TOXICOLOGICAL SCIENCES **108(1)**, 4–18 (2009)
doi:10.1093/toxsci/kfn263
Advance Access publication January 6, 2009

REVIEW

Oxidative Stress in Developmental Origins of Disease: Teratogenesis, Neurodevelopmental Deficits, and Cancer

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Received May 23, 2008; accepted December 11, 2008

In the developing embryo and fetus, endogenous or xenobiotic-enhanced formation of reactive oxygen species (ROS) like hydroxyl radicals may adversely alter development by oxidatively damaging cellular lipids, proteins and DNA, and/or by altering signal transduction. The postnatal consequences may include an array of birth defects (teratogenesis), postnatal functional deficits, and diseases. In animal models, the adverse developmental consequences of *in utero* exposure to agents like thalidomide, methamphetamine, phenytoin, benzo[*a*]pyrene, and ionizing radiation can be modulated by altering pathways that control the embryonic ROS balance, including enzymes that bioactivate endogenous substrates and xenobiotics to free radical intermediates, antioxidative enzymes that detoxify ROS, and enzymes that repair oxidative DNA damage. ROS-mediated signaling via Ras, nuclear factor kappa B and related transducers also may contribute to altered development. Embryopathies can be reduced by free radical spin trapping agents and antioxidants, and enhanced by glutathione depletion. Further modulatory approaches to evaluate such mechanisms *in vivo* and/or in embryo culture have included the use of knockout mice, transgenic knock-ins and mutant deficient mice with altered enzyme activities, as well as antisense oligonucleotides, protein therapy with antioxidative enzymes, dietary depletion of essential cofactors and chemical enzyme inhibitors. In a few cases, measures anticipated to be protective have conversely enhanced the risk of adverse developmental outcomes, indicating the complexity of development and need for caution in testing therapeutic strategies in humans. A better understanding of the developmental effects of ROS may provide insights for risk assessment and the reduction of adverse postnatal consequences.

Key Words: **teratogenesis; neurodevelopmental deficits; cancer; oxidative stress; reactive oxygen species; phenytoin; benzo[a]pyrene; methamphetamine; thalidomide; ionizing radiation.**

Exposure of the developing embryo or fetus to some environmental agents like gamma irradiation and thalidomide is known to produce anatomical anomalies leading to *in utero* death or structural birth defects, commonly termed teratogenesis. Perhaps less well appreciated is that such environmental exposures also can cause functional disorders that persist postnatally and into adult life. The spectrum of such postnatal consequences is growing, and more recently is thought to include disorders of the immune system, brain function, obesity and diseases such as diabetes and cancer, to name a few. The nature and underlying mechanisms of the developmental basis for such postnatal consequences have long served as the focus of national scientific societies like the American Teratology Society (www.teratology.org), and a growing awareness of the extensive range of consequences has led more recently to the founding of the International Society for Developmental Origins of Health and Disease (www.dohadsoc.org). Such societies provide useful information about this emerging field. For the purposes of this review, all such structural and functional abnormalities are collectively referred to as teratogenesis.

This review is based largely on a keynote lecture presented in 2007 at the 11th International Congress of Toxicology in Montreal, and focuses upon molecular and biochemical mechanisms underlying the adverse effects of reactive oxygen species (ROS) and oxidative stress on embryonic and fetal development. This review does not address receptor-mediated mechanisms like the binding of xenobiotics to the retinoic acid receptor, nor the bioactivation of xenobiotics to electrophilic reactive intermediates that bind covalently (irreversibly) to cellular macromolecules (proteins, DNA), which is reviewed elsewhere (Juchau *et al.*,

An earlier version of this review was presented as a keynote lecture at the 11th International Congress of Toxicology, Montreal, Quebec, July 2007. Proceedings, No. K3.0, 2007.

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1998; Wells and Winn, 1996; Wells *et al.*, 1997; Winn and Wells, 1995). In the latter case, xenobiotics like phenytoin and benzo[*a*]pyrene can be bioactivated by enzymes like the cytochromes P450 (CYPs), but the developing embryo and fetus have relatively low levels of most CYP isozymes, and electrophilic reactive intermediates generally are too unstable to be transported out of the maternal liver, across the placenta and into the conceptus (embryo or fetus and related tissues). Accordingly, despite some supporting evidence (Juchau *et al.*, 1998; Wells and Winn, 1996; Wells *et al.*, 1997; Winn and Wells, 1995), it is difficult to ascribe a substantial contribution of this mechanism to teratogenesis during the earlier period of organogenesis, although a role may emerge during the later fetal period as the expression of some CYPs increases, particularly in humans.

The data presented herein from the authors' laboratory are expanded and extended by results from other laboratories, but do not constitute a comprehensive review of the relevant literature. Primary references are provided only for more recent publications, whereas reviews are cited to provide references for most of the older literature. Recent reviews also are cited where possible to direct the reader to related primary references describing new ROS-related teratological research beyond the scope of this review. A relatively limited number of ROS-initiating teratogenic agents are discussed herein as examples, including the sedative drug thalidomide, the illicit drug methamphetamine, the antiepileptic drug (AED) phenytoin, the environmental chemical benzo[*a*]pyrene and ionizing radiation. These agents each have both different and corroborating advantages as models, and differing developmental effects. Most of the studies discussed are in mouse models, which offer the advantage of genetically altered strains for testing mechanisms. Other species are used when necessary, as exemplified by studies of thalidomide teratogenicity, to which rabbits, like humans, but not rodents, are susceptible. Virtually no similar information is known for humans. To examine both molecular mechanisms and their developmental consequences, a combination of approaches is discussed, including *in vitro* studies with isolated enzymes and macromolecular targets (proteins, DNA), cellular models, whole embryo culture and studies of pregnant animals.

Key times of susceptibility include the period of organogenesis, when organs are developing (in the mouse, approximately gestational days [GDs] 8–15 of a 20-day pregnancy), and the fetal period (GDs 16–20). Exposures during the embryonic period typically result in embryonic death or structural birth defects, whereas exposure during the later fetal period result in functional deficits such as neurodevelopmental deficits, although there are numerous exceptions to these generalizations. Exceptions might be anticipated with xenobiotics that are cleared slowly from the embryo and remain in high concentration during the fetal period, and similarly in cases of irreversible damage to embryonic DNA, which in the absence of adequate repair persists in the fetus. Developmental outcomes of *in utero* exposures covered herein include major structural abnormalities (e.g., cleft palates and exencephaly) and their consequences (e.g., fetal death [resorptions]), postnatal functional abnormalities like neurodevelopmental deficits (e.g., motor coordination deficits), and adult disease exemplified by cancer. Such adverse outcomes constitute a relatively small portion of the known spectrum of developmentally initiated disorders, and we still have much to learn about both the full spectrum and the underlying mechanisms.

What follows is a brief review of oxidative stress, followed by sections on how teratogenic agents can initiate oxidative stress, antioxidative mechanisms for eliminating potentially toxic ROS, and protective pathways for repairing oxidative DNA damage. A final section briefly covers a rapidly emerging field elucidating developmental effects of ROS through signal transduction.

ROS AND OXIDATIVE STRESS

ROS such as hydrogen peroxide and hydroxyl radicals are formed via a variety of physiological and pathophysiological reactions (Fig. 1) (Halliwell and Gutteridge, 1999), and ROS formation can be enhanced by radiation and xenobiotics, including drugs and environmental chemicals (Fantel, 1996; Hitchler and Domann, 2007; Wells and Winn, 1996; Wells *et al.*, 1997, 2005; Winn and Wells, 1995). These short-lived ROS can play physiological roles in signal transduction, but in excess can contribute to the mechanisms of disease by dysregulation of signal transduction and/or by oxidative damage to cellular macromolecules (lipids, proteins, DNA, RNA, carbohydrates) that exceeds the cellular capacity for regeneration or repair (Fig. 2). As elaborated later under signal transduction, dysregulation of signal transduction and/or macromolecular lesions can adversely alter cellular function

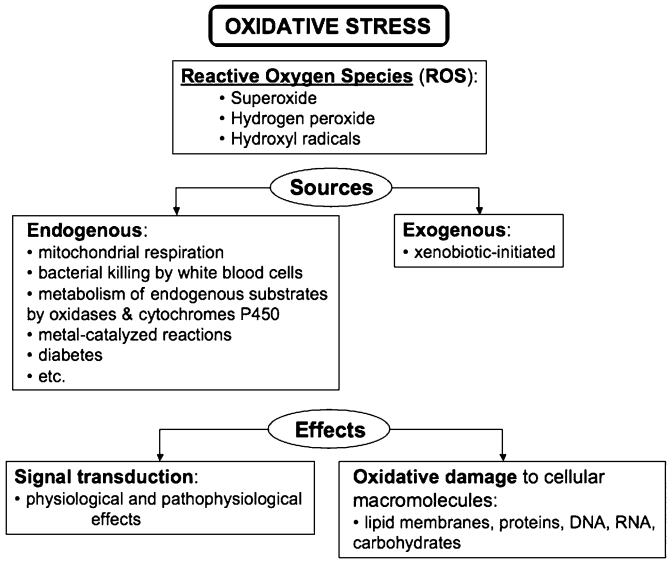

FIG. 1. Sources of ROS and the general mechanisms by which oxidative stress can alter cellular function.

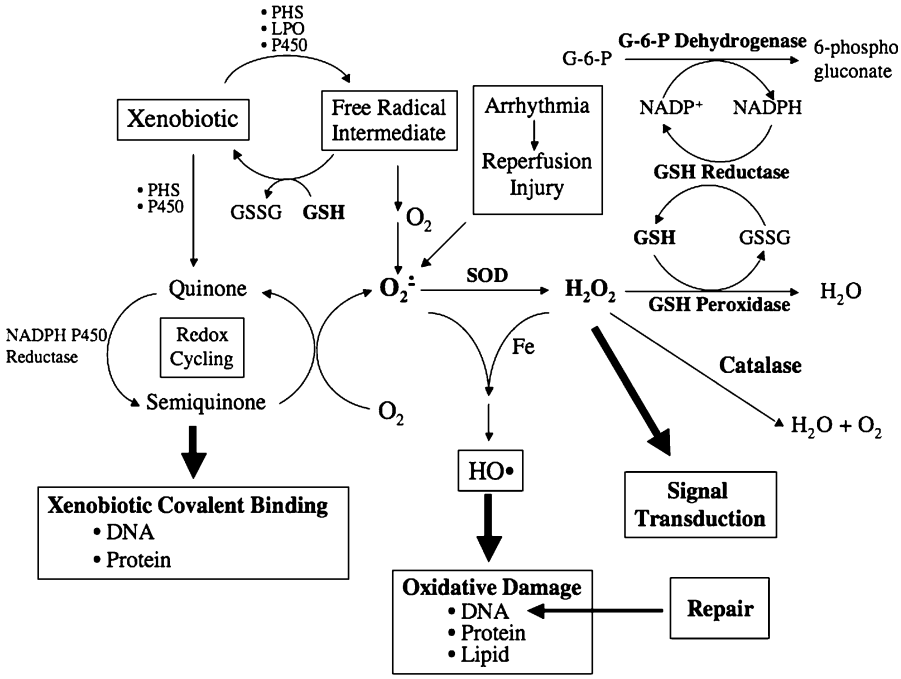

FIG. 2. Biochemical pathways for the formation, detoxification and cellular effects of xenobiotic free radical intermediates and ROS. Abbreviations: Fe, iron; GSSG, glutathione disulfide; H_2O_2, hydrogen peroxide; $HO^{\bullet}$, hydroxyl radical; $NADP^{+}$, nicotinamide adenine dinucleotide phosphate; $O_2^{\bullet}$, superoxide; P450, cytochromes P450 (modified from: Wells *et al.*, Mutat. Res. 396, 65–78, 1997).

or trigger apoptotic or necrotic cellular death. A battery of antioxidants (e.g., vitamins C and E) and antioxidative enzymes offer either direct or indirect protection against ROS. Although the levels of most antioxidative enzymes with the exception of glucose-6-phosphate dehydrogenase (G6PD) are low in the embryo, there is some evidence that they nevertheless may provide protection against at least constitutive or physiological levels of ROS, if not drug-enhanced ROS formation.

XENOBIOTIC-ENHANCED ROS FORMATION

Xenobiotic-enhanced ROS formation can occur via several mechanisms (Fig. 2). Some xenobiotics or their hydroxylated metabolites can redox cycle, whereby a single xenobiotic molecule can generate an amplified production of ROS (Halliwell and Gutteridge, 1999; Juchau *et al.*, 1992). The potential for this mechanism has been demonstrated in embryo culture, which lacks a maternal contribution (Juchau *et al.*, 1992). Usually, such hydroxylated metabolites would be formed and conjugated with water soluble endogenous substrates like glucuronic acid in the maternal liver, and the glucuronide-xenobiotic conjugate eliminated in the maternal urine without reaching the embryo (Wells *et al.*, 2005). If so, the redox cycling mechanism may be most likely to contribute to teratogenesis when the mother has a deficiency in enzymes like the UDP-glucuronosyltransferases (UGTs), which catalyze glucuronidation. Also, in some cases, the xenobiotic free radical intermediate formed during redox cycling may covalently bind to cellular macromolecules, forming a xenobiotic-macromolecular adduct that alters cellular function.

Unlike the low to negligible levels of most CYPs, the embryo has high levels of enzymes with or associated with peroxidase activities, like prostaglandin H synthases (PHSs) and lipoxygenases (LPOs), which can convert teratogens like phenytoin and related AEDs, benzo[*a*]pyrene, and methamphetamine to free radical intermediates that initiate ROS formation (Jeng *et al.*, 2006; Parman *et al.*, 1998; Wells *et al.*, 2005). This is discussed later under embryonic xenobiotic bioactivation.

Some xenobiotics like phenytoin, structurally related AEDs and several antiarrythmic agents have been shown to reduce embryonic heart rate (Danielsson *et al.*, 2007; Shanks *et al.*, 1989), and ROS formation associated with reperfusion following restoration of normal heart rate has been implicated in the teratological mechanism of these agents (Danielsson *et al.*, 2007). Because embryopathies have been observed at lower phenytoin concentrations that do not reduce embryonic heart rate (Shanks *et al.*, 1989), this reperfusion mechanism for ROS generation seems most likely to contribute at higher xenobiotic concentrations.

EMBRYOPATHIC ROLE OF ROS AND REACTIVE NITROGEN SPECIES

The embryopathic potential of constitutive levels of ROS in the absence of drug treatment is revealed in a number of genetically altered mouse strains, including mutant mice

deficient in the antioxidative enzymes G6PD and catalase, and knockout mice lacking the ataxia telangiectasia mutated (ATM) protein important in DNA damage response and repair. These examples are discussed later.

Some of the evidence supporting an embryopathic role of ROS in the molecular mechanism of teratogenesis for a number of xenobiotics (phenytoin and structurally related AEDs, benzo[*a*]pyrene, methamphetamine) is elaborated later, but includes (1) enhanced hydroxyl radical formation and/or oxidation of embryonic proteins, glutathione (GSH) and/or DNA by these xenobiotics; (2) protection by inhibitors of PHS or LPO (acetylsalicylic acid [ASA], eicosatetraynoic acid [ETYA]) that catalyze xenobiotic bioactivation to a free radical intermediate, and similar protection in PHS knockout mice; (3) protection by free radical spin trapping agents (phenylbutylnitrone [PBN], salicylate); (4) enhanced embryopathies in mutant mice deficient in antioxidative enzymes (G6PD, catalase), and in mice with antioxidative activities reduced by nutritional deficiency (GSH peroxidases reduced by selenium deficiency) or inhibitors (GSH reductase inhibited by bis-chloroethylnitrosourea [BCNU] treatment); (5) protection by protein therapy with antioxidative enzymes (superoxide dismutase [SOD], catalase), and by antioxidants (vitamin E, caffeic acid); and (6) enhanced embryopathies with depletion of GSH (Wells and Winn, 1996; Wells *et al*., 1997, 2005; Winn and Wells, 1995). Additional support for ROS in the mechanism of teratogenesis is revealed by an accident of nature in the species-specific susceptibility to thalidomide teratogenicity. Thalidomide causes embryonic oxidative DNA damage in the rabbit, which is susceptible to birth defects similar to those observed in thalidomide-exposed humans, but does not increase DNA oxidation in mice, which are not susceptible to thalidomide teratogenicity (Parman *et al*., 1999). Both the enhanced embryonic DNA damage and teratogenicity caused by thalidomide in rabbits are inhibited by pretreatment of the pregnant doe with the free radical spin trapping agent PBN. These species-dependent differences with thalidomide show that ROS-mediated alterations in signal transduction and/or macromolecular damage, possibly including DNA oxidation, may play a critical role in the molecular mechanism of teratogenesis.

Reactive nitrogen species (RNS) might be expected to have adverse developmental effects on their own and/or in concert with ROS (Fig. 3). As with ROS, these effects could be mediated at the molecular level via dysregulation of signal transduction and/or macromolecular damage, as elaborated later under signal transduction. Although less is known about the developmental effects of RNS, there is evidence that they can contribute to the teratological mechanism of some xenobiotics (Fantel and Person, 2002), including phenytoin (Kasapinovic *et al*., 2004), although at least in the case of phenytoin RNS effects cannot fully account for its embryopathic effects in nitric oxide synthase knockout mice.

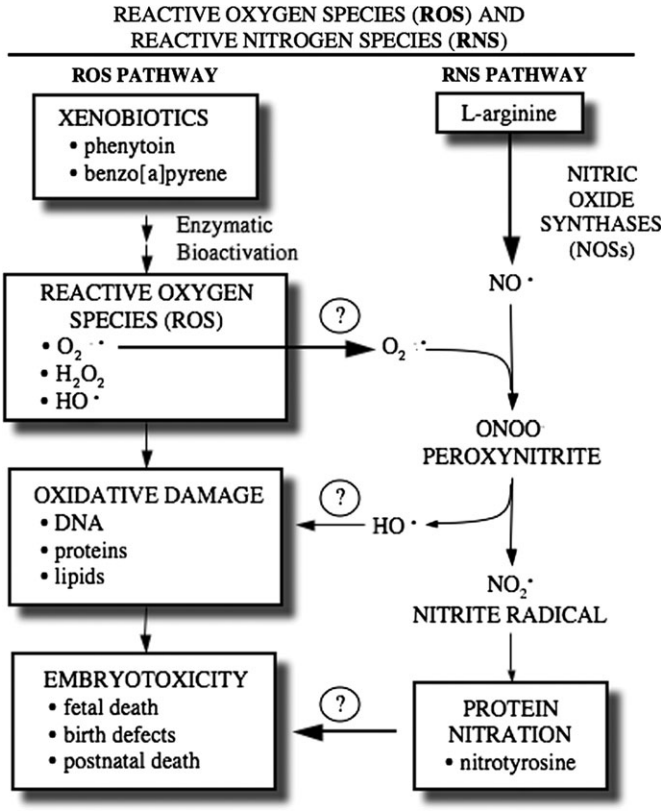

FIG. 3. Potential interactions between the pathways for ROS and RNS (from: Kasapinovic *et al*., Free Radic. Biol. Med. 37, 1703–1711, 2004).

EMBRYONIC XENOBIOTIC BIOACTIVATION

For drugs like phenytoin and structurally related AEDs, benzo[*a*]pyrene and thalidomide, xenobiotic bioactivation to a free radical intermediate by PHS or LPO within the embryo or fetus can be a critical determinant of teratogenesis (Fig. 4). PHS catalyzes the bioactivation of at least phenytoin and related AEDs to free radical intermediates, and the levels of PHS protein are high in both the embryo and fetus (Parman and Wells, 2002; Parman *et al*., 1998). This includes not only the PHS-1 isozyme that is constitutive in virtually all adult tissues, but also the PHS-2 isozyme that in most adult tissues is nonconstitutive. The teratogenicity of phenytoin and structurally related AEDs in mice, and thalidomide in rabbits, can be blocked by pretreatment of the pregnant dam or doe with the PHS inhibitor ASA, and benzo[*a*]pyrene embryopathies are reduced in PHS-2 knockout mice (Wells *et al*., 2005), consistent with an initiating role for PHS-catalyzed xenobiotic bioactivation.

ANTIOXIDANTS

The teratogenicity of phenytoin can be reduced by pretreatment of the pregnant dam with the water soluble

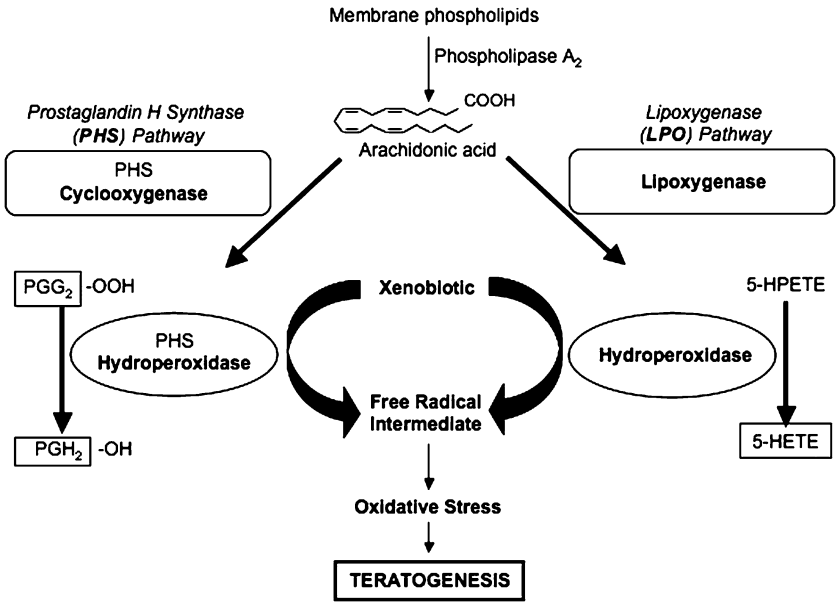

FIG. 4. Bioactivation of xenobiotics via the PHS and LPO pathways—postulated role in teratogenesis. The hydroperoxidase component of embryonic and fetal PHSs, and hydroperoxidases associated with LPOs, can oxidize xenobiotics to free radical intermediates that initiate the formation of ROS causing oxidative stress (modified from: Yu and Wells, Toxicol. Appl. Pharmacol. 131, 1–12, 1995).

antioxidant caffeic acid and with the lipid soluble vitamin E (Wells *et al*., 2005; Winn and Wells, 1995), consistent with an embryopathic role for ROS. However, the actions of such antioxidants can be complex, and vitamin E in particular has numerous activities unrelated to its antioxidant effects, all of which are dose-, time- and tissue/cell-dependent, so differences in the design of animal studies, or in the environmental as well as genetic characteristics of subjects in human studies, may produce conflicting results. At higher doses, vitamin E conversely can enhance oxidative DNA damage in a tissue-selective fashion (Chen and Wells, 2006), and enhance phenytoin embryopathies including fetal death (Wells *et al*., 2005), illustrating the potential dangers inherent in designing protective strategies.

In considering the fetal basis of adult diseases, the low embryonic and fetal levels of most antioxidative enzymes theoretically would be expected to leave them at higher risk for the *in utero* initiation of ROS-related diseases manifested in later postnatal life. Because ROS can initiate oxidative DNA damage leading to mutations, as well as enhance the process of cancer promotion, it might be expected that some postnatal cancers might be initiated and/or promoted by ROS *in utero*. This subject is comprehensively reviewed elsewhere (Winn and Wan, 2006). In our laboratory, alterations in the fetal and embryonic redox environment were investigated in a p53 knockout mouse model that exhibits a high, gene dose-dependent incidence of spontaneous postnatal tumors. When p53-deficient dams were fed a diet supplemented during pregnancy with low-dose (0.1%) vitamin E, the incidence of postnatal tumors in the heterozygous p53-deficient offspring was reduced, consistent with a role for constitutive ROS in the *in utero* initiation and/or promotion of cancer (Chen *et al*., in press). Conversely, when the same p53 knockout model received dietary supplementation during pregnancy with a very high dose of vitamin E (10%), despite a reduction in fetal death, both the heterozygous and homozygous p53-deficient offspring exhibited an enhanced rate of postnatal tumorigenesis (Chen and Wells, 2006). The high-dose vitamin E exposure resulted in tissue-dependent differences in vitamin E levels and oxidative DNA damage, with paradoxical increased oxidative DNA damage in some but not all tissues, suggesting the potential for a cell-specific role of oxidative stress in the increased rate of tumorigenesis. It nevertheless should be remembered that vitamin E has numerous effects unrelated to its antioxidant activity. As with the teratological studies above, the converse, dose-dependent modulatory effects of vitamin E dietary supplementation illustrate both the developmental complexity and a cautionary requirement in considering therapeutic strategies.

ANTIOXIDATIVE ENZYMES

The embryonic level of most antioxidative enzymes (Fig. 2) is around only 5% of maternal activity (Wells *et al*., 2005). Early organogenesis stage embryos are particularly sensitive to toxic insult during the transition phase from anaerobic to aerobic metabolism coinciding with the maturation of mitochondrial structure and function. This may reflect the observations that low levels of antioxidant enzyme activities

increase as organogenesis proceeds, and that early in organogenesis the embryo may not be able to respond as effectively to oxidative imbalances (Choe *et al*., 2001; Hansen, 2006). One notable exception is G6PD, the embryonic and fetal activities of which are equal to or greater than maternal activity, suggesting an important role in development (Nicol *et al*., 2000). This possibility is not widely appreciated, and most textbooks of medicine limit the potential clinical consequences of G6PD deficiencies in humans to enhanced red blood cell hemolysis following exposure to oxidant drugs such as antimalarial agents. However, even pregnant mutant G6PD-deficient mice with no xenobiotic exposure have a significantly enhanced incidence of fetal death, and in the surviving offspring, enhanced neonatal death (Nicol *et al*., 2000). These observations show the embryopathic potential of even physiological levels of ROS in the absence of normal antioxidative protection. This possibility is consistent with the results of preliminary studies focused upon another antioxidative enzyme, catalase, which unlike G6PD is present in the embryo with only about 5% of maternal activity. Although such enzymatic levels might be considered too low for antioxidative protection, preliminary studies found that mutant acatalasemic embryos with even lower activity had a higher incidence of developmental abnormalities in embryo culture (Perstin and Wells, 2007). Similar evidence for the embryopathic potential of constitutive ROS is found in *atm* knockout mice discussed next under DNA repair.

Antioxidative enzymes can be similarly protective against ROS-initiating teratogens, implicating ROS in their teratological mechanisms. Mutant G6PD-deficient embryos are more susceptible to the embryopathic effects of phenytoin, and the risk is both G6PD gene dose-dependent and phenytoin dose-dependent (Nicol *et al*., 2000). This is particularly remarkable because some medical texts in discussing the risk for red blood cell hemolysis in G6PD-deficient patients list phenytoin as one of the drugs that is safe (for adults) to take. The results from G6PD-deficient mice suggest that this would not be wise advice for pregnant women. Inhibition of other antioxidative enzymes by dietary depletion of selenium, reducing GSH peroxidase, and by chemical inhibition with BCNU, inhibiting GSH reductase, similarly enhance susceptibility to phenytoin teratogenesis (Wells *et al*., 2005; Winn and Wells, 1995). Conversely, protein therapy in embryo culture using catalase or SOD stabilized by conjugation with polyethylene glycol (PEG) provided enzyme dose-dependent protection against both embryonic oxidative DNA damage and embryopathies caused by phenytoin and benzo[*a*]pyrene (Wells *et al*., 2005; Winn and Wells, 1995). Perhaps more interesting from the perspective of potential therapeutic strategies, *in vivo* pretreatment of pregnant dams with PEG-conjugated catalase substantially enhanced the embryonic activity of catalase, and provided catalase dose-dependent protection against phenytoin teratogenicity (Winn and Wells, 1999). Once again, however, a cautionary insight was revealed by the converse enhancement in phenytoin teratogenicity by maternal pretreatment with PEG-SOD, possibly due to enhanced hydrogen peroxide production via the exogenous SOD that exceeded the capacity of the constitutive downstream catalase (Fig. 2).

OXIDATIVE DNA DAMAGE RESPONSE AND REPAIR

Recent comprehensive reviews discuss the repair of oxidative DNA damage (David *et al*., 2007), and more particularly the potential role of DNA damage detection and repair in teratogenesis (Hales, 2005). One of the most prevalent of about 20 forms of oxidative DNA damage caused by hydroxyl radicals is the formation of 8-hydroxyguanine, or its physiologically prevalent keto form, 7,8-dihydro-8-oxoguanine, commonly termed 8-oxoguanine (8-oxoG) (Fig. 5). There are several mechanisms whereby the accumulation of 8-oxoG may contribute to teratological outcomes. 8-OxoG formation in dividing developing cells may lead to transversion mutations, which can affect the expression and activity of proteins required for normal development and function. In addition to these effects, the 8-oxoG lesion may alter gene transcription via several potential mechanisms. Conflicting evidence exists regarding the ability of 8-oxoG or 8-oxoG-derived G:C to T:A transversions to disrupt the function of RNA polymerase II. Stalling of basal transcriptional machinery by the introduction of 8-oxoG lesions to the DNA template has been observed (Viswanathan and Doetsch, 1998), but other studies suggest that only DNA helix-distorting changes (single-strand breaks, pyrimidine dimers) and not oxidized bases may be capable of blocking DNA and RNA polymerases (Kathe *et al*., 2004). These experimental discrepancies may be explained by recent observations that the repair level and/or the transcriptional

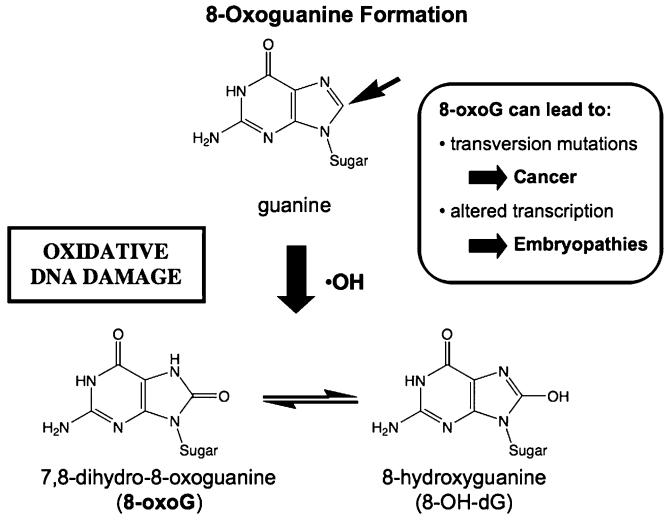

FIG. 5. Reaction of hydroxyl radicals ($HO^{\bullet}$) with guanine residues of DNA to form the molecular lesion 7-8,dihydro-8-oxoguanine (8-oxoG). If not repaired, this oxidative damage can cause mutations and/or altered gene transcription, which may lead to cancer and/or embryopathies.

arrest caused by a unique 8-oxoG lesion varies according to the promoter strength and nucleotidic sequence surrounding the lesion (Pastoriza-Gallego *et al*., 2007). Transcriptional mutagenesis via 8-oxoG bypass has been documented in mammalian systems (Charlet-Berguerand *et al*., 2006), and this mechanism has been shown to lead to phenotypic changes in bacterial systems (Viswanathan *et al*., 1999). 8-OxoG lesions may also effect the expression of specific genes via their ability to regulate the binding efficiency of transcription factors such as nuclear factor kappa B (NF-κB) to specific promoter elements (Hailer-Morrison *et al*., 2003). The potential for 8-oxoG lesions to affect specific cellular pathways is supported by recent observations that the *in vivo* accumulation of 8-oxoG is not randomly distributed throughout the genome, and that there are susceptible genomics sites that are likely to be cell type and stimulus specific (Toyokuni, 2008). Accumulation of oxidative DNA damage can also lead to apoptosis, and a number of cellular studies have implicated human 8-oxo-guanine glycosylase-1 (hOGG1) protein as an inhibitor of oxidative stress-induced apoptosis (Harrison *et al*., 2005; Hyun *et al*., 2003; Youn *et al*., 2007).

All of the model agents discussed herein (phenytoin and structurally related AEDs, benzo[*a*]pyrene, thalidomide, methamphetamine, gamma irradiation) enhance embryonic and fetal 8-oxoG levels (Jeng *et al*., 2005; Wells *et al*., 2005). Of the agents investigated, all also enhance the oxidation of GSH, proteins and lipids, as might be expected, and the focus herein on oxidative DNA damage and 8-oxoG in particular does not preclude teratological contributions from other oxidative DNA and RNA lesions, or other oxidatively damaged cellular macromolecules.

One reason for the focus upon oxidative DNA damage, as distinct from oxidative damage to other cellular macromolecules (proteins, lipids), or ROS-mediated signal transduction, is that several different yet complementary approaches can be used to determine the causative role of oxidative DNA damage in the molecular mechanism of teratogenesis. These approaches involve the modulation of several important proteins that detect and respond to DNA damage (p53, ATM), or play a direct role in the repair of oxidative DNA damage, and 8-oxoG in particular (OGG1, Cockayne Syndrome B [CSB], formamidopyrimidine DNA glycosylase [FPG]) (Fig. 6). In the studies below, the levels of key proteins controlling rate-limiting steps in the response to and repair of 8-oxoG were decreased in knockout mice, or increased in stably transfected cells and transgenic mice with germ cell transmission of the relevant gene, the latter of which include bacterial *fpg* and human *OGG1*. If 8-oxoG is a pathogenic molecular lesion, then genetically altered cells and mice with an increased expression of these protective proteins contributing to oxidative DNA damage response and repair should exhibit reduced cytotoxicity or teratogenesis, whereas those with decreased protein levels should exhibit enhanced teratogenesis. In addition to implicating oxidative DNA damage in the mechanism of teratogenesis, these approaches also differentiate the contribution of oxidative DNA damage

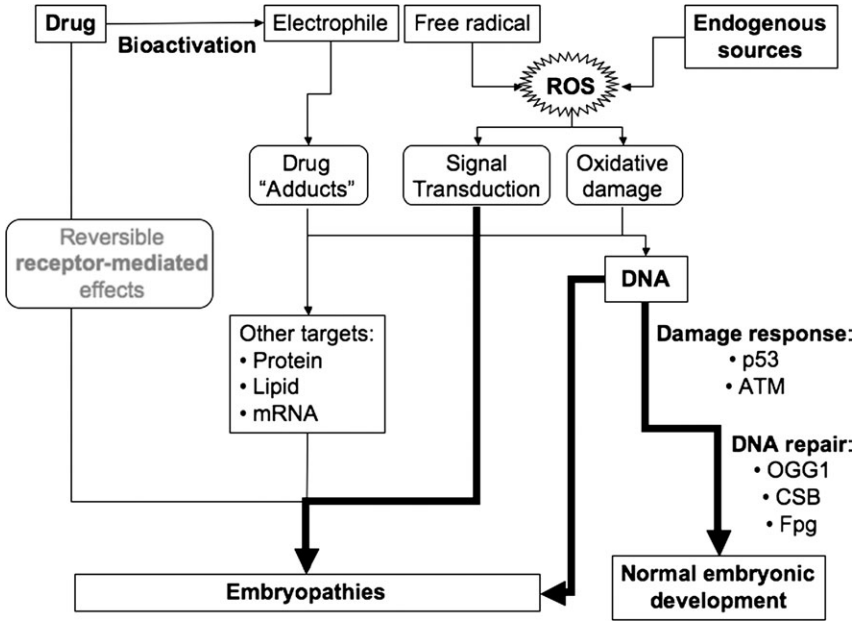

FIG. 6. Strategy for distinguishing competing mechanisms potentially leading to teratogenesis. Changes in teratological outcomes resulting from modifying the pathways involved in DNA damage response and repair help distinguish the role of oxidative DNA damage from oxidative damage to other cellular macromolecules (protein, RNA, lipids, carbohydrates), as well as from ROS effects via signal transduction. Similarly, changes in teratological outcomes due to modifications in antioxidants and antioxidative enzymes help distinguish the role of ROS from mechanisms involving electrophilic xenobiotic reactive intermediates, and reversible, receptor-mediated interactions.

from other potential mechanisms, including oxidative damage to other cellular macromolecules, ROS-mediated signal transduction, drug-macromolecular adducts and reversible, drug receptor-mediated interactions (Fig. 6).

OXIDATIVE DNA DAMAGE RESPONSE

As with several antioxidative pathways discussed above, DNA damage and repair pathways may be important in protecting the developing embryo from constitutive oxidative stress in the absence of teratogen exposure. This may be the case for at least one protein involved in ROS sensing and the DNA damage response, ATM (Fig. 7), which is highly expressed even at the early embryonic stage (Bhuller *et al.*, 2006). In the absence of teratogen exposure, even heterozygous (+/−) ATM-deficient knockout embryos in culture exhibited significant developmental abnormalities, with homozygous (−/−) ATM-deficient littermates more severely affected (Bhuller and Wells, 2006). A similar *atm* gene dose-dependent increase in embryopathies was observed in +/− and −/− ATM-deficient fetuses *in vivo* for some but not all parameters following maternal treatment with only the saline vehicle (Bhuller *et al.*, 2006). Similarly in another *in vivo* study, the untreated −/− ATM-deficient control mice had a lower fetal body weight than their +/− and +/+ littermates (Laposa *et al.*, 2004). The observed enhanced embryopathies in ATM-deficient mice in the absence of teratogen exposure may, similar to deficiencies in antioxidative enzymes, reveal the pathogenic potential of constitutive oxidative stress, in this case allowing an accumulation of oxidative DNA damage, possibly in selective tissues and/or cell types.

When ROS formation and oxidative DNA damage are enhanced by xenobiotics, the full pathogenic potential of oxidative DNA damage, and perhaps 8-oxoG, is revealed, along with the developmental importance of activities of DNA damage response and repair pathways as risk factors that determine individual susceptibility. With respect to ROS sensing and DNA damage response (Fig. 7), when pregnant *atm* knockout mice are exposed to a low, normally non-teratogenic single dose of gamma radiation (0.5 Gy), −/− ATM-deficient fetuses exhibit a significant increase in fetal and neonatal death and structural tail anomalies (Laposa *et al.*, 2004). If the maternal radiation dose is raised to 2.0 Gy, even the +/− ATM-deficient fetuses are affected, with an overall *atm* gene dose-dependent pattern of severity. Similarly, when *p53* knockout mice with a deficient DNA damage response are treated with benzo[*a*]pyrene, a *p53* gene dose-response in the incidence of fetal embryopathies is observed, with homozygous p53-deficient fetuses most severely affected (Nicol *et al.*, 1995). A similar outcome has been reported for 4-hydroperoxycyclophosphamide, the activated analog of the anticancer drug cyclophosphamide, in mouse embryo limb culture (Moallem and Hales, 1998). On the other hand, an ocular birth defect caused by 2-chloro-2′-deoxyadenosine is decreased in p53-deficient fetuses (Wubah *et al.*, 1996), revealing considerable drug-dependent variability in the role of p53, possibly due in part to differences in the molecular actions of each drug, and specifically in their ability to enhance oxidative stress. Another contributing factor may be drug- and/or dose-dependent differences in triggering functions like the balance of the competing roles of p53 in directing apoptosis with severe damage, and DNA repair with more subtle damage (Fig. 7). Overall, the enhanced risk observed in ATM- and p53-deficient fetuses exposed to constitutive oxidative stress, phenytoin, benzo[*a*]pyrene, cyclophosphamide, and ionizing radiation suggest that at least some forms of oxidative DNA damage, and possibly 8-oxoG, may constitute an embryopathic molecular lesion, with the activities of DNA damage response proteins like ATM and p53 constituting potential risk factors.

OXIDATIVE DNA DAMAGE REPAIR

Perhaps most discriminating are studies investigating the role of proteins that function more directly in the repair of oxidative DNA damage, and particularly the 8-oxoG lesion.

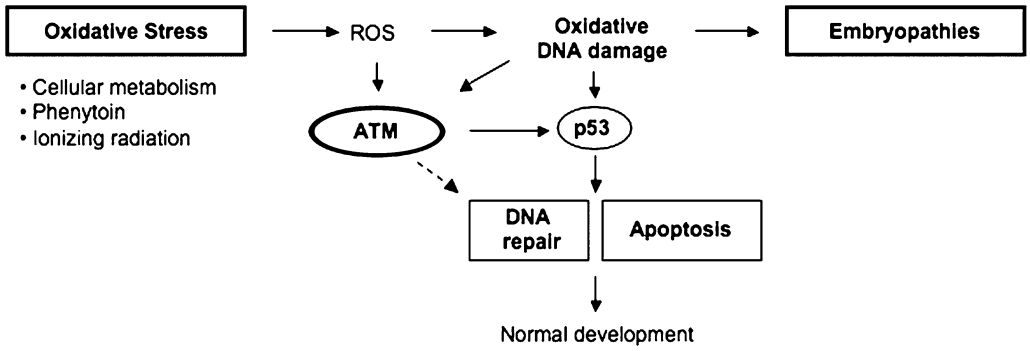

FIG. 7. The potential relation of the ATM and p53 proteins in the cellular DNA repair response or apoptosis following DNA damage initiated by oxidative stress (from: Bhuller and Wells, Toxicol. Sci. 93, 156–163, 2006).

OGG1 is a critical protein in the base excision repair pathway involved in the rate-limiting step for the repair of 8-oxoG, the excision of 8-oxoG (Fig. 6), whereas FPG is the bacterial homolog for this repair (David *et al*., 2007). If 8-oxoG is an embryopathic molecular lesion, decreased OGG1 should enhance the toxicity of ROS-initiating xenobiotics, whereas overexpression of OGG1 or FPG should be protective.

Human embryonic kidney cells stably transfected with constructs expressing either human *OGG1* (hOGG1) or FPG exhibited increased DNA repair activity (repair of 8-oxoG), and resistance to the formation of 8-oxoG and cytotoxic effects caused by ROS (hydrogen peroxide) and ROS-initiating xenobiotics including menadione, platinum drugs and phenytoin (Preston *et al*., 2007, 2009). These results directly implicate 8-oxoG as a cytotoxic molecular lesion, and OGG1 activity as a determinant of susceptibility, both of which may be similarly relevant to teratogenesis.

Similar developmental results were observed *in vivo* in pregnant mice treated with methamphetamine, which in CD-1 mice causes embryonic 8-oxoG formation and neurodevelopmental deficits reflected by postnatal motor coordination deficits (Jeng *et al*., 2005), the latter of which is measured using a rotarod apparatus. When pregnant *Ogg1* knockout mice were treated on GD 17 with a single dose of methamphetamine, 8-oxoG levels in fetal brain were increased in the −/− and +/− OGG1-deficient fetuses in an *Ogg1* gene dose-dependent fashion compared with their +/+ *Ogg1*-normal littermates (Wong *et al*., 2008). Similarly, in offspring that were delivered and evaluated postnatally for rotarod performance, female (but not male) −/− and +/− OGG1-deficient offspring exposed *in utero* to a single dose of methamphetamine exhibited *Ogg1* gene dose-dependent deficits in motor coordination from 6 weeks postnatally up to at least 12 weeks. In pregnant wild type mice on GD 17, OGG1 activity in fetal brain and liver was about twofold higher than that in maternal tissues, and was the sole contributor to 8-oxoG repair, consistent with an important role in protecting the fetus from this molecular lesion (McCallum and Wells, 2007). These results suggest that 8-oxoG formation plays a causal role in the postnatal motor coordination deficits produced by *in utero* exposure to methamphetamine, and that variations in OGG1 activity are a likely determinant of risk.

The CSB protein also is involved in the repair of 8-oxoG, although the mechanisms underlying this action are unclear, and may include transcription-coupled repair. Consistent with the above observations in *Ogg1* knockouts, when pregnant *Csb* knockout mice were treated on GD 17 with methamphetamine, the female −/− CSB-deficient fetuses exhibited enhanced brain 8-oxoG levels and postnatal motor coordination deficits (Wong *et al*., 2005), although the severity of both the molecular lesion and functional deficit was less than that in *Ogg1* knockouts. CSB accordingly appears to be embryoprotective in cases of xenobiotic-enhanced oxidative DNA damage, although less so than OGG1.

These cellular and *in vivo* results focusing on OGG1 and CSB provide the most direct evidence to date that 8-oxoG constitutes an embryopathic molecular lesion, and that deficiencies in OGG1, and perhaps CSB, may constitute important risk factors for developmental abnormalities.

DNA double strand breaks (DSBs), which can be initiated by ROS as well as via other mechanisms, are a further type of DNA damage with potential embryopathic consequences (Hales, 2005). DSBs can be repaired by homologous recombination, and studies of phenytoin in cell culture have found that concentrations of phenytoin which increase ROS formation also increase DSBs and the activity of homologous recombination (Winn *et al*., 2003), implicating DSBs in the embryopathic mechanism and homologous recombination as another repair-related risk factor. Interestingly, another teratogenic AED, valproic acid, was found to increase ROS formation and DSBs and homologous recombination in cell culture without an apparent increase in 8-oxoG levels (Defoort *et al*., 2006), suggesting the possibility of some overlap with phenytoin in the involvement of oxidative stress, with differences in the resulting macromolecular lesions.

ROS IN SIGNAL TRANSDUCTION AND DEVELOPMENT

As recently reviewed (Janssen-Heininger *et al*., 2008), a rapidly expanding literature has implicated ROS and RNS in signal transduction that is tightly and temporally regulated, and is selective to cell types, subcellular organelles, and even to the microenvironments within individual proteins and lipids. Physiologically relevant RNS-mediated signal transduction is attributed to reactions of nitric oxide and nitrosylated thiols, particularly *S*-nitrosoglutathione, with cysteine residues of key proteins resulting in reversibly *S*-nitrosylated residues that alter cellular function. Although at physiological RNS concentrations such reactions are readily enzymatically reversible, excessive *S*-nitrosylation could have pathophysiological consequences. This reversible mechanism is different from the adverse cellular outcomes attributed to macromolecular damage caused by highly reactive species like peroxynitrite and nitrogen dioxide that produce less reversible oxidative molecular changes including the formation of 3-nitrotyrosine (Fig. 3).

In the case of ROS-mediated signal transduction, physiological effects are attributed to less reactive and more diffusible hydrogen peroxide, which selectively oxidizes sulfhydryl groups of specific cysteine residues on proteins resulting in a variety of reversible molecular modifications, including the formation of protein-protein (Pr-Pr) and glutathione-protein disulfides (GS-Pr, mixed disulfides). Although enzymatically reversible at physiological concentrations of hydrogen peroxide, higher exposures could lead to excessive *S*-oxidation with pathological consequences. As recently reviewed (Hansen, 2006), the oxidation state of such protein sulfhydryls is

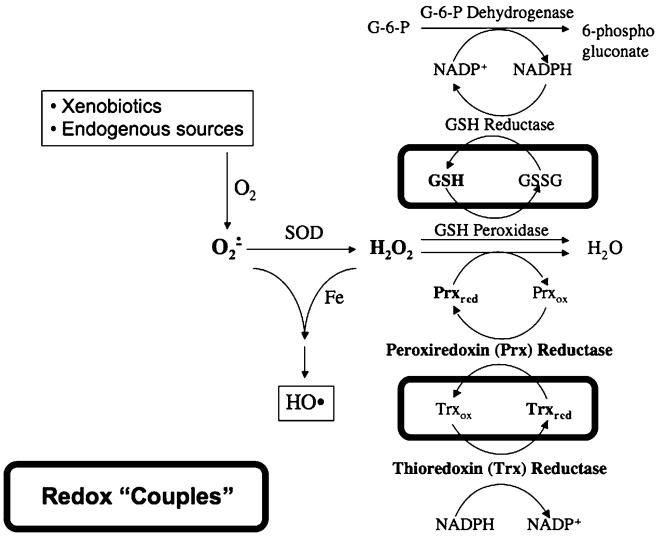

FIG. 8. Redox couples modulating the cellular effects of endogenous or xenobiotic-enhanced oxidative stress. Redox couples in addition to those circled include NADPH/$NADP^+$ and Prx_{red}/Prx_{ox} shown above, and cysteine/cystine (not shown). Abbreviations: ox, oxidized; red, reduced (see Fig. 2 for remaining abbreviations).

determined by the redox state of the cell, which is modulated by the ratios of several compartmentalized redox "couples" (Fig. 8) including cysteine/cystine, GSH/glutathione disulfide and $thioredoxin_{reduced}$ (Trx_{red})/$thioredoxin_{oxidized}$ (Trx_{ox}), with cysteine concentrated in the plasma, GSH differentially in the cytosol and mitochondria and Trx in the nucleus. As development of the embryo proceeds from a reducing to an oxidizing environment during embryogenesis, these redox couples are thought to serve as reversible redox switches regulating cellular proliferation, differentiation, apoptosis and necrosis (Fig. 9) (Hansen, 2006).

A major signal transduction mechanism in development may involve ROS-dependent alterations in epigenetic regulation, or DNA modifications independent of changes in sequence, as reviewed elsewhere (Hitchler and Domann, 2007). Mechanisms involving DNA methylation and histone modifications can work alone or coordinately. The first process involves the methylation of cytosines in CpG dinucleotides in DNA, which generally results in gene silencing. The second process involves a variety of posttranslational modifications in histone tails that can cause either gene activation or silencing. In the latter case, histone methylation and acetylation are among several posttranslational modifications known to effect development. For example, methylation of particular lysines and arginines in histone tails can activate or inhibit gene transcription depending upon the specific amino acid modified. These reactions are reversible, catalyzed by histone demethylases and histone deacetylases, thereby providing a dynamic system with the plasticity necessary for development. Pertinent to oxidative stress, a number of histone demethylases function as oxidases that require oxygen as a cofactor in the demethylation process, and hence serve as oxygen sensors in the microenvironment of genes within the nucleus. Interestingly, the production of hydrogen peroxide during the demethylation reaction may have other signaling consequences, as discussed below under macromolecular damage in signal transduction. The activity of these oxidases (histone demethylases) will accordingly vary with the redox state of specific cell types and the gestational period as development proceeds from the reducing environment of the fertilized egg to an oxidizing environment beginning in the mouse around GD 10, during embryogenesis.

ROS-initiated decreases in GSH levels can reduce DNA and histone methylation reactions indirectly by diverting homocysteine to the synthesis of GSH, and away from synthesis of

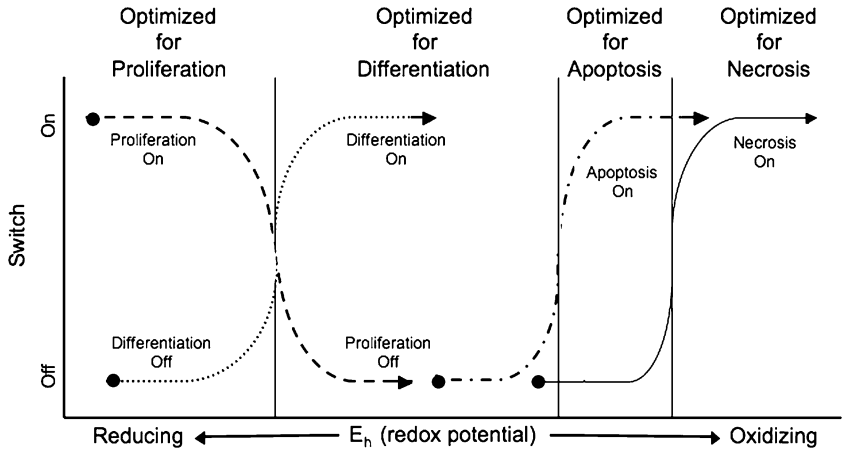

FIG. 9. Developmental redox switching during proliferation, differentiation and cell death. A reducing environment early in development favors cellular proliferation. As development moves through the period of organogenesis, the environment becomes more oxidizing, shutting off proliferation and turning on differentiation. A further increase in the oxidizing environment, either physiologically or xenobiotic-enhanced, can activate cellular death (from: Hansen, Birth Defects Res. C 78, 293–307, 2006).

methionine and ultimately *S*-adenosylmethionine (SAM), the essential cofactor for methylation reactions (Hitchler and Domann, 2007). Decreased levels of GSH also reduce the activity of SAM synthase, further depleting SAM concentrations.

Another oxygen-dependent process involves the hypoxia-inducible factor (HIF) family of transcription factors, as reviewed elsewhere (Hitchler and Domann, 2007). Under lower oxygen tension, HIFs form heterodimers with the aromatic nuclear translocator protein and bind to hypoxia response elements in gene promoters. HIF stability and transactivation are negatively and posttranslationally regulated by hydroxylation of proline and asparagine residues, respectively catalyzed by oxygen-dependent prolyl hydroxylase (PHD) and an asparaginyl hydroxylase termed Factor Inhibiting HIF (FIH). Higher oxygen tensions promote HIF hydroxylation and inactivation, suppressing gene transcription, which could have teratological consequences. Also, as oxidases similar to histone demethylases discussed above, PHD- and FIH-catalyzed hydroxylations produce hydrogen peroxide, which has other signaling consequence discussed below.

A final example is the sirtuin family of protein deacetylases, which can deacetylate some transcription factors, thereby increasing the transcription of some genes, and deacetylate histones, thereby suppressing the transcription of other genes, as reviewed elsewhere (Hitchler and Domann, 2007). Sirtuins are NAD+-dependent, so increasing oxygen tension enhances the NAD+/NADH ratio, resulting in enhanced deacetylation. Depending upon the target, the resulting hypoacetylation could cause the activation (via transcription factor deacetylation) of some developmentally important genes and suppression (via histone deacetylation) of others.

In all of the above cases, with pre-existing genetic imbalances in the pathways that control ROS levels (Fig. 2), and/or exposure to ROS-initiating xenobiotics, one would expect enhanced epigenetic dysregulation to exacerbate adverse developmental outcomes.

As with RNS, ROS-mediated signal transduction is distinct from the pathophysiological consequences initiated by highly reactive hydroxyl radicals, which unlike hydrogen peroxide cause less reversible macromolecular damage including the oxidation of lipids, carbohydrates, proteins (e.g., formation of protein carbonyl groups) and a variety of oxidative lesions in DNA (e.g., 8-oxoG, Fig. 5) and RNA, discussed previously under DNA repair. However, as noted below, even the level of oxidative ''lesions'' like 8-oxoG at least under physiological conditions may constitute a tightly regulated mechanism of signal transduction.

ROS-INITIATED MACROMOLECULAR "DAMAGE" IN SIGNAL TRANSDUCTION

It seems possible that even so-called oxidative damage to proteins and DNA damage might not only constitute an irreversible macromolecular lesion with pathological consequences, but also in some cases may contribute to pathways normally associated with signal transduction. For example, in the case of protein oxidation, cysteine sulfhydryls can be more highly oxidized (higher than in Pr-Pr and GS-Pr disulfide formation) during oxidative stress, forming sulfinic (SO_2H) and sulfonic (SO_3H) acids, termed ''overoxidation'' or ''hyperoxidation,'' which are not readily reversed. However, the sulfinyl residue of inactivated peroxiredoxin can be repaired by sulfiredoxins, which are cysteine sulfinyl reductases, regenerating active enzyme, thereby regulating signal transduction (Janssen-Heininger *et al.*, 2008). Excessive hyperoxidation and/or reduced repair by sulfiredoxins resulting from genetic predisposition and/or xenobiotic exposure might have teratological consequences.

Similarly in the case of oxidative DNA damage, the hydroxylation of DNA (forming 8-oxoG) might constitute a physiological transduction signal, with DNA repair serving a regulatory function. For example, in the demethylation of lysine 4 of histone 3 by lysine-specific demethylase 1 (LSD1), this enzyme functions as a flavin (FAD)–dependent oxidase, converting oxygen to hydrogen peroxide (Forneris *et al.*, 2005). LSD1-catalyzed demethylation could thereby cause localized 8-oxoG formation that selectively alters gene transcription. Recently, controlled DNA ''damage'' and repair process has been implicated in the regulation of estrogen-induced gene transcription, in which estrogen-initiated demethylation of histone proteins produces hydrogen peroxide release with 8-oxoG formation, and recruitment of the repair protein OGG1 in a controlled process that tightly regulates transcription (Perillo *et al.*, 2008). This mechanism could participate in epigenetic modifications that regulate normal development, in which case an enhancement in localized 8-oxoG formation caused by xenobiotics could lead to epigenetic dysregulation, with teratological consequences.

ROS-DEPENDENT SIGNAL TRANSDUCTION IN TERATOGENESIS

Several recent reviews have comprehensively addressed the rapidly expanding body of evidence for ROS-mediated signal transduction in teratogenesis (Hansen, 2006; Hitchler and Domann, 2007; Kovacic and Pozos, 2006; Kovacic and Somanathan, 2006) and transplacental carcinogenesis (Winn and Wan, 2006). We have investigated the potential contribution of ROS-mediated signal transduction in the mechanism of phenytoin embryopathies. During oxidative stress, Ras and NF-κB are frequently found to be elevated in various cellular and animal models, with NF-κB activation occurring downstream of Ras activation. In mouse embryo culture, phenytoin enhanced the embryonic levels of activated Ras protein and embryopathies (Winn and Wells, 2002). Both outcomes were blocked by preincubation with alpha-hydroxyfarnesylphosphonic acid,

a farnesyltransferase inhibitor that prevents posttranslational Ras activation, suggesting that Ras activation contributes to the embryopathic mechanism of phenytoin (Fig. 10). Similarly, phenytoin was found in embryo culture to enhance embryonic NF-κB signaling in a transgenic mouse expressing an NF-κB–dependent *lacZ* reporter gene (Kennedy *et al*., 2004). Pre-incubation of embryos with an antisense oligonucleotide for NF-κB blocked the elevation of embryonic NF-κB activity by phenytoin, and provided concentration-dependent protection against phenytoin embryopathies, suggesting that NF-κB activation contributes to the mechanism of phenytoin embryopathies. These studies together implicate ROS-mediated signal transduction in the mechanism of phenytoin teratogenicity, possibly via pathways that include Ras upstream to NF-κB, or via different pathways involving Ras and NF-κB, respectively. Although early studies suggested that ROS were mediators of NF-κB activation (Schreck *et al*., 1991), current knowledge of the complexity of the NF-κB cascade and the multiplicity of redox-sensitive targets within it suggest that the modulation of this pathway by ROS is likely to be highly stimulus- and cell type specific (Pantano *et al*., 2006). The complexity of ROS modulation of NF-κB signaling specificity extends to sub-cellular compartments within cells, as nuclear translocation of NF-κB can be stimulated by ROS but within the nucleus NF-κB must be fully reduced to bind DNA efficiently (Matthews *et al*., 1992; Pantano *et al*., 2006). A key role for NF-κB signaling in vertebrate limb outgrowth has been identified by transdominant inhibition of NF-κB activity, which causes severe disruption in developing chick limb bud outgrowth with associated reductions in expression of *Twist*, *Sonic hedgehog*, and *Fgf-8*, whereas

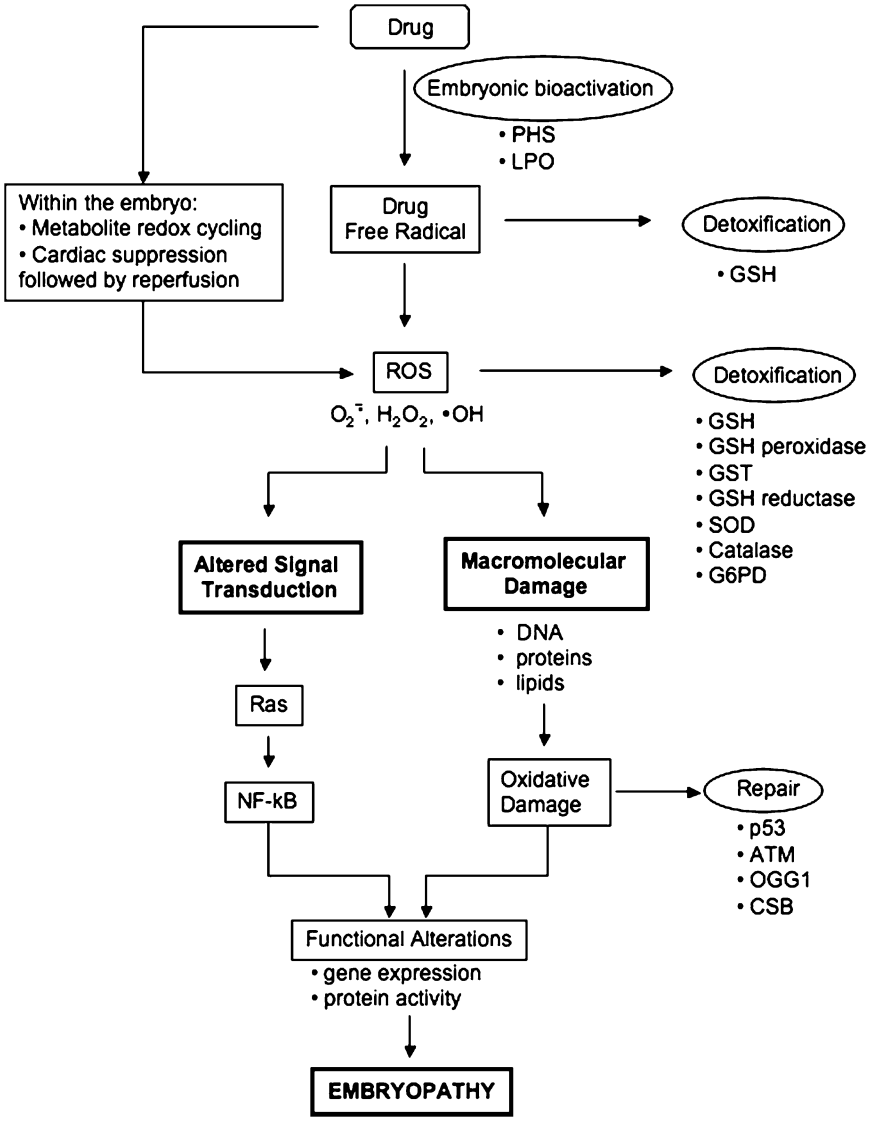

FIG. 10. Potential contribution of Ras and NF-κB proteins in signal transduction pathways initiated by drug-enhanced formation of ROS (see Figs. 2 and 6 for remaining abbreviations) (modified from: Kennedy *et al*., Mol. Pharmacol. 66, 404–412, 2004).

derepressing the bone morphogenetic protein-4 gene (Bushdid *et al*., 1998; Kanegae *et al*., 1998). Hansen and colleagues have used rat and rabbit limb bud cells to examine dysregulation of this signaling pathway as a potential mechanism of thalidomide teratogenesis. A thalidomide-dependent rabbit-specific reduction in NF-κB activity, which could be partially blocked by coadministration of N-acetylcysteine or PBN, was found *in vitro* when limb bud cells were transfected with a NF-κB green fluorescent protein reporter construct (Hansen *et al*., 2002a) *In situ* hybridization following *in utero* treatment with thalidomide revealed a rabbit-specific reduction in expression of *Twist*, and Fgf-*8*, which could be blocked by intravenous cotreatment with PBN, suggesting that thalidomide-initiated redox shifts in rabbit lead to dysregulation of NF-κB signaling resulting in reductions in expression of key genes necessary for limb outgrowth (Hansen *et al*., 2002a). Further *in vitro* studies in limb bud cell cultures revealed that thalidomide initiated more ROS in limb cells from rabbits than rats, and that the ROS initiation in rabbit was preferentially localized in the nucleus and associated with a selective reduction in nuclear GSH content (Hansen *et al*., 2002b).

Recent studies suggest that the molecular mechanism of thalidomide-initiated limb malformations involves a ROS-dependent upregulation of apoptotic pathways (Knobloch *et al*., 2007, 2008b). These studies using primary embryo fibroblasts from chicks and human as well as chicken embryos have shown that thalidomide upregulates expression of bone morphogenetic proteins (Bmps) resulting in both the hyper-expression of the secreted *Wingless* and *INT-1* antagonist Dickkopf1 (Dkk1), and enhanced activity of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) that suppresses the phosphatidylinositide 3-OH kinase/protein kinase B (Akt) pathway. Together these events increase glycogen synthase kinase 3 beta (Gsk3β) producing down regulation of β-catenin–mediated transcriptional activation, ultimately leading to capase-dependent apoptosis via the intrinsic mitochondrial and Fas death receptor pathway (Knobloch *et al*., 2007, 2008b). PBN cotreatment abbrogated both thalidomide-induced cell death and upregulation of *Bmp4* and *Dkk*1 expression in primary chick limb bud cultures (Knobloch *et al*., 2007). Inhibition of NF-κB activity via nuclear ROS maybe the mechanism of thalidomide-induced expression of *Bmp4* based on previous studies showing its upregulation following transdominat inhibition of NF-κB activity in chick limb buds (Bushdid *et al*., 1998). A further study using embryo fibroblasts to delineate the molecular basis for the species susceptibilty to thalidomide teratogenesis suggests that mouse embryo fibroblasts (MEFs) are resistant to the effects of thalidomide due to an approximately fivefold higher level of GSH compared with sensitive species, and that pharmacological depletion of GSH sensitizes MEFs to thalidomide-initiated superoxide anion formation and apoptosis that is observed both in human and chick embryo fibroblasts (Knobloch *et al*., 2008a).

Other proteins involved in developmental ROS-mediated signal transduction are discussed in the cited reviews, and many more will no doubt continue to be identified. The contribution of such transduction proteins may vary with the transduction pathway, cell type, tissue, and developmental stage, as well as the source of oxidative stress. For a definitive understanding and the development of therapeutic strategies, it will be important to determine which proteins play a causal role in the mechanism of teratogenesis, as opposed to merely being altered, for both constitutive and xenobiotic-enhanced oxidative stress. For some ROS-initiated adverse developmental outcomes, changes in both signal transduction and macromolecular damage may contribute to the teratological mechanism, and the relative contributions may vary with the xenobiotic, tissue and gestational period.

CONCLUSIONS

Evidence from molecular studies *in vitro* combined with results from complementary studies in embryo culture and *in vivo* suggest that ROS and RNS in animal models contribute to both constitutive origins of teratogenesis as well as a broad spectrum of xenobiotic-initiated adverse developmental outcomes, including structural birth defects, traditionally referred to as teratogenesis, as well as neurodevelopmental deficits and cancer in later postnatal life. At least some of the pathways that contribute to the levels of embryonic ROS and oxidative macromolecular damage, including embryonic xenobiotic bioactivation, antioxidant activity and repair of oxidative macromolecular damage, or at least repair of oxidative DNA damage, modulate the risk of teratogenesis in animal models. However, we still know relatively little about the full nature of the determinants of susceptibility, particularly with regard to signal transduction. It is likely that other important contributing pathways, as well as ROS-independent mechanisms for the same xenobiotics, remain to be discovered, and that more than one mechanism may contribute to the same adverse developmental outcome. The results from animal studies provide a basis for similar evaluations in humans, for whom little information is available.

FUNDING

Canadian Institutes of Health Research (CIHR) grant, the National Cancer Institute of Canada grant, and the National Institute of Environmental Health Science grant (R21-ES013848) supported research from the PGW laboratory; CIHR/Rx&D Health Research Foundation doctoral scholarship to C.J.J.L. and postdoctoral fellowships to G.P.M. and T.J.P.; and Novartis doctoral fellowship from the Society of Toxicology (U.S.A.) to A.W.W.

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