

Review

Molecular and biochemical mechanisms in teratogenesis involving reactive oxygen species[☆]

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Abstract

Developmental pathologies may result from endogenous or xenobiotic-enhanced formation of reactive oxygen species (ROS), which oxidatively damage cellular macromolecules and/or alter signal transduction. This minireview focuses upon several model drugs (phenytoin, thalidomide, methamphetamine), environmental chemicals (benzo[a]pyrene) and gamma irradiation to examine this hypothesis in vivo and in embryo culture using mouse, rat and rabbit models. Embryonic prostaglandin H synthases (PHSs) and lipoxygenases bioactivate xenobiotics to free radical intermediates that initiate ROS formation, resulting in oxidation of proteins, lipids and DNA. Oxidative DNA damage and embryopathies are reduced in PHS knockout mice, and in mice treated with PHS inhibitors, antioxidative enzymes, antioxidants and free radical trapping agents. Thalidomide causes embryonic DNA oxidation in susceptible (rabbit) but not resistant (mouse) species. Embryopathies are increased in mutant mice deficient in the antioxidative enzyme glucose-6-phosphate dehydrogenase (G6PD), or by glutathione (GSH) depletion, or inhibition of GSH peroxidase or GSH reductase. Inducible nitric oxide synthase knockout mice are partially protected. Inhibition of Ras or NF-κB pathways reduces embryopathies, implicating ROS-mediated signal transduction. Atm and p53 knockout mice deficient in DNA damage response/repair are more susceptible to xenobiotic or radiation embryopathies, suggesting a teratological role for DNA damage, consistent with enhanced susceptibility to methamphetamine in *ogg1* knockout mice with deficient repair of oxidative DNA damage. Even endogenous embryonic oxidative stress carries a risk, since untreated G6PD- or ATM-deficient mice have increased embryopathies. Thus, embryonic processes regulating the balance of ROS formation, oxidative DNA damage and repair, and ROS-mediated signal transduction may be important determinants of teratological risk.

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Keywords: Teratogen; Reactive oxygen species; Prostaglandin H synthase

Abbreviations: ASA, acetylsalicylic acid; ATM, ataxia telangiectasia mutated; COX, cyclooxygenase (PHS component); CYP, cytochromes P450; ETYA, eicosatetraenoic acid; G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; iNOS, inducible nitric oxide synthase; IR, ionizing radiation (gamma irradiation); LPO, lipoxygenase; NOS, nitric oxide synthase; NO[•], nitric oxide; NF-κB, nuclear factor kappa B; Ogg1, oxoguanine glycosylase 1; 8-Oxo-dG, 8-Oxo-2'-deoxyguanosine; P450, cytochrome P450; PBN, alpha-phenyl-N-t-butyl nitrene; PHS, prostaglandin H synthase (cyclooxygenase and hydroperoxidase components); RNS, reactive nitrogen species; ROS, Reactive oxygen species; UGT, UDP-glucuronosyltransferase.

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Introduction

This minireview is based upon a presentation in a workshop on *New Approaches in the Assessment of Developmental Toxicology* presented at the 10th International Congress of Toxicology in Tampere, Finland (July 2004). The potential involvement of reactive oxygen species (ROS) in teratogenesis is reviewed with respect to the use of mutant, transgenic and knockout mice and antisense oligonucleotides, in determining underlying pharmacological mechanisms of embryopathy. The use of genetically modified strains avoids the often unknown effects attending the use of chemical inhibitors, although such genetic alterations are not without their own compensating changes that may confound interpretation of the data. Short-term exposure to antisense oligonucleotides to inhibit protein expression, when practical, is highly specific and generally less complicating than genetically modified mice since less time is available for embryonic compensation. Mouse, rat and rabbit models together with multiple levels of complexity, including the use of purified proteins, cell culture, embryo culture and *in vivo* studies, are employed comprehensively to examine the potential relevance of molecular and biochemical mechanisms to teratological outcomes. Embryo culture, while methodologically demanding, allows for an assessment of embryonic determinants in the absence of maternal factors, along with more precise control of exposure levels, among other advantages. Its major disadvantage compared to *in vivo* studies is that the ultimate teratological relevance cannot be established within the period during which embryos can be viably cultured. Several models of ROS-initiating teratogens with differing advantages are employed, including drugs (phenytoin, thalidomide, methamphetamine), environmental chemicals (benzo[a]pyrene) and ionizing radiation (IR), the latter of which is highly penetrating and does not require enzymatic bioactivation. Details beyond the scope of this minireview, including earlier

citations of the primary literature, are provided in the cited reviews. Most of the data discussed herein are from animal models. Little is known about the role of ROS in human teratogenesis.

Overview

General mechanisms

Many drugs and environmental chemicals exert their toxic effects by binding reversibly to a receptor, evoking an embryopathic response that is enhanced with an increasing concentration of the xenobiotic in the plasma and at the tissue receptor, with the effect declining as the plasma concentration decreases due to drug metabolism and elimination (Fig. 1). In this case, enhanced risk is commonly associated with excessive xenobiotic exposure levels and/or a deficiency in quantitatively major maternal pathways of metabolism and elimination, such as glucuronidation, that keep a xenobiotic and/or its teratogenic stable metabolites from reaching the embryo.

Alternatively, a number of xenobiotics, sometimes termed “proteratogens”, that are relatively non-toxic can be enzymatically bioactivated within the embryo to highly toxic, electrophilic or free radical reactive intermediates (Fig. 1) (Juchau et al., 1992; Fantel, 1996; Wells and Winn, 1996; Wells et al., 1997b). If not detoxified, xenobiotic electrophilic reactive intermediates can bind covalently (irreversibly) to embryonic cellular macromolecules (e.g. proteins, DNA), while xenobiotic free radical reactive intermediates can react directly or indirectly with molecular oxygen to initiate the formation of ROS, such as superoxide anion, hydrogen peroxide and hydroxyl radicals. These ROS, if not detoxified by antioxidants or antioxidative enzymes, may oxidatively damage cellular macromolecules such as lipids, proteins, RNA and DNA. Macromolecular damage, if not repaired, may interfere

TERATOGENESIS: BIOCHEMICAL AND MOLECULAR DETERMINANTS

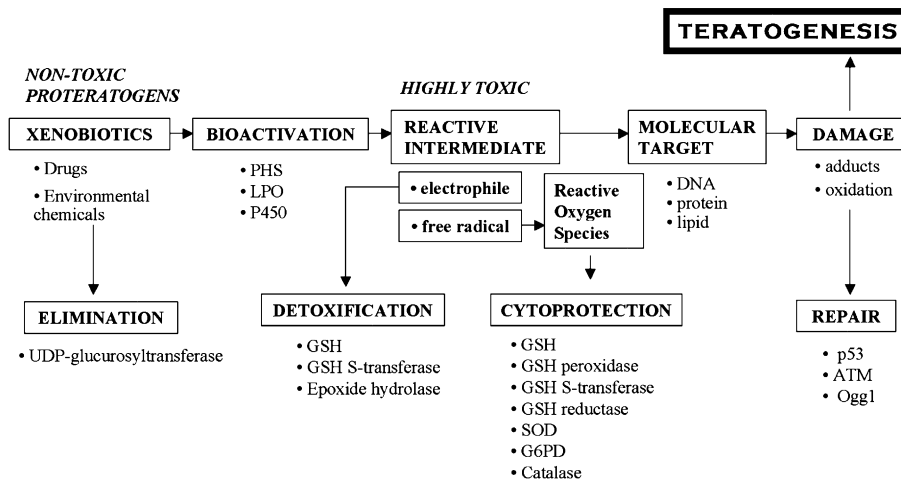


Fig. 1. Postulated balance of biochemical pathways involved in the mechanism and determinants of risk for chemical teratogenesis mediated by reactive oxygen species (ROS). If not eliminated via maternal pathways of drug metabolism, xenobiotics are transferred to the embryo where they are bioactivated to toxic reactive intermediates. Risk is determined by the balance between embryonic bioactivation and the pathways of maternal elimination and embryonic reactive intermediate detoxification, ROS cytoprotection and repair of macromolecular damage. Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; LPO, lipoxygenase; PHS, prostaglandin H synthase; P450, cytochrome *P*450 (CYP); SOD, superoxide dismutase; ATM, ataxia telangiectasia mutated; Ogg1, oxoguanine glycosylase 1 (modified from Wells and Winn, 1996).

with embryonic and/or fetal development. Development also may be adversely affected by the reversible reaction of ROS with transduction proteins, thereby altering embryonic

or fetal signal transduction pathways (Fig. 2). The focus of this minireview is the potential role of ROS in the mechanism of teratogenesis.

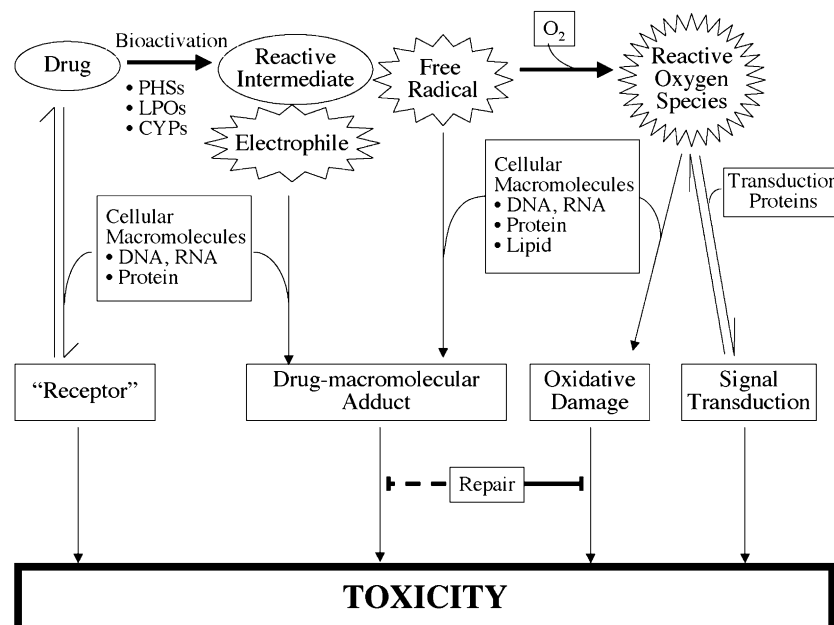


Fig. 2. Potential competing mechanisms involved in chemical teratogenesis. A given teratogen often will exhibit multiple reactions, including binding reversibly to receptors, forming covalent drug-macromolecular adducts, initiating reactive oxygen species (ROS)-mediated oxidative damage to most cellular macromolecules and enhancing ROS-mediated signal transduction. The individual teratological contributions of each of these pathways can be discriminated in part by determining: (1) the effects of free radical xenobiotic intermediates and ROS as distinct from effects mediated by drug-macromolecular adducts or receptor-mediated pathways, using free radical spin trapping agents and altering levels of antioxidants or antioxidative enzymes; (2) the consequences of damage to DNA, as distinct from proteins and lipids, by altering pathways of DNA repair, and particularly those pathways that selectively repair oxidative DNA damage and (3) the effect of blocking key proteins involved in ROS-mediated signal transduction pathways. Abbreviations: PHSs, prostaglandin H synthases; LPOs, lipoxygenases; CYPs, cytochromes *P*450.

Developmental background

Teratogenesis, derived from the Greek term “monster”, often is viewed as referring to structural birth defects, such as a child exposed in utero to the drug thalidomide, and born without arms or legs. However, in the broader context, teratogenesis includes both structural and functional birth defects, which can include lethal events resulting in the death of an infant before or shortly after birth. Both structural and functional defects in this context refer to permanent changes in the child resulting from in utero exposure. Functional birth defects can include deficits in brain function, such as low I.Q. scores or behavioral abnormalities, abnormalities in the immune system, permanent alterations in biochemical pathways (biochemical imprinting) and postnatal cancer, to name a few.

The research reviewed herein is focused upon the developmental periods following implantation of the conceptus in the maternal uterus (postimplantation). During the embryonic period, which lies within the first trimester of pregnancy, the structural development of most organs (organogenesis) is completed. Subsequently, during the fetal period, tissue differentiation and functional development take place. One of the hallmarks of teratological risk is that an organ is susceptible to abnormal development usually only if exposed during the critical “window” or time period during which the organ is being structurally formed (embryonic period) or functionally developing (fetal period). Although there are exceptions, exposure to a teratogen during the embryonic period generally results in structural birth defects, while exposure during the fetal period results in functional or biochemical defects.

Pharmacological background

With ROS-mediated teratogenesis, the pathogenic pathway of xenobiotic bioactivation, oxidative macromolecular damage and altered signal transduction is balanced or protected against by pathways for the detoxification of the xenobiotic reactive intermediate, antioxidative pathways for detoxifying ROS and pathways for the repair of oxidatively damaged cellular macromolecules (Wells and Winn, 1996; Wells et al., 1997b). Accordingly, embryopathic risk is theoretically determined by the balance between the pathogenic and embryoprotective pathways. If the pathogenic determinants exceed the capacity of one or more of the critical protective pathways, then even at therapeutic drug concentrations or supposedly safe exposure levels of environmental chemicals, embryopathies can be initiated. This is quite different from embryopathies initiated by the reversible binding of a xenobiotic to its receptor, wherein embryopathies will usually occur only when the embryo is exposed to higher concentrations of the xenobiotic. The risk of ROS-mediated teratogenesis can be enhanced by either genetic or environmental determinants that increase embryonic xenobiotic bioactivation or decrease one or more of the

embryoprotective pathways. In some cases, maternal pathways of a different nature can indirectly contribute to risk, as discussed below.

It is important to note that evidence for a role for ROS in the mechanism of chemical teratogenesis also can provide insights into the mechanism of spontaneous embryopathies occurring in the absence of xenobiotic exposure. Thus, even endogenous or physiological levels of ROS may be embryopathic in animals deficient in critical pathways for ROS detoxification or pathways for repairing oxidative macromolecular damage. Examples of this are discussed later, including mutant mice deficient in the antioxidative enzyme glucose-6-phosphate dehydrogenase (G6PD), and knockout mice lacking the ataxia telangiectasia mutated (ATM) protein involved in sensing and repairing oxidative DNA damage.

Most ROS are too unstable to travel beyond the cell (Halliwell and Gutteridge, 1999), so the teratologically relevant processes of embryopathic xenobiotic bioactivation and reactive intermediate detoxification, ROS formation and their associated protective pathways involving antioxidants and antioxidative enzymes, and pathways for the repair of oxidative DNA damage, all lie exclusively within the embryo. Thus, maternal activities of these pathways do not contribute directly to the mechanism of ROS-mediated teratogenesis, nor to the determinants of risk. This concept is important not only to understanding teratological mechanisms, but also to the design of human studies in the field of molecular epidemiology. Nevertheless, as discussed later, even in the case of ROS-mediated teratogenesis, maternal and extra-embryonic factors may still contribute indirectly to embryopathic risk via pathways of drug elimination that alter embryonic exposure, or via production of diffusible factors that alter embryonic determinants of oxidative macromolecular damage or embryonic signal transduction mediated by ROS and/or reactive nitrogen species (RNS).

For the model teratogens discussed herein, all except ionizing radiation can: (1) bind reversibly to receptors; (2) be bioactivated by CYPs to electrophilic reactive intermediates that form covalent adducts with proteins and DNA and (3) be bioactivated by PHSs and other enzymes to free radical intermediates that may bind to DNA, or react directly or indirectly with molecular oxygen to initiate the formation of ROS (Fig. 2). In the latter case, ROS may both oxidatively damage cellular macromolecules and reversibly react with transduction proteins to alter signal transduction. Finally, embryonic lipids, protein, GSH and DNA are all oxidatively damaged by ROS-initiating teratogens. In the face of these simultaneous events, the studies discussed herein attempt to address the question of which of the above mechanisms actually play a causal role in the molecular mechanism of teratogenesis. In part, the approaches take advantage of interventions that are relatively selective for discerning: (1) the requirement for xenobiotic bioactivation as distinct from

reversible, receptor-mediated interactions; (2) free radical as distinct from electrophilic reactive xenobiotic intermediates; (3) production of ROS as distinct from xenobiotic covalent binding; (4) repair of oxidative DNA damage as distinct from DNA-xenobiotic adducts or the oxidation of other cellular macromolecules (lipid, proteins) or GSH and (5) ROS-mediated alterations in signal transduction as distinct from oxidative damage to cellular macromolecules.

Mechanisms and risk factors

Maternal and extra-embryonic determinants

Perhaps the most straightforward mechanism by which maternal determinants can contribute indirectly to the risk of ROS-dependent teratogenicity is via pathways that eliminate the parent compound or its stable metabolites before they can be transported across the placenta to the embryo. Most xenobiotics and/or their stable metabolites are conjugated with hydrophilic endogenous substrates such as glucuronic acid or sulfate, rendering them sufficiently water-soluble to be readily excreted in the maternal urine (Fig. 1). For such teratogens, a deficiency in the relevant conjugating pathway, either for genetic or environmental reasons, will increase the maternal plasma concentration of the xenobiotic/stable metabolite (proteratogen) and hence the amount that is transported to the embryo. This issue may be particularly important for the placental transfer of catechol metabolites, as discussed later under ROS formation.

In the case of benzo[a]pyrene, its hydroxylated metabolites are conjugated with glucuronic acid, catalyzed primarily by hepatic UDP-glucuronosyltransferases (UGTs) (Hu and Wells, 1992). The potential teratological impact of maternal genetic determinants is evident in mutant Gunn rats, which have a genetic deficiency in the UGT1 family (Wells et al., 2004) resulting in a decrease in the glucuronidation of the hydroxylated metabolites of benzo[a]pyrene (Kim and Wells, 1996a; Wells and Winn, 1996). In pregnant Gunn dams treated with a low, normally non-carcinogenic and non-teratogenic dose of benzo[a]pyrene (25 mg/kg ip), there was a substantial 2.9-fold increase in fetal resorptions (in utero death of the fetus) compared to untreated Gunn controls (Fig. 3, upper panel). This low dose of benzo[a]pyrene did not alter the incidence of resorptions in control Wistar rats, the parent strain of the Gunn rat with normal UGT activity (Fig. 3), nor did it alter fetal body weight in either the Gunn or Wistar strains (data not shown), indicating a subtle embryopathic effect. The substantial embryopathic impact of this maternal UGT deficiency reflects the fact that glucuronidation generally is the major route for elimination of many metabolites, including those of benzo[a]pyrene, whereas the bioactivating pathway for this and many other

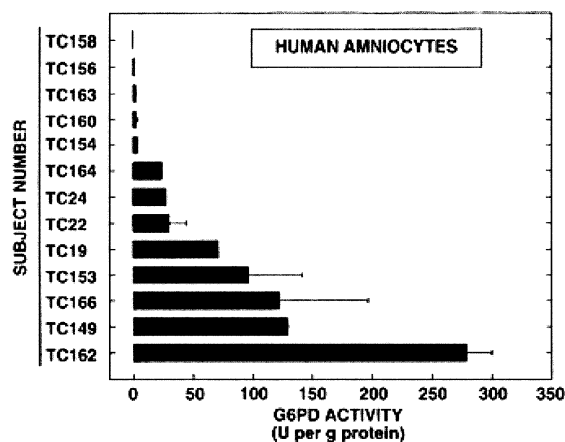
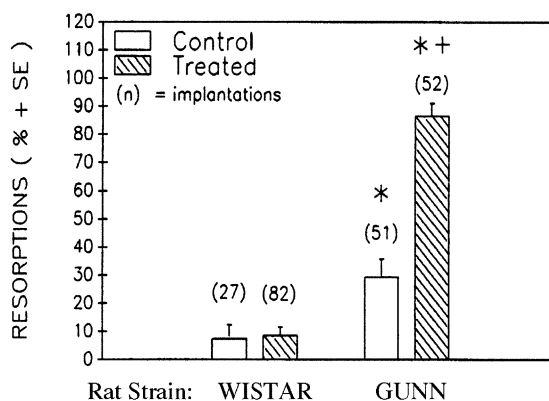


Fig. 3. Potential maternal and fetal determinants of risk. Upper panel: effect of a genetic deficiency in maternal glucuronidation on benzo[a]pyrene-initiated in utero resorptions (fetal death). Pregnant mutant Gunn rats with a hereditary deficiency in the UDP-glucuronosyltransferase (UGT) 1 family, or the parent Wistar strain with normal UGT activity, were treated with benzo[a]pyrene (25 mg/kg ip) or its corn oil vehicle (control) on gestational day (GD) 10. Dams were killed on GD 20 for examination of the uterus and fetuses. Implantations include resorptions and fetuses delivered alive. Asterisks indicate a difference from Wistar rats with the same treatment, and the plus symbol indicates a difference from the Gunn control group ($P < 0.05$). Lower panel: Interindividual variability in the activity of glucose-6-phosphate dehydrogenase (G6PD) in amniocytes obtained from human subjects. Fetal amniocytes were obtained from term amniotic membranes of volunteer mothers who had ($n = 8$) or had not ($n = 5$) taken an anticonvulsant drug throughout pregnancy. Of those women taking anticonvulsants, 4 took phenytoin, 2 took phenobarbital, 1 took carbamazepine and 1 took phenytoin plus primidone. No correlation was apparent between anticonvulsant exposure and G6PD activity. Cells were cultured to confluency, aliquotted and kept frozen until assayed for G6PD activity. For each subject, 4 amniocyte aliquots were homogenized in PBS buffer (pH 7.4), sonicated for 5 min to ensure membrane lysis and analyzed as described elsewhere (Nicol et al., 2000). All results were standardized with respect to total protein content and reported in International Units (U) per gram (g) of protein (U/g). Values represent the mean \pm SD.

proteratogens is a quantitatively minor pathway. Hence, a relatively small decrease in glucuronidation may result in a major percentage increase in xenobiotic bioactivation. The potential embryopathic importance of this maternal deficiency is reflected in the fact that the benzo[a]pyrene dose employed in this study was only about 10% of a typical

dose (250 mg/kg/ip) used to initiate cancer in adult rats. Similarly, in vitro, UGT-deficient rat skin fibroblasts are more susceptible to oxidative DNA damage and micronucleus formation (a form of genotoxicity) initiated by phenytoin and its major hydroxylated metabolite, HPPH (Kim et al., 1997c), and by benzo[a]pyrene (Kim and Wells, 1996b). A preliminary report describes a similarly enhanced in vivo hydroxyl radical formation and embryopathic risk in pregnant UGT-deficient RHA and Gunn rats treated with phenytoin (Kim and Wells, 1998). Genetic deficiencies in the various UGT isozymes in humans are proving to be relatively common (Wells et al., 2004), and these together with environmentally compromised activities of UGT and sulfotransferase isozymes might be important risk determinants if a pregnant woman was exposed to a teratogen eliminated primarily by that particular isozyme.

Another potential indirect mechanism by which maternal and other extra-embryonic pathways can modulate ROS-mediated teratogenesis is via the production of diffusible factors that can traverse placental and cellular membranes into the embryo and alter embryonic determinants of oxidative macromolecular damage or signal

transduction. This is exemplified by the peroxynitrite pathway, in which nitric oxide synthases (NOSs) produce relatively stable and readily diffusible nitric oxide (NO^\bullet) (Halliwell and Gutteridge, 1999; Marnett et al., 2003). Once in the embryo, NO^\bullet can alter embryonic signal transduction pathways, or react with superoxide to produce directly embryopathic hydroxyl radicals and the RNS peroxynitrite (ONOO^-), the latter of which can initiate the hydroxylation and nitration of embryonic proteins and DNA, among other reactions (Fig. 4). Any of these processes could adversely affect embryonic development. In embryo culture studies using knockout mice lacking the inducible form of NOS (iNOS, NOS2), iNOS-deficient embryos were partially protected from the embryopathic effects of both benzo[a]pyrene and phenytoin, suggesting that the peroxynitrite pathway plays a contributory, albeit not essential, role in the respective teratological mechanisms, and that iNOS is constitutively expressed in embryos (Kasapinovic et al., 2004). However, analysis of iNOS protein levels showed that iNOS is not measurably expressed in the embryo itself, indicating that NO^\bullet generated by iNOS in the ectoplacental cone can diffuse to the embryo and react with superoxide generated in the

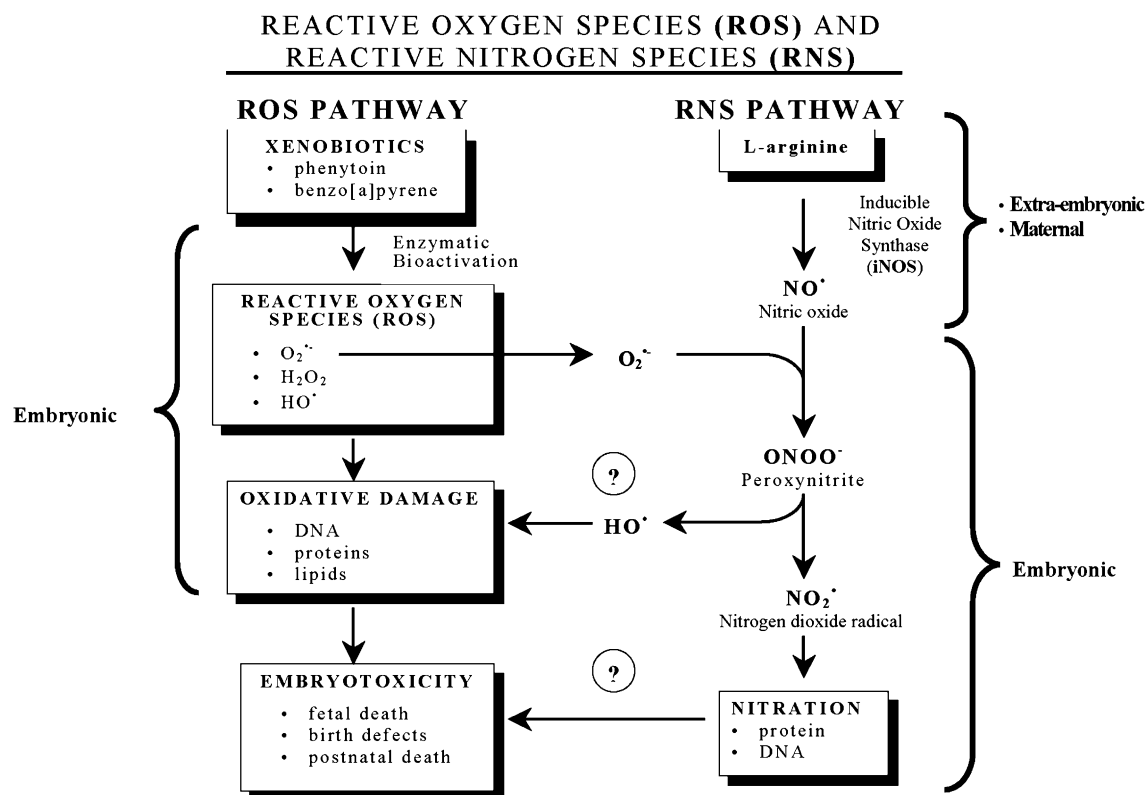


Fig. 4. Postulated interactions between the pathways for formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Among the nitric oxide synthases (NOSs), inducible nitric oxide synthase (iNOS) appears to be expressed during organogenesis only in extra-embryonic (ectoplacental cone) and maternal tissues, producing relatively stable nitric oxide that diffuses into the embryo where it reacts with embryonically generated superoxide anion to produce highly reactive peroxynitrite (see text). The embryopathic roles of the neuronal and endothelial forms of NOS have yet to be shown. Among other reactions, peroxynitrite can initiate oxidative damage to cellular macromolecules, oxidize soluble (e.g. GSH) and protein thiols, cause the nitration of aromatic amino acids (tyrosine, tryptophan, phenylalanine) in proteins and similarly cause the nitration and in some cases deamination of DNA bases (e.g. guanine, adenine), all of which could have embryopathic consequences.

embryo by the ROS-initiating teratogens (Fig. 2), thereby enhancing their teratogenicity.

The embryonic and maternal roles of other NOSs have yet to be established, although increased limb malformations and other anomalies have been observed with NOS inhibitors, suggesting detrimental developmental effects with either too much or too little NO[•] (Fantel, 1996; Fantel and Person, 2002).

It would not be surprising if other indirect maternal determinants, including placental xenobiotic transporters and maternally generated cytokines, may prove to be important in modulating the teratological risk for particular ROS-initiating teratogens.

Embryonic ROS formation

During the embryonic period including organogenesis, most isoenzymes of the cytochromes P450 family (CYPs), which after birth and particularly in adulthood catalyze the bioactivation of many xenobiotics, are not expressed prenatally (Juchau et al., 1992, 1998). The few CYP isoenzymes that are measurably expressed during organogenesis (e.g. CYP1A1, CYP1B1, CYP2E1, CYP3A7) are low in human embryos and very low in rodent embryos (Juchau et al., 1992, 1998; Wells and Winn, 1996; Wells et al., 1997b). Although even these low levels appear to be sufficient for the embryonic bioactivation of some proteratogens (Juchau et al., 1998), for a number of reasons detailed elsewhere (Winn and Wells, 1995), including low embryonic CYP levels, substrate specificity and an embryopathic enhancement by pretreatment with CYP inhibitors in mouse models, CYPs do not appear to

contribute measurably to the embryonic bioactivation of most of the model teratogens discussed herein, at least in mouse models. In the later fetal period, however, the expression of prenatally expressed CYP isozymes increases, particularly in humans, as distinct from rodents. Increases in human fetal CYP1B1, CYP3A7 and CYP2E1 are particularly noteworthy, and the latter may be relevant to ROS production and functional teratogenesis, including intellectual and behavioral deficits, resulting from exposure to substrates like ethanol in the last trimester of pregnancy.

In contrast to low embryonic CYP expression, another family of enzymes that catalyze xenobiotic bioactivation, the prostaglandin H synthases (PHSs), is relatively highly expressed during both the embryonic and fetal periods, and hence likely candidates for embryonic and fetal xenobiotic bioactivation leading to both structural and functional teratogenesis. During the synthesis of prostaglandins, the hydroperoxidase component of PHS can co-oxidize xenobiotics to free radical intermediates that initiate ROS formation (Marnett, 1990; Wells and Winn, 1996; Wells et al., 1997b), and a similar reaction can occur via the embryonic lipoxygenase (LPO) pathway (Yu and Wells, 1995; Wells and Winn, 1996) (Fig. 5). Both PHS-1 (Winn and Wells, 1997) and PHS-2 (Parman and Wells, 2002) are constitutively expressed in mouse embryos, even though the latter is non-constitutive in most adult tissues. Using purified enzyme in an in vitro system, PHS can catalyze the bioactivation of several proteratogens to xenobiotic free radical intermediates, directly characterized by electron paramagnetic resonance spectrometry. These proteratogen substrates include phenytoin and several structurally

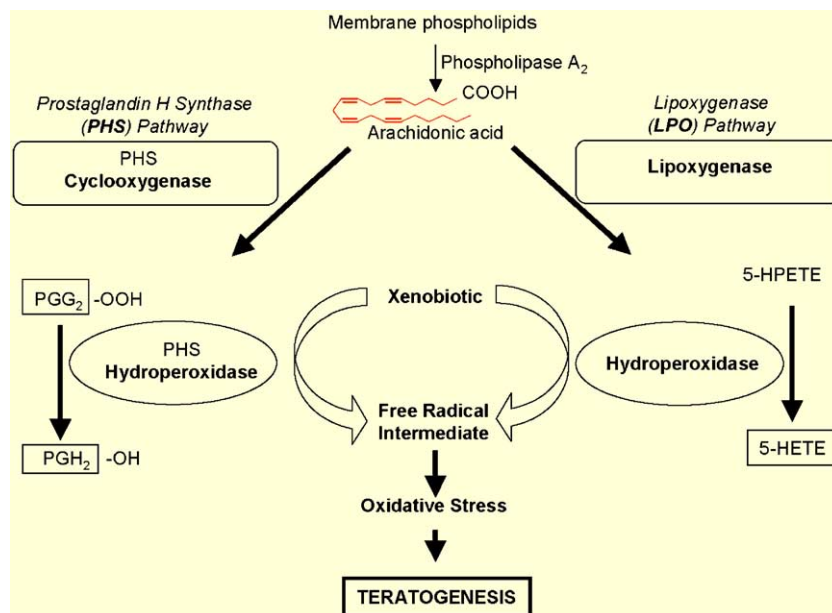


Fig. 5. Xenobiotic bioactivation by prostaglandin H synthases (PHSs) and lipoxygenases (LPOs). Cyclooxygenase and hydroperoxidase are the two catalytic components of PHS. During the synthesis of prostaglandins and eicosanoids, hydroperoxidases can oxidize xenobiotics to free radical intermediates that react directly or indirectly with oxygen to produce reactive oxygen species and oxidative stress.

related anticonvulsant drugs (phenobarbital, mephentyoin and its *N*-demethylated metabolite nirvanol, trimethadione and its *N*-demethylated metabolite dimethadione), benzo[a]pyrene and several amphetamine analogs, including methamphetamine (Wells and Winn, 1996; Wells et al., 1997a, 1997b; Parman et al., 1998; Jeng and Wells, 2004). The xenobiotic free radical intermediates initiate the formation of ROS, characterized by salicylate trapping of hydroxyl radicals (Kim and Wells, 1996c), which oxidize lipids, protein, glutathione (GSH) and DNA (Wells and Winn, 1996; Wells et al., 1997a; Winn and Wells, 1997). These reactions all are PHS-dependent, and are blocked by the PHS inhibitors acetylsalicylic acid (ASA, aspirin), indomethacin, eicosatetraenoic acid (ETYA) and/or the free radical spin trapping agent phenylbutylnitron (PBN) (Wells and Winn, 1996; Wells et al., 1997a; Parman et al., 1998, 1999). In vivo and/or in embryo culture, embryonic DNA oxidation and teratogenicity caused by phenytoin and structurally related anticonvulsant drugs in mice (Wells and Winn, 1996), and by the sedative/antileptotic drug thalidomide in rabbits (Arlen and Wells, 1996), are blocked by a single pretreatment with the PHS inhibitors ASA and/or ETYA. Similarly, PHS-2 knockout mice are less susceptible to benzo[a]pyrene-initiated fetal resorptions and teratogenicity (Parman and Wells, 2002). These results suggest that embryonic PHS-catalyzed bioactivation may play an important role in the mechanism of teratogenesis for some ROS-initiating teratogens. There is evidence in vivo and in embryo culture of a similar role

for embryonic LPOs in xenobiotic bioactivation and teratogenesis (Yu and Wells, 1995; Wells and Winn, 1996), and additional embryonic bioactivating enzymes may yet be discovered.

Another mechanism for embryonic ROS formation is via the redox cycling of catechol metabolites (Fig. 6). These hydroxylated metabolites may be produced by embryonic CYPs (Juchau et al., 1992, 1998), although it is not clear to what extent this mechanism contributes during organogenesis, particularly in rodents with very low embryonic activities of most CYPs. The contribution of this mechanism may increase during the fetal period as expression of some CYPs increases, particularly in human embryos. Alternatively, the embryo may be exposed via placental transfer of maternally generated hydroxylated metabolites, the latter being particularly likely with higher maternal xenobiotic exposure levels or when relevant maternal conjugating activity is deficient, as discussed earlier.

Lastly, embryonic ROS formation may be enhanced via a reperfusion phenomenon similar to that described in adult cases of cardiac dysfunction or stroke (Halliwell and Gutteridge, 1999). In development, the suppressive effect of some drugs on the rate or extent of myocardial contractility of the embryonic heart may cause hypoxia followed by reperfusion and the generation of ROS. This mechanism has been implicated in the teratogenicity of several teratogenic anticonvulsant drugs, including phenytoin, phenobarbital, trimethadione and carbamazepine, as

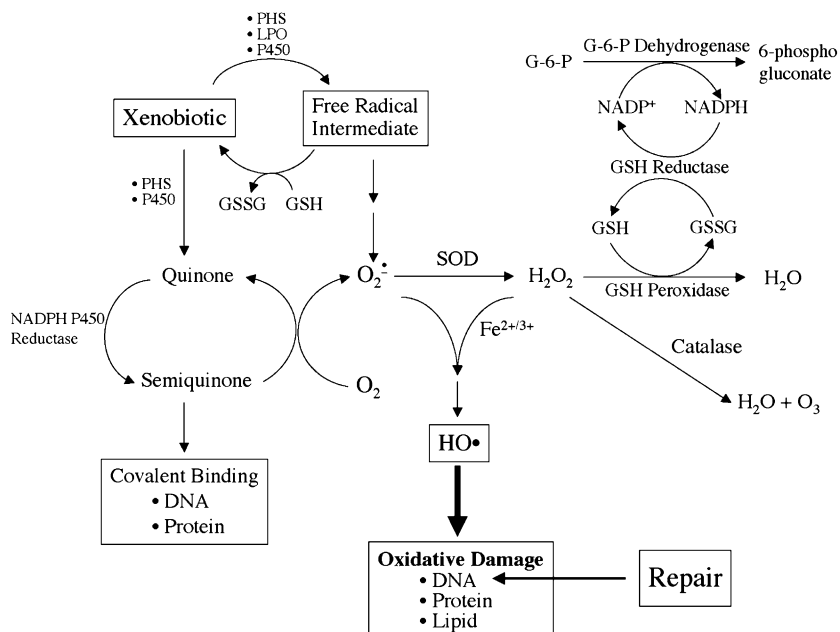


Fig. 6. Pathways contributing to the formation and detoxification of reactive oxygen species (ROS). In addition to the embryonic enzymatic bioactivation of xenobiotics to free radical intermediates, and the redox cycling of quinone metabolites, ROS also may be produced in the embryo via a hypoxia–reperfusion reaction secondary to the suppression of embryonic heart rate and/or contractility by some teratogens (see text). Abbreviations: G-6-P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; GSH, glutathione; GSSG, glutathione disulfide; LPO, lipoxigenase; PHS, prostaglandin H synthase; P450, cytochrome *P*450 (CYP); SOD, superoxide dismutase.

well as several NOS inhibitors and calcium channel blockers (Fantel, 1996; Azarbayjani and Danielsson, 2002; Fantel and Person, 2002). With anticonvulsant drugs like phenytoin, the initiating mechanism of cardiosuppression is postulated to involve drug inhibition of a specific potassium ion current known as the rapid activating component of delayed rectified potassium channels (I_{kr}) (Azarbayjani and Danielsson, 2002). Trimethadione has a lower cardiosuppressive effect than its dimethadione metabolite (Azarbayjani and Danielsson, 2002), consistent with their relative embryopathic potencies (Wells et al., 1989), although other mechanisms including substrate affinity for PHS-catalyzed bioactivation also may explain this difference (Wells et al., 1989). The potential importance of embryonic cardiosuppression due to such reversible, receptor-driven mechanisms would be expected to be increasingly relevant at higher maternal drug doses and fetal exposure, or in cases where maternal pathways for drug metabolism and elimination are deficient. Risk would be increased with sustained exposure, as in chronic maternal drug therapy, and lower with sporadic exposure, as in some cases of ROS-initiating drugs of abuse. On the other hand, the teratological relevance of this mechanism at lower embryonic xenobiotic concentrations is unclear. In the first studies to show that therapeutic concentrations (10–20 $\mu\text{g/ml}$) of phenytoin exerted a negative chronotropic effect on embryonic hearts, a maximal decrease in heart rate was achieved at a drug concentration (10 $\mu\text{g/ml}$) that was relatively non-embryopathic (Shanks et al., 1989). Also, in embryo culture at a higher but still therapeutic concentration of 20 $\mu\text{g/ml}$, phenytoin is equally embryopathic when added to the culture for only 4 h, or when included throughout the entire 24-h culture period, the latter precluding the opportunity for a reperfusion effect. The relative cardiac effects of several other ROS-initiating teratogens would be worth examining to further test the hypoxia/reperfusion hypothesis at lower xenobiotic concentrations, including: (1) other ROS-initiating teratogens that may not inhibit embryonic heart rate at clinically relevant concentrations (e.g. benzo[a]pyrene, thalidomide); (2) HPPH, the major hydroxylated metabolite of phenytoin, which is equipotent in causing oxidative DNA damage, genotoxicity and embryopathies (Kim et al., 1997c); (3) the stereoselectively embryopathic *l*-isomers of mephentoin and its more toxic nirvanol metabolite (Wells et al., 1982) and (4) some ROS-initiating teratogens like methamphetamine (Jeng et al., 2005) that at least in adults are cardiac stimulants rather than depressants.

In comparing the potential teratological relevance of ROS formation via peroxidase (e.g. PHS)-dependent xenobiotic bioactivation to the mechanisms involving catechol redox cycling and hypoxia/reperfusion, a number of distinctions are noteworthy. Phenytoin, HPPH (Wells and Winn, 1996; Kim et al., 1997c; Winn and Wells, 1997; Parman et al., 1998) and benzo[a]pyrene (Kim and Wells, 1996b; Kim et al., 1997a; Winn and Wells, 1997) initiate

PHS-dependent free radical formation, DNA oxidation and chromosomal damage using purified protein incubations and in cell culture, precluding a dependence on CYP-dependent catechol formation or suppression of cardiac function in these systems. In vivo and in embryo culture, the reduction in xenobiotic teratogenicity/embryopathy in PHS knockout mice (benzo[a]pyrene) (Parman and Wells, 2002) and in animals pretreated, or in embryos cocultured, with a PHS and/or LPO inhibitor (phenytoin, thalidomide, benzo[a]pyrene) (Arlen and Wells, 1996; Wells and Winn, 1996; Wells et al., 1997a; Winn and Wells, 1997) suggests that PHS-dependent ROS formation may play a critical role in teratogenesis, particularly at lower xenobiotic concentrations.

Oxidative damage to cellular macromolecules

Phenytoin, benzo[a]pyrene, thalidomide and methamphetamine all cause ROS-mediated oxidative damage to embryonic lipids, proteins, GSH and DNA (Wells and Winn, 1996; Wells et al., 1997a; Winn and Wells, 1997; Jeng et al., 2005), all of which potentially may play a role in teratogenesis. The teratological importance of ROS-mediated oxidative damage is consistent with the observations that the same strategies which reduce PHS-dependent hydroxyl radical formation and oxidative damage to cellular macromolecules (PHS knockout mice, PHS inhibitors, free radical spin trapping agents [PBN]) caused by these teratogens also reduce their teratogenicity (Arlen and Wells, 1996; Kim and Wells, 1996c; Kim et al., 1997a, 1997b; Wells et al., 1997a; Winn and Wells, 1997; Parman et al., 1998, 1999; Winn and Wells, 1999; Parman and Wells, 2002). Further evidence for the teratological importance of ROS-mediated oxidative macromolecular damage is provided by studies with thalidomide (Parman et al., 1999). Administration of thalidomide to rabbits, which like humans are susceptible to thalidomide teratogenicity, causes a substantial increase in embryonic DNA oxidation. In contrast, thalidomide does not increase embryonic DNA oxidation in mice, which are resistant to thalidomide teratogenicity. As noted above, pretreatment of rabbits with the free radical spin trapping agent PBN blocks both thalidomide-initiated embryonic DNA oxidation and teratogenicity. Finally, as discussed below, the protective effect afforded by embryonic antioxidants and antioxidative enzymes corroborates the teratological importance of ROS, and is consistent with a role for ROS-mediated oxidative macromolecular damage in the mechanism of teratogenesis.

Antioxidants and antioxidative enzymes

The ability of antioxidants (GSH, caffeic acid, vitamin E), iron chelators (desferoxamine), free radical spin trapping agents (PBN) and antioxidative enzymes to reduce the oxidative macromolecular damage and teratogenicity

caused by phenytoin, benzo[a]pyrene and thalidomide (using PBN) suggests a critical role for ROS in the mechanism of teratogenesis (Kim and Wells, 1996a; Ozolins et al., 1996; Wells and Winn, 1996; Kim et al., 1997c; Wells et al., 1997a; Winn and Wells, 1997, 1999; Parman et al., 1999; Nicol et al., 2000). As a corollary, interindividual variation in embryonic antioxidant levels and the activities of particular antioxidative enzymes would be expected to constitute important determinants of teratological risk. Various antioxidative enzymes (Figs. 1 and 6) have been found to protect against ROS-initiating teratogens like phenytoin and benzo[a]pyrene. Embryopathies are enhanced in mutant mice deficient in glucose-6-phosphate dehydrogenase (G6PD) (Nicol et al., 2000), by GSH depletion or inhibition of GSH synthesis, and by inhibition of GSH peroxidase or GSH reductase activities (Ozolins et al., 1996; Wells and Winn, 1996; Wells et al., 1997a). The protection afforded by these antioxidative enzymes is remarkable, since the embryonic activity of most is less than 5% of maternal activity (Ozolins et al., 1996; Wells and Winn, 1996; Wells et al., 1997a; Winn and Wells, 1997). The one known exception is embryonic G6PD, which is expressed at or above adult levels, and hence likely is a major embryoprotective enzyme (Nicol et al., 2000). This pathway may have particular clinical relevance, since G6PD deficiencies constitute the most common human enzymopathy. The deficiencies characterized in adults are consistent with a 200-fold range in G6PD activity found in human amniocytes (embryonic cells) obtained from a small group of patients (Fig. 3, lower panel), suggesting that interindividual variability in human embryonic G6PD activity may constitute an important determinant of teratological risk.

One cautionary note: preliminary reports suggest that alterations in the redox environment of the embryo and fetus may have contrasting effects depending upon the dose of antioxidant used and type of embryopathy characterized. In the case of cancer, the constitutively low level of most embryonic antioxidative enzymes suggests that endogenous ROS in biochemically-compromised embryos might provide the basis for an in utero origin for some spontaneous postnatal cancers. In cancer-prone *p53* knockout mice, postnatal cancer in the offspring was reduced by a low (0.1%) maternal dietary dose of the antioxidant vitamin E prior to and up to the end of pregnancy (Chen and Wells, 2004). However, a high dietary vitamin E dose (10%), while affording some protection against short-term embryopathies like fetal resorptions, in contrast enhanced the incidence of postnatal cancer in the offspring (Chen and Wells, 2005). Particularly in the latter case, the mechanism underlying the in utero enhancement of postnatal tumorigenesis by high-dose vitamin E may involve effects other than its antioxidative properties.

The protective effects of antioxidants and antioxidative enzymes corroborate an important role for ROS in the mechanism of teratogenesis for these xenobiotics. While the

data are consistent with a similar teratologically important role for oxidative macromolecular damage, they do not exclude a potential contribution from ROS-mediated signal transduction, discussed later.

Repair of oxidative DNA damage

Given that ROS oxidize all embryonic cellular macromolecules (lipids, proteins, GSH, RNA, DNA), one way of determining the causal relevance of a particular oxidized molecular target is to determine whether changes in its repair alter the embryopathic effects of a ROS-initiating teratogen (Fig. 2). Evidence to that effect also supports the importance of oxidative damage as distinct from drug-macromolecular adducts, ROS-mediated signal transduction or reversible, receptor-mediated interactions of the parent compound. Perhaps the current most definitive target for evaluation is DNA, since the pathways for repair of oxidative DNA damage are different from those for drug-DNA adducts and are relatively well understood compared to those for other macromolecular targets. As in the case of antioxidative enzymes, evidence of a modulatory role for specific pathways involved in the detection and repair of oxidative DNA damage would provide insights not only for the mechanism of teratogenesis, but also for a potential determinant of individual teratological risk.

Phenytoin, benzo[a]pyrene, thalidomide and methamphetamine all cause oxidative DNA damage as reflected by an increase in the level of 8-oxo-2'-deoxyguanosine (8-oxo-dG). This particular oxidative lesion is mutagenic, but perhaps more important for development, can reduce the rate and fidelity of transcription, which could be embryopathic. In vivo repair of 8-oxo-dG in the embryos of mice treated with phenytoin is high, matching that in maternal tissues, with a return to baseline levels within 24 h (Liu and Wells, 1995). DNA damage is detected by a pathway involving the ataxia telangiectasia mutated (ATM) protein, with the *p53* protein involved in both detection and repair via ATM-dependent and ATM-independent pathways. The teratogenicity of ionizing radiation (IR) and benzo[a]pyrene is enhanced in *Atm* (Laposa et al., 2004) and *p53* (Nicol et al., 1995) knockout mice, and a preliminary report indicates a similar enhancement in some phenytoin embryopathies in *p53* knockout mice (Wells and Winn, 1996; Laposa et al., 1997). ATM deficiencies may have particular clinical relevance, since even heterozygous *Atm* knockout mice exhibited enhanced susceptibility to the embryopathic effects of IR, and the incidence of *Atm* heterozygosity in the human population is relatively high (about 2%). These results suggest that DNA damage plays a role in the embryopathic mechanism, and that the activities for damage detection and repair are determinants of risk. More specifically, oxoguanine glycosylase 1 (Ogg1) is the rate-limiting enzyme in the direct repair of 8-oxo-dG, and

is expressed during the embryonic and fetal periods. Preliminary reports indicate that pregnant *ogg1* knockout mice treated with a single dose of methamphetamine show enhanced levels of 8-oxo-dG in several areas of fetal brain within a few hours, and the *null* offspring exhibit enhanced long-term postnatal neurodevelopmental deficits reflected by dysfunctional motor coordination (Wong et al., 2004). This is the most direct evidence to date that the 8-oxo-dG lesion in DNA plays a causal role in the embryopathic mechanism of ROS-initiating teratogens, and further suggests that variations in embryonic *Ogg1* activity may constitute a determinant of risk.

The results from knockout mice deficient in the detection and repair of oxidative DNA damage also suggest a specific teratological contribution from this macromolecular lesion, as distinct from oxidative damage to embryonic proteins or lipids, and as distinct from ROS-mediated signal transduction, the formation of covalent xenobiotic-DNA and xenobiotic-protein adducts and the reversible binding of the xenobiotic to an embryonic receptor. Although implicating oxidative DNA damage in the teratological mechanism, these studies do not exclude embryopathic contributions from the oxidation of lipids and proteins, the causal effects of which have yet to be elucidated.

ROS-mediated signal transduction

In addition to causing oxidative damage to embryonic cellular macromolecules, ROS may interact reversibly with transduction proteins to enhance signal transduction pathways at critical times during structural and/or functional development (Fig. 2). This hypothesis also would be consistent for the previously discussed evidence for ROS formation and antioxidative protection. One such ROS-related signal transduction pathway includes the successive activation of Ras and nuclear factor-kappa B (NF- κ B) proteins. Phenytoin enhances the expression of Ras in embryo culture, and blocking phenytoin-enhanced embryonic Ras expression by coincubation with a farnesyl-protein transferase inhibitor also inhibited phenytoin embryopathies (Winn and Wells, 2002), indicating a role for Ras-dependent signal transduction in the teratological mechanism. Using a transgenic reporter mouse with NF- κ B-dependent beta-galactosidase expression, phenytoin was shown in embryo culture to enhance the expression of embryonic NF- κ B (Kennedy et al., 2004). Preincubation with NF- κ B antisense oligonucleotides blocked both the phenytoin-enhanced NF- κ B expression and phenytoin embryopathies. These studies suggest that ROS-mediated signal transduction involving Ras and NF- κ B plays a role in the mechanism of phenytoin teratogenicity. NF- κ B expression has also been implicated in the mechanism of thalidomide teratogenicity (Hansen et al., 2002), and may be similarly involved in the embryopathic mechanism for other ROS-initiating teratogens.

Conclusions

The embryopathic effects of ROS-initiating teratogens may involve both oxidative damage to embryonic cellular macromolecules and enhanced embryonic signal transduction (Fig. 2). Teratogenicity likely depends to a large extent upon a balance between the pathogenic pathways of xenobiotic bioactivation, oxidative macromolecular damage and signal transduction on one hand, and on the other, the protective pathways of maternal elimination, embryonic detoxification of xenobiotic reactive intermediates and reactive oxygen species, and embryonic pathways for the detection and repair of oxidative DNA damage (Fig. 1). Individual risk would be expected to increase in proportion to the number of pathways altered by either genetic or environmental mechanisms, and the magnitude of each alteration, with embryopathies occurring at doses or exposure levels and plasma concentrations of xenobiotics that may fall within the therapeutic or generally safe range. Whether these insights from animal models are relevant to human teratogenesis remains to be determined.

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