0.1 M PB solution, pH 7.4, 5 mM EGTA, and 2 mM $MgCl_2$). Embryos were then washed three times for 15 min in "washing solution" (0.1 M PB, pH 7.4, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40). Embryos were then stained with 1 mg/ml X-gal solution (0.1 M PB, pH 7.4, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide) from 6 h to overnight at 37°C. Pictures of embryos were taken using a dissecting microscope (Carl Zeiss GmbH, Jena, Germany) attached to a digital camera (Coolpix 995; Nikon, Tokyo, Japan). LacZ expression was indicated by the appearance of visible blue precipitate.

β-Galactosidase Quantification. To quantify the amount of lacZ expression, the digital images of embryos showing a blue color indicative of β-galactosidase activity were analyzed using Adobe Photoshop and Scion Image (Scion Corporation, Frederick, MD), based on the program NIH Image for MacIntosh by Wayne Rasband.

Using Photoshop 5.5 (Adobe Systems, Mountain View, CA), blue spots on embryo digital pictures were highlighted using the "Select Similar" function and then filled with black. These black areas were cut and copied into a new document to create a "blue spots" black and white image, where the black areas represented the total area of blue spots appearing in the original image of the embryo. Next, the total area of the embryo was filled in with black and used to create a second black and white image, "total area", in which the black area represented the total area of the embryo.

Scion Image was then employed to determine the total number of black pixels in both the "blue spots" and "total embryo area" black and white images. The amount of β-galactosidase expression was obtained by expressing the number of black pixels of "blue spots" as a percentage of the number of black pixels in the "total area" of the embryo.

Embryoprotection by Antisense NF-κB Oligonucleotides. To study the influence of NF-κB activation in phenytoin embryopathy, NF-κB activation was inhibited in embryo culture by p65 NF-κB antisense phosphorothioate oligonucleotides, which interfere with translation of p65 by extending over the initiation sites of NF-κB mRNAs (Du et al., 1999). Antisense NF-κB (p65) and control sense and nonsense oligonucleotides were dissolved in 0.15 M phosphate-buffered saline (PBS; 0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, and 0.0015 M KH_2PO_4 and had the following sequences: antisense p65 oligonucleotide, 5′-GGG AAA CAG ATC GTC CAT GGT CAG-3′; sense oligonucleotide, 5′-CTG ACC ATG GAC GAT CTG TTT CCC-3′; and nonsense oligonucleotide, 5′-CGG GCC ATG AAG AGT CGC CGC GCT-3′ (Du et al., 1999).

CD-1 embryos of equal somite development were cultured in the presence of antisense NF-κB oligonucleotides (2.5–25 μM), or one of the sense or nonsense oligonucleotide or vehicle (PBS) controls for 1 h at 37°C, after which phenytoin (20 μg/ml; 80 μM) or its vehicle (0.002 N NaOH) was added, antisense or oligonucleotide control concentration was reduced by half, and the embryos were cultured for 24 h at 37°C. After 24 h, developmental (anterior neuropore closure, turning and somite development) and morphologic parameters (yolk sac diameter, crown rump length) were evaluated.

To determine the extent of NF-κB inhibition by antisense oligonucleotides, κB-lacZ embryos were cultured with antisense NF-κB and control oligonucleotides under conditions identical to those described above for CD-1 embryos. At the end of the specified culture period, the embryos were tested for β-galactosidase expression.

To lessen the costly use of antisense oligonucleotides, the embryo culture media volume was reduced to maintain an adequate antisense concentration while using minimal antisense. Five embryos could be cultured in 4 ml of "embryo media" with adequate antisense for 1 h before the addition of phenytoin or its vehicle with an additional 4 ml of "embryo media" necessary for normal development.

Statistical Analysis. Statistical significance between treatment groups was established using a commercial software program (SigmaStat 2.03). Binomial data were analyzed using the 32 χ^2 test, and numerical data were compared by using a one-factor analysis of variance. The minimum level of significance throughout was $p < 0.05$.

Results

Validation of the Embryo Culture Model in B6SJL Mice. A B6SJL (C57BL/6 x SJL) transgenic mouse carrying a κB-dependent reporter gene was employed to determine whether NF-κB is altered during phenytoin embryopathy and, if so, by how much. This mouse contains a κB-lacZ transgene, where the lacZ gene is inserted downstream of κB-promoter sites (Schmidt-Ullrich et al., 1996). Whole-mount β-galactosidase staining was used to determine lacZ expression, which directly reflects NF-κB activity, because lacZ expression is driven by NF-κB activation.

Because B6SJL embryos had not previously been characterized for susceptibility to phenytoin embryopathy, our first concern was to determine whether wild-type B6SJL embryos show the typical anomalies associated with phenytoin exposure in other strains using the embryo culture model (Winn and Wells, 1995). In this method, embryos are removed from the pregnant dam and cultured for 24 h with a therapeutic concentration of phenytoin (20 μg/ml; 80 μM) or its vehicle control (0.002 N NaOH) and examined for alterations in morphological and developmental parameters. The embryo culture model is particularly useful in that it permits the evaluation of embryos exposed to precise concentrations of different compounds in the absence of maternal effects. This model also allows the selection of embryos at the same stage of development, which cannot be achieved in vivo.

B6SJL embryos explanted on GD 9.0 and cultured with phenytoin were susceptible to characteristic phenytoin embryopathies. Compared with embryos cultured in vehicle control, phenytoin-exposed B6SJL embryos showed decreases of 54% in anterior neuropore closure, 59% in turning, 11% in yolk sac diameter, 19% in crown rump length, and 22% in somite development ($p < 0.05$) (Fig. 1). The phenytoin-initiated embryopathies in B6SJL embryos were qualitatively and quantitatively similar to those in the well characterized CD-1 strain.

Phenytoin-Initiated NF-κB Activation. To examine NF-κB activity under the same exposure conditions resulting in phenytoin embryopathy, whole-mount β-galactosidase staining was performed on κB-lacZ embryos, where NF-κB activity was indicated by the appearance of visible blue substrate. κB-lacZ embryos explanted on GD 9.0 and incubated with phenytoin for 2, 4, 6, 12, and 24 h showed increased NF-κB activity compared with κB-lacZ embryos incubated with vehicle control for equal periods (Figs. 2 and 3). A 2-fold increase in NF-κB activity occurred between 4 and 12 h of phenytoin exposure ($p < 0.01$). The absolute enhancement in NF-κB activity by phenytoin remained constant after 4 h, although the percentage difference declined after 12 h because of increased baseline NF-κB activity during that developmental period (Fig. 3).

Tissue-specific localization of phenytoin-initiated NF-κB–dependent β-galactosidase expression was examined with respect to nontarget tissues (torso) and target tissues (head, somites, heart), the latter reflecting tissues exhibiting embryopathies observed in embryo culture and/or in vivo at or after birth (Fig. 4). The head is of primary interest because maternal treatment of pregnant rats with phenytoin produces behavioral deficits in the offspring (Adams et al., 1990). Likewise, intellectual deficits are perhaps the most prevalent anomaly in the children of pregnant epileptic women who take phenytoin during pregnancy (Vanoverloop et al., 1992). Phenytoin caused the greatest increase in NF-κB–dependent β-galactosidase expression in the head, with a 177% increase over vehicle controls at 4 h and a 90% increase at 24 h ($p < 0.01$). The approximately 3-fold increase in head expression caused by phenytoin at 4 h was substantially greater than the overall 2-fold increase determined by whole-body analysis at this time. As indicated above, the lower percentage enhancement by phenytoin in all target tissues at 24 h was caused in part by an increase of ~3-fold in constitutive expression over this period. The increased expression by phenytoin in the head may contribute to the accompanying decrease in anterior neuropore closure, as well as the deficits in brain function observed postnatally in rodents and humans. A lesser enhancement in expression by phenytoin was evident in the somites, where increases of 55 and 47% were observed in phenytoin-exposed embryos at 4 and 24 h,

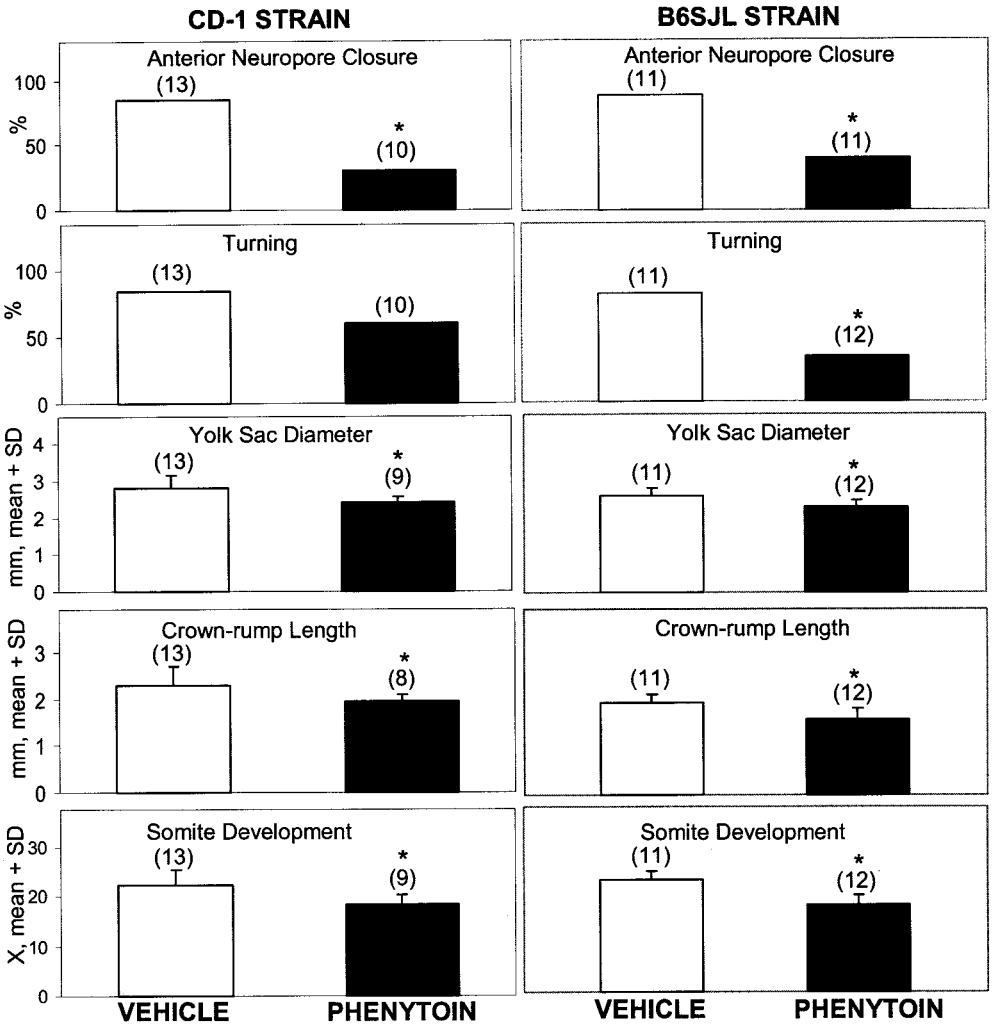

Fig. 1. Phenytoin embryopathy in CD-1 and B6SJL embryos. GD 9.5 CD-1 (left) or GD 9.0 B6SJL (right) mouse embryos at the same stage of development were incubated for 24 h at 37°C in the presence of phenytoin (20 μg/ml, 80 μM) or the vehicle (0.002 N NaOH). The number of embryos is given in parentheses. Asterisks indicate the embryopathic effects of phenytoin ($p < 0.05$).

respectively ($p < 0.05$). The somites give rise to bones, muscle, and skin, and the phenytoin-enhanced NF-κB activity in these structures may contribute to the decreased turning seen in embryo culture and the development of structural anomalies in vivo. Exposure during pregnancy to phenytoin infrequently causes cardiac anomalies in mice and humans (Kaneko, 1991; Wells et al., 1997b). In embryo culture, phenytoin did not alter NF-κB–dependent β-galactosidase expression in the heart at 4 h, and the phenytoin-initiated 42% increase at 24 h was not statistically significant ($p = 0.164$). The marginal nature of the increase in heart tissue may in part explain the low incidence of cardiac anomalies in vivo associated with maternal phenytoin treatment. NF-κB–dependent β-galactosidase expression was not enhanced by phenytoin in nontarget tissues (Fig. 2).

Overall, the tissue-selective enhancement by phenytoin in NF-κB–dependent β-galactosidase expression was 3.4-fold higher in the head compared with the somites at 4 h and 1.9-fold higher at 24 h.

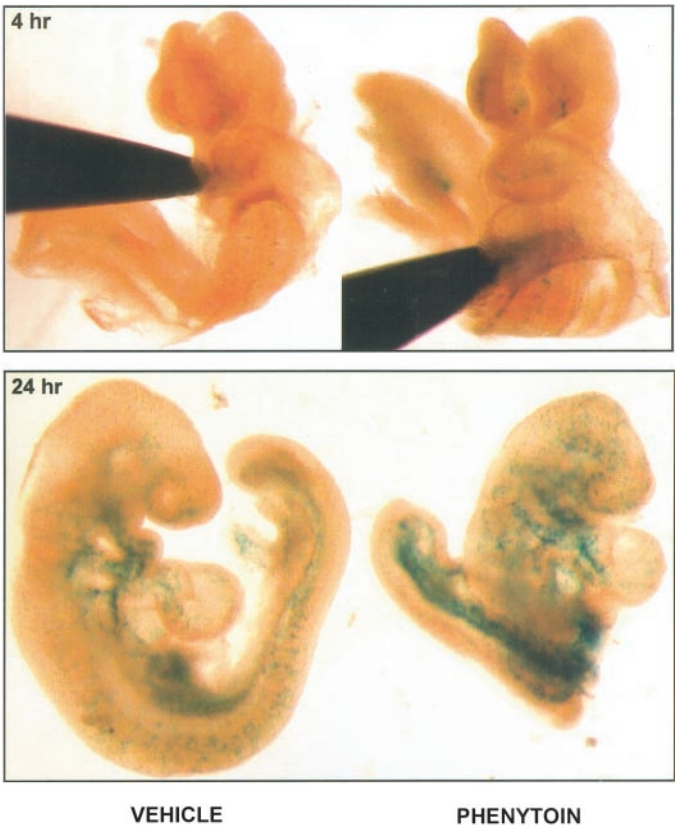

Fig. 2. NF-κB–dependent β-galactosidase expression in κB-lacZ embryos. κB-lacZ embryos were incubated with vehicle (0.002 N NaOH) (left) or phenytoin (20 μg/ml, 80 μM) (right) for 4 h (top) and 24 h (bottom) and then stained for β-galactosidase expression.

Protection by NF-κB Inhibition. To study the role of NF-κB in the mechanism of phenytoin embryopathy, NF-κB activation was inhibited in embryo culture by the addition of antisense oligonucleotides. NF-κB is most commonly found as a heterodimer consisting of p50 and p65 subunits. In this study, antisense p65 was employed, because antisense p65 has been used successfully in other pathological studies and because p65 is required for transduction of signals initiated by oncogenic Ras transformations (Higgins et al., 1993; Finco et al., 1997).

Antisense NF-κB oligonucleotides (2.5–25 μM) almost completely blocked the embryopathic effects of phenytoin on anterior neuropore closure, turning, and somite development in a concentration-dependent manner ($p < 0.05$) but did not protect against decreases in yolk sac diameter and crown-rump length in CD-1 embryos (Fig. 5). No protective effects were observed using sense or nonsense oligonucleotides (25 μM), or antisense vehicle controls (PBS).

To determine the efficacy of NF-κB inhibition by antisense oligonucleotides, whole-mount β-galactosidase staining was

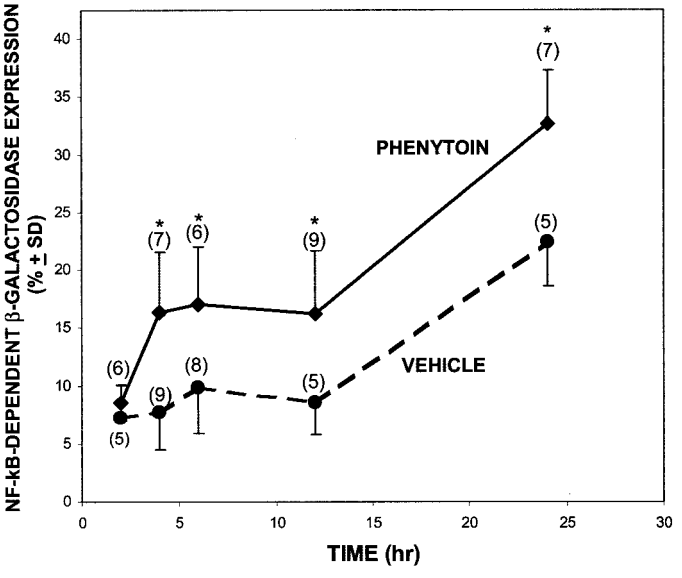

Fig. 3. Whole-body quantification of NF-κB–dependent β-galactosidase expression in κB-lacZ embryos. β-Galactosidase was quantified by X-gal staining in κB-lacZ embryos after incubation with vehicle (0.002 N NaOH) or phenytoin (20 μg/ml, 80 μM) for 2, 4, 6, 12, and 24 h. The number of embryos is indicated in parentheses. Asterisks indicate the effects of phenytoin on β-galactosidase expression ($p < 0.01$).

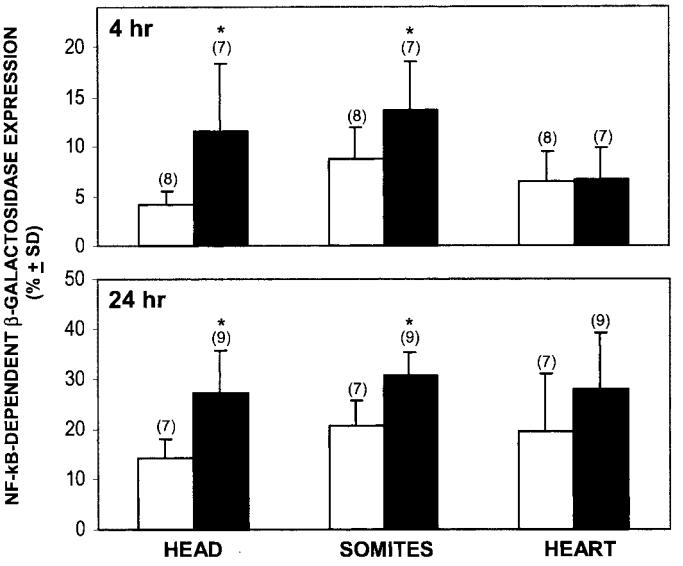

Fig. 4. Localized quantification of NF-κB–dependent β-galactosidase expression in selected target tissues of κB-lacZ embryos. Using the same embryos shown in Fig. 3, β-galactosidase was quantified in selected tissues at 4 h (top) and 24 h after exposure to phenytoin or its vehicle. The number of embryos is indicated in parentheses. Asterisks indicate the effects of phenytoin on β-galactosidase expression ($p < 0.05$).

performed on κB-lacZ embryos explanted on GD 9.0 and incubated with 25 μM antisense NF-κB or antisense vehicle (PBS) control together with phenytoin or its vehicle (NaOH) for 4 or 24 h (Figs. 6 and 7). Antisense NF-κB oligonucleotides effectively blocked phenytoin-enhanced embryonic NF-κB activity at both 4 and 24 h ($p < 0.001$) but did not seem to reduce constitutive NF-κB activity in the vehicle control embryos.

Discussion

The substantial tissue-selective increase in NF-κB activity caused by phenytoin in embryonic target tissues and the concentration-dependent inhibition of phenytoin-initiated NF-κB activity and embryopathies by antisense NF-κB oligonucleotides provide the first direct evidence that NF-κB may play a critical role in mediating phenytoin teratogenicity. The increase of >3-fold in NF-κB activity in the head compared with the somites is consistent with the higher prevalence of intellectual deficits in children exposed in utero to phenytoin. The magnitude of NF-κB–dependent β-galactosidase expression caused by phenytoin further correlates with the infrequent occurrence of cardiac anomalies and with an absence of anomalies in nontarget tissues, suggesting that NF-κB activity plays a causative role in phenytoin embryopathies.

An embryopathic contribution via enhanced NF-κB activity also is consistent with previous evidence for the involvement of embryonic PHS-catalyzed drug bioactivation and ROS formation in phenytoin teratogenesis, because other studies have demonstrated a relationship between increased ROS and NF-κB activation. Antioxidative enzymes such as superoxide dismutase (SOD) and catalase inhibit tumor necrosis factor-mediated activation of NF-κB in cell lines (Manna et al., 1998), and we have shown in embryo culture and in vivo that administration of either enzyme increases

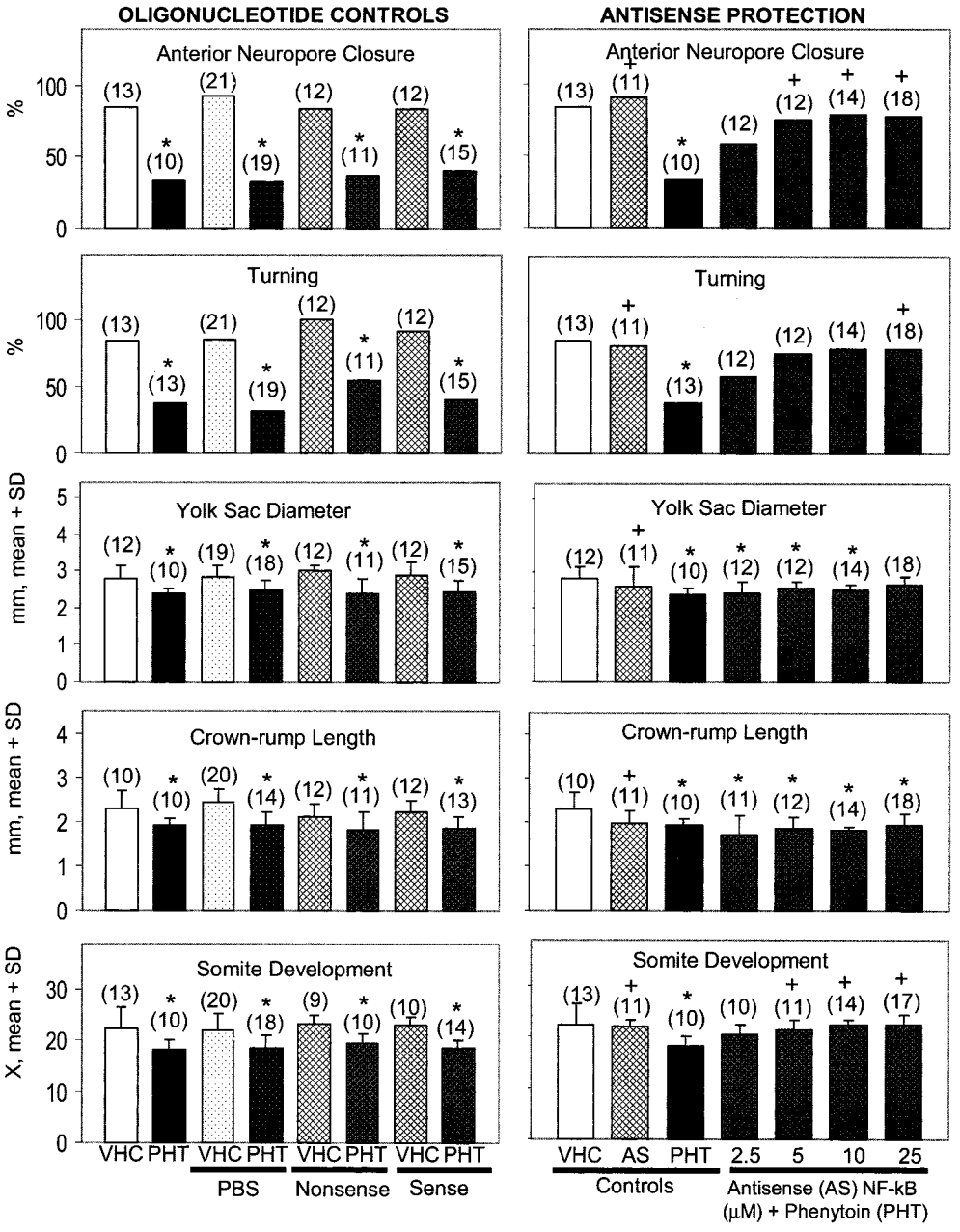

Fig. 5. Inhibition of phenytoin embryopathy in CD-1 embryos by antisense NF-κB oligonucleotides. GD 9.5 embryos were cultured for 24 h at 37°C with phenytoin (PHT) (20 μg/ml, 80 μM) or its vehicle (VHC) (0.002 N NaOH), with or without preincubation for 1 h with antisense NF-κB (2.5, 5, 10, and 25 μM) or the controls [antisense vehicle (PBS), nonsense or sense oligonucleotides (25 μM)]. The number of embryos is indicated in parentheses. *, difference from vehicle control ($p < 0.05$); +, difference from control embryos exposed to phenytoin alone ($p < 0.05$). Hatched marks indicate the presence of oligonucleotides.

embryonic antioxidative activity and decreases phenytoin-initiated oxidative DNA damage and embryopathies (Winn and Wells, 1995, 1999). Thus, SOD and catalase may reduce phenytoin embryopathies in part through inhibition of NF-κB activation. NF-κB is also inhibited by several antioxidants including vitamin E derivatives (Suzuki and Packer, 1993), and we have found that vitamin E also reduces phenytoin embryotoxicity (Wells et al., 1997a). On the other hand, the glutathione-depleting agent buthionine sulfoximine (BSO) enhances NF-κB activation (Sen et al., 1997), and we have found that BSO potentiates phenytoin embryopathies in embryo culture (Wells et al., 1997a). Thus, BSO may increase phenytoin embryotoxicity in part through increased NF-κB activation.

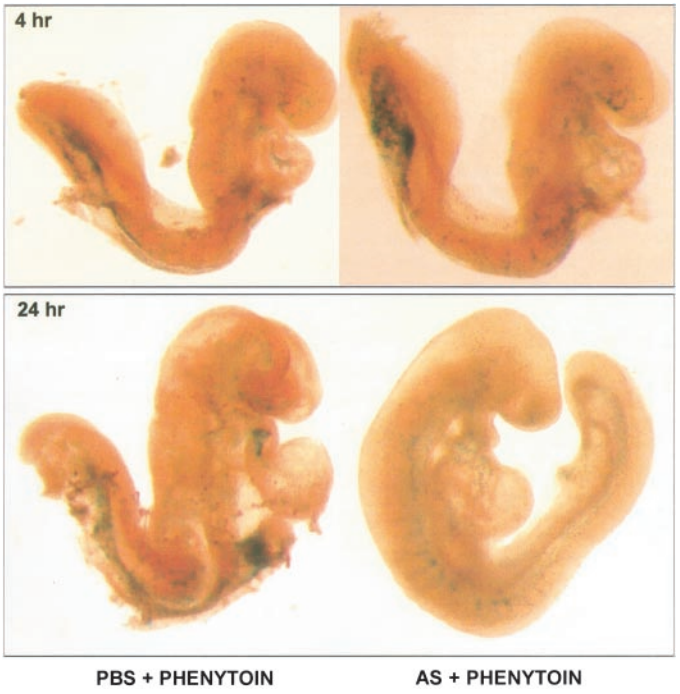

Fig. 6. Inhibition of phenytoin-enhanced NF-κB–dependent β-galactosidase expression in κB-lacZ embryos by antisense NF-κB oligonucleotides. κB-lacZ embryos were incubated with antisense NF-κB (25 μM) or its vehicle (PBS) in the presence of phenytoin (20 μg/ml, 80 μM) for 4 h (top) or 24 h (bottom) and stained for β-galactosidase expression.

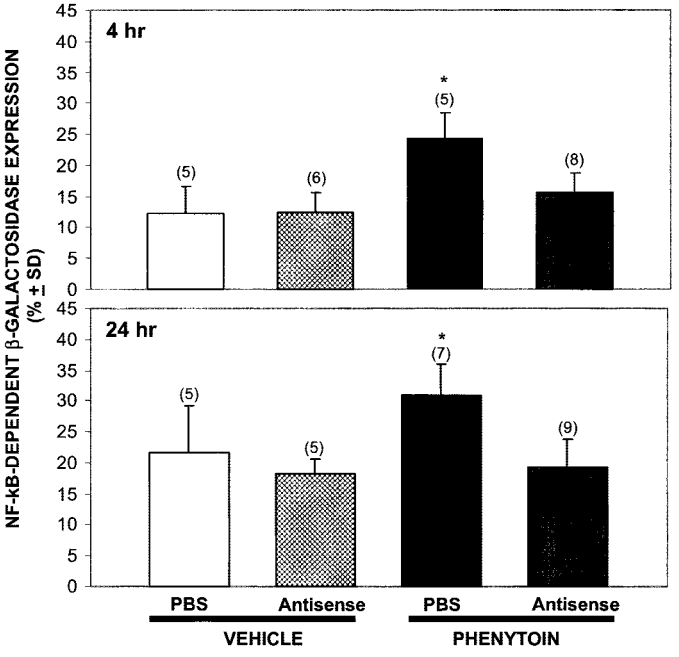

Fig. 7. Quantification of phenytoin-enhanced NF-κB–dependent β-galactosidase expression and its inhibition by antisense NF-κB oligonucleotides in κB-lacZ embryos. κB-lacZ GD 9.0 embryos were preincubated for 1 h with antisense NF-κB (25 μM) or antisense vehicle (PBS) and then cultured for 4 h (top) or 24 h (bottom) at 37°C with phenytoin (20 μg/ml, 80 μM) or its vehicle (0.002 N NaOH). The number of embryos is indicated in parentheses. Asterisks indicate a difference from all other embryo groups. Hatched marks indicate the presence of antisense oligonucleotide.

These results of the study reported herein are also consistent with a previous observation that Ras signaling, which lies upstream to NF-κB in some signaling cascades, may be mechanistically important in phenytoin teratogenesis (Winn and Wells, 2002). Several relationships have been demonstrated between Ras and NF-κB: 1) Ras can activate NF-κB, 2) NF-κB is required for an oncogenic effect of Ras-mediated cellular transformation, and 3) Ras is required for NF-κB activation by oxidants (Finco et al., 1997; Janssen-Heininger et al., 1999). Therefore ROS-mediated stimulation of Ras signaling and downstream NF-κB activation may constitute an important pathway mediating phenytoin embryopathy (Fig. 7). This mechanism may play a similar role in the embryopathic effects of endogenous oxidative stress and other ROS-initiating teratogens such as benzo[*a*]pyrene and thalidomide.

Antisense oligonucleotides are short sequences of chemically modified, synthetic DNA designed to specifically inhibit expression of a protein product encoded by a target gene. The oligonucleotide sequence is designed to hybridize via Watson-Crick base pairing with a complementary mRNA sequence that can either: 1) activate RNase H to selectively cleave the mRNA portion of the mRNA-oligonucleotide complex, 2) interfere with ribosomal binding and sliding, 3) inhibit mRNA splicing, or 4) form a triple helix with DNA to inhibit translation (Potts and Sadler, 1997; Cooper et al., 1999). Antisense used in this study is believed to interfere with ribosomal binding by extending over the initiation sites of NF-κB p65 mRNAs (Du et al., 1999). The controls are necessary to assure that the biological effects are a result of the specific antisense sequence and not caused by nonspecific effects of oligonucleotides, such as binding to growth factors (Guvakova et al., 1995; Potts and Sadler, 1997). Herein, the controls included nonsense oligonucleotides that are the same length as the antisense but contain a sequence not complementary to any known mRNA and sense oligonucleotides that consist of a sequence antiparallel to that of the antisense.

Potential ways in which NF-κB may exert its harmful effects include alterations in gene expression and/or inhibition of repair processes. NF-κB is expressed in the embryo at least as early as GD 8.5 and through to GD 12.5 (Schmidt-Ullrich et al., 1996; Li et al., 2000). In utero exposure to phenytoin can alter the expression of genes potentially important in normal neural tube closure and craniofacial development (Bennett et al., 1997; Gelineau-van Waes et al., 1999), which may be related to changes in NF-κB activity. For example, the reported decrease in transforming growth factor (TGF)-β expression by phenytoin (Bennett et al., 1997) may lead to increased NF-κB activity, because TGF-β (in particular TGF-β1) can reduce NF-κB activity partly through increased NF-κB inhibition (Arsura et al.,

1996). TGF-β2 may be critical for normal neural tube closure (Bennett et al., 1997), which is consistent with results in this study demonstrating that antisense inhibition of NF-κB reduces the embryopathic effects of phenytoin on anterior neuropore closure. Expression of *c-jun*, which is important for embryonic cellular proliferation, is also reported to be decreased by in utero phenytoin exposure (Bennett et al., 1997). This downstream suppression may result from NF-κB activation, because NF-κB can decrease activity of the proapoptotic c-Jun amino-terminal kinase cascade (De Smaele et al., 2001).

Some studies have found that NF-κB activation can inhibit the p53 tumor suppressor gene (Wadgaonkar et al., 1999; Webster and Perkins, 1999), although others have found that NF-κB and p53 may act synergistically (Benoit et al., 2000). NF-κB and p53 are believed to compete with each other through competition for a limited supply of transcriptional coactivators (p300 and cAMP response element-binding protein) that are needed for maximal activity (Wadgaonkar et al., 1999). Several lines of evidence suggest that endogenous oxidative stress and a number of ROS-initiating teratogens, including phenytoin, may exert their teratological effects in part through ROS-mediated oxidative DNA damage (Wells et al., 1997a,b; Winn and Wells, 1999; Nicol et al., 2000). DNA repair may be mediated by p53, which can recognize DNA damage and trigger appropriate repair and apoptotic pathways (Wells et al., 1997b). We have previously shown that p53 can function in vivo as a "teratological suppressor", because p53-deficient knockout mice are more susceptible to ROS-initiating teratogens such as phenytoin (Laposa and Wells, 1995) and benzo[*a*]pyrene (Nicol et al., 1995). Increased NF-κB activation could decrease p53-mediated repair and increase susceptibility to phenytoin-initiated DNA damage. This may contribute to the protective effects of antisense NF-κB inhibition on phenytoin embryopathy, whereby inhibition of NF-κB may alleviate its inhibitory effects on p53 and enhance DNA repair activity.

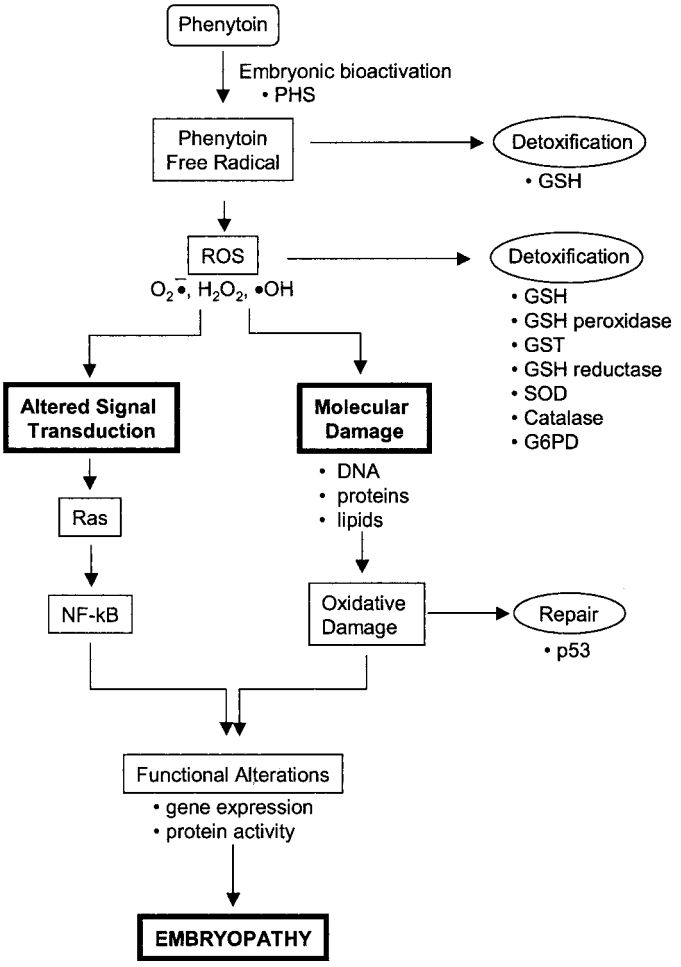

Fig. 8. Postulated molecular mechanisms of phenytoin embryopathy. Phenytoin can be bioactivated in the embryo by such enzymes as prostaglandin H synthase to a free radical intermediate, which, if not detoxified, can initiate the formation of ROS. Under some conditions, ROS also may be initiated via the redox cycling of catechol metabolites of phenytoin and/or reperfusion effects caused by the cardiosuppressive effects of phenytoin. If not similarly detoxified by protective mechanisms, ROS can inappropriately activate signal transduction cascades, which may involve Ras and NF-κB signaling or cause oxidative damage to cellular macromolecules, both of which may contribute to the type and severity of phenytoin embryopathies. A similar mechanism may be involved in the embryopathic effects of other ROS-initiating teratogens like benzo[*a*]pyrene and thalidomide. In the absence of adequate embryonic protective pathways, embryopathies may also be initiated by ROS formed during endogenous oxidative stress. G6PD, glucose 6-phosphate-dehydrogenase; GSH, glutathione; GST, glutathione-*S*-transferase.

The protective effect of antisense NF-κB oligonucleotides against phenytoin embryopathies observed herein may also involve suppression of PHS-2 (also known as cyclooxygenase-2) expression. PHS-catalyzed drug bioactivation has been shown to contribute to ROS formation, oxidative DNA damage, and teratogenicity of several teratogens including phenytoin, benzo[*a*]pyrene, and thalidomide (Wells et al., 1997b; Parman et al., 1998, 1999; Parman and Wells, 2002). PHS-2 contains an NF-κB promoter site, and NF-κB inhibitors can suppress PHS-2 expression by COX-2 activators (D'Acquisto et al., 1997). Therefore, the increased NF-κB activity in embryonic target tissues after exposure to phenytoin may in turn increase PHS-2 expression, thereby enhancing the formation of phenytoin free radical reactive intermediates and ROS formation. On the other hand, this contribution would also be consistent with the observed inhibition of phenytoin embryopathy by antisense NF-κB oligonucleotides, wherein inhibition of NF-κB may decrease PHS-2 expression, thus reducing phenytoin bioactivation and consequent embryopathy. However, the extent of an embryopathic contribution via the up-regulation of PHS-2 expression is not clear. The time course for the synthesis of a teratologically sufficient increase in the amount of PHS-2 protein in embryos has yet to be determined, and embryopathies are caused by phenytoin after as little as a single dose in vivo or a 4-h exposure in embryo culture. Up-regulation of PHS-2 would be more likely to exert an embryopathic contribution in vivo with long-term exposure to phenytoin throughout pregnancy.

Control embryos exposed only to antisense NF-κB oligonucleotides seemed to develop normally, presumably because constitutive activation was unaffected (Fig. 7). The basal level of NF-κB activity in unstimulated cells is caused by high turnover of the NF-κB regulatory molecule inhibitory κB (Rice and Ernst, 1993), and would be regulated by the level of existing inhibitory κB protein rather than mRNA. Thus, antisense oligonucleotides, which block at the mRNA level, would not be expected to inhibit basal NF-κB activity.

In conclusion, the studies herein suggest that NF-κB is a critical component in the mechanism of embryopathies caused by the ROS-initiating teratogen phenytoin, consistent with a similarly important upstream role previously shown

for Ras activation. Hence, the mechanisms underlying phenytoin embryopathies seem to include ROS-initiated enhancement in embryonic signal transduction as well as oxidative damage to cellular macromolecules. These novel results may provide new insights into risk factors and therapeutic interventions for embryopathies caused by phenytoin and potentially by other endogenous and exogenous sources of enhanced and/or unprotected embryonic oxidative stress. The remarkable protective efficacy of antisense NF-κB oligonucleotides raises the intriguing possibility of similar approaches for therapeutic strategies during pregnancy.

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