

# Chapter 1

## Impact of Oxidative Stress on Development

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### Introduction

The developing embryo and fetus, collectively termed the conceptus, are highly susceptible to the adverse effects of oxidative stress initiated by endogenous processes and by xenobiotics that enhance the formation of reactive oxygen species (ROS). This susceptibility is due in part to the high conceptual rates of cellular division and differentiation and the complex processes involved in the formation of organ structures and development of functional systems, including brain activity. Alterations in these processes can result in in utero or perinatal death, or structural and functional birth defects, termed “teratogenesis.” The instability of ROS, and particularly hydroxyl radicals, means that proximate formation of ROS within the conceptus, rather than distal maternal formation, plays a critical role in teratogenesis. Susceptibility is compounded by relatively high levels of embryonic and fetal enzymes involved in ROS formation and xenobiotic bioactivation to free radical

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## Human Development and “Critical Periods” for Drug Exposure

GESTATIONAL TIME (weeks)	1 - 2	3	4	5	6	7	8	12	16	20 - 36	38	
STAGE	From fertilization to blastocyst; Implantation	<b>Embryonic</b> Period					<b>Fetal</b> Period					
DEVELOPMENTAL PROCESS	Cellular division	Cellular differentiation and organogenesis					Histological differentiation and functional development					
TERATOLOGICAL CONSEQUENCE	Prenatal death	Major morphological abnormalities					Functional defects and minor morphological abnormalities					
ORGAN SUSCEPTIBILITY	Usually not susceptible to teratogens in first two weeks	Heart										
Central nervous system												
Arms												
Eyes												
Legs												
Teeth												

**Fig. 1.1** Human development and critical periods for exposure to developmentally toxic agents (Modified from Wells et al. [98]). Exposure to toxic agents during the first 2 weeks following fertilization can result in either the death of the conceptus or a morphologically normal child, or possibly birth defects resulting from genetic or epigenetic changes. Exposure during the embryonic period, which encompasses the period of cellular differentiation and organogenesis, can result in either *in utero* death or major gross morphological birth defects. Exposure during the fetal period, which encompasses histological differentiation and functional development, can result in altered functions of organs and biochemical pathways and systems

intermediates and conversely low levels of protective antioxidative enzymes. Enhanced ROS levels can adversely affect development by altering signal transduction and/or by oxidatively damaging conceptual cellular macromolecules such as lipids, proteins, and DNA, the latter of which may be mitigated by DNA repair enzymes. The risk of teratogenesis is therefore largely determined at the conceptual level, at which in mouse models those littermates with an unfavorable imbalance among pathways of ROS formation and detoxification, and DNA repair, exhibit greater structural and/or functional birth defects than littermates with a favorable balance.

### Embryonic and Fetal Development

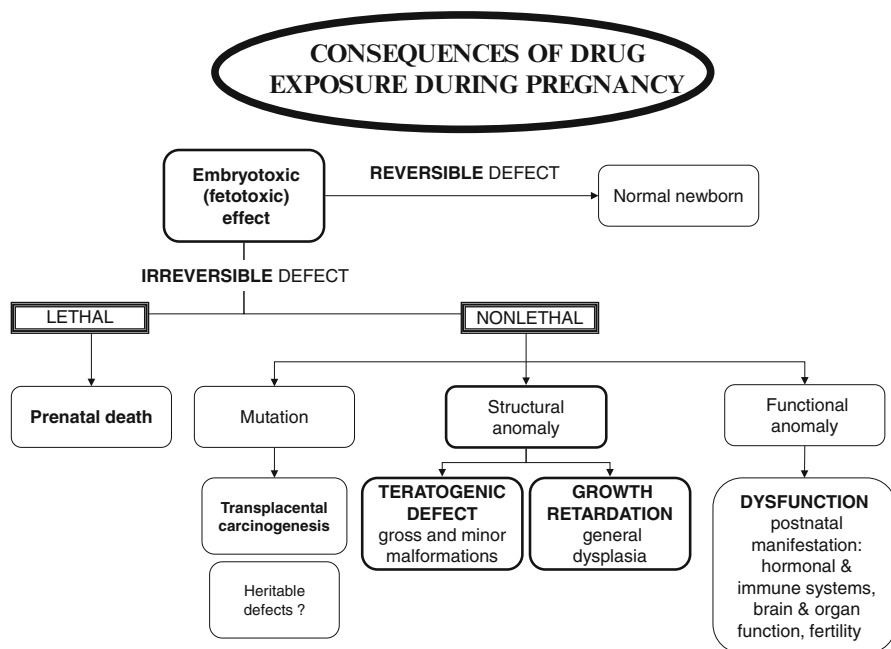
Following fertilization, development in the human begins with a 2-week period of conceptual cell division, followed by an embryonic period of organ development and a fetal period of functional development (Fig. 1.1). Human exposure to

developmentally toxic agents during the initial 2 weeks following fertilization traditionally has been believed to result in either the death of the conceptus or a normal child, since cells have not yet begun to differentiate and a sufficient residual of unaffected cells can compensate for the lost cells. However, this concept was largely based upon teratogens that caused structural birth defects by receptor-mediated processes, and it is likely that exposure to agents that damage DNA via either genetic or epigenetic mechanisms may alter later developmental processes. Later effects also could arise from early exposure to teratogens with long half-lives of elimination. Exposure during the *embryonic* period, which encompasses the period of cellular differentiation and organogenesis, largely results in in utero death or major structural birth defects (shortened or missing limbs, cleft palate, spinal bifida, etc.), although postnatal functional deficits can be initiated by exposure to some xenobiotics during this period. The risk of developmental toxicities exhibits a remarkable pattern not found in other areas of toxicology, namely, critical periods of susceptibility, exemplified in Fig. 1.1 by the filled bars representing periods in which specific organs are at maximal risk for alterations leading to major structural defects [61, 104]. Exposure to teratogens outside of the critical period for a given organ typically will not affect the structural development of that organ, although prior exposure to a xenobiotic with slow elimination may result in toxic concentrations being sustained into the critical period. Exposures in the later component of the critical period, exemplified by the open bars, which for some organs extend into the fetal period, result in only minor structural defects, and exposures beyond the critical period have no teratogenic effect. Exposures during the *fetal* period, during which histological differentiation and functional development occur, typically result in altered functions of organs and biochemical pathways and systems like the immune system. Susceptibility to the initiation of postnatal cancer by transplacental carcinogens at least in mouse models is highest during this period. Fetal exposures can be particularly important for organs like the brain, in which functional development continues throughout gestation and postnatally until around the age of 20 years in humans. As the risk of functional teratogenesis from fetal exposures has become more evident, the traditional focus on the first trimester of pregnancy, encompassing the embryonic period, has shifted to a more balanced view of developmental risk encompassing the entire duration of pregnancy and even into the postnatal period.

The broad spectrum of developmental outcomes is shown in Fig. 1.2 and can vary by the nature of the developmental toxin, the exposure level, and gestational timing of the exposure.

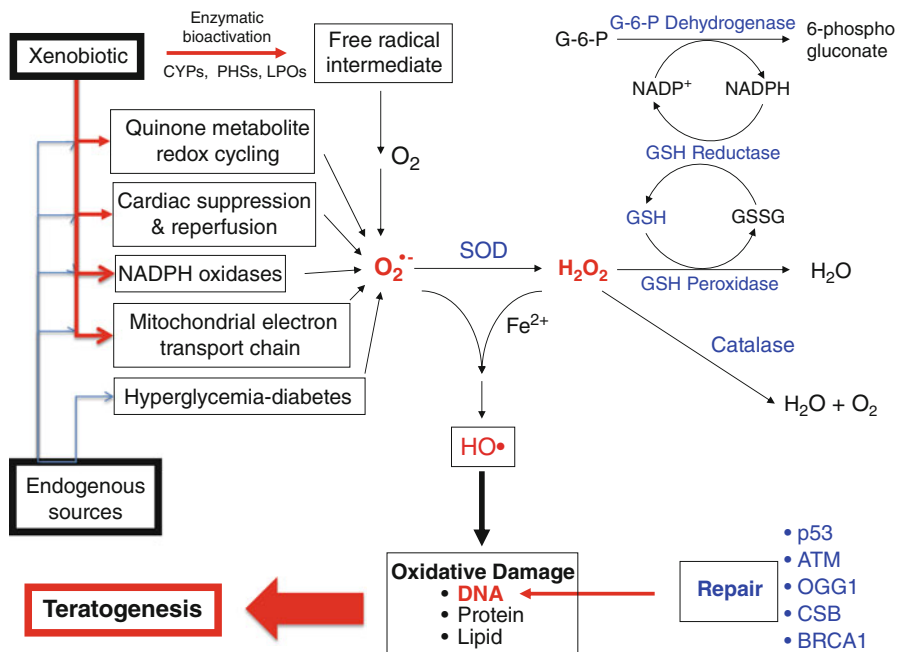
## Conceptual Reactive Oxygen Species Formation

Developmental toxicity can be initiated via a spectrum of mechanisms that are not necessarily mutually exclusive, including the receptor-mediated effects of xenobiotics and macromolecular damage caused by the bioactivation of xenobiotics to electrophilic and free radical intermediates. Teratological mechanisms involving



**Fig. 1.2** Consequences of exposure to developmentally toxic agents during pregnancy. A reversible defect will result in a normal newborn. An irreversible defect, if lethal, can lead to prenatal death. If the irreversible defect is nonlethal, three general consequences are possible: (1) mutations leading to transplacental carcinogenesis or heritable defects; (2) structural or gross morphological anomalies, either teratogenic defects or growth retardation; or (3) functional anomalies, including a spectrum of postnatal metabolic dysfunctions (Modified from Neubert et al. [65])

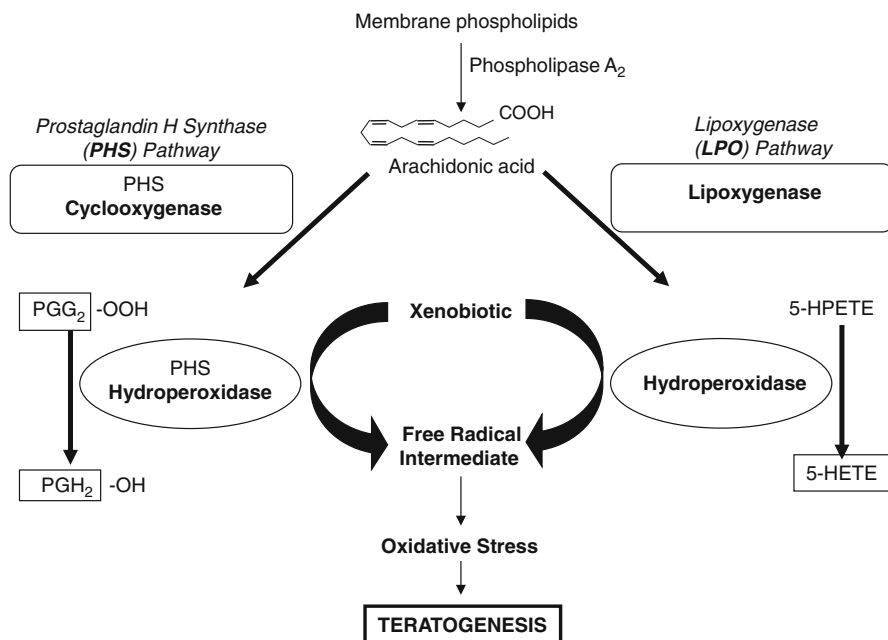
receptor-mediated processes and the covalent binding of xenobiotic electrophilic reactive intermediates to cellular macromolecules have been discussed elsewhere ([97–99, 120]). Reactive oxygen species (ROS) and oxidative stress can be initiated proximally in the embryo and fetus by xenobiotics and agents like ionizing radiation via several mechanisms (Fig. 1.3). These include redox cycling of quinone metabolites of xenobiotics, cardiac suppression by xenobiotics followed by reperfusion (e.g., phenytoin), activation and/or induction of ROS-producing NADPH oxidases (NOXs) (e.g., ethanol and methanol), and interruption of the mitochondrial electron transport chain. A number of endogenous pathways can lead to ROS formation, including redox cycling (e.g., quinone metabolites of dopamine in the brain), as well as enhanced ROS formation in diseases like diabetes [25, 99, 116]. In diabetes, high sugar levels and possibly additional factors can dysregulate the mitochondrial electron transport chain [114] as well as induce NOXs [28], both of which result in enhanced ROS formation. Xenobiotics can also be converted to ROS-initiating free radical intermediates by enzymes like prostaglandin H synthases (PHSs) and lipoxygenases (LPOs) (Fig. 1.4) as well as by cytochromes P450 (CYPs). Unlike CYPs, PHSs and LPOs are highly expressed in the



**Fig. 1.3** Biochemical pathways for endogenous and xenobiotic-enhanced formation and detoxification of reactive oxygen species (ROS) and repair of oxidatively damaged cellular macromolecules. Teratogenesis is postulated to result from ROS-mediated alterations in signal transduction (not shown) and/or embryonic or fetal macromolecular damage. If embryonic or fetal ROS formation exceeds the proximal capacity for ROS detoxification and/or repair of cellular macromolecules, this imbalance can result in enhanced teratogenesis, even at a therapeutic drug concentration or generally “safe” exposure level for an environmental chemical. *Blue arrows* indicate pathways through which ROS are formed endogenously. Many of the same ROS-forming pathways that are enhanced by xenobiotics are also responsible for endogenous ROS formation. Abbreviations: *ATM* ataxia telangiectasia mutated, *BRCA1* breast cancer 1, *CSB* Cockayne syndrome B, *CYPs* cytochromes P450, *Fe* iron, *G-6-P* glucose-6-phosphate, *GSH* glutathione, *GSSG* glutathione disulfide, *OGG1* oxoguanine glycosylase 1, *PHSs* prostaglandin H synthases, *SOD* superoxide dismutase (Modified from Wells et al. [100])

embryo and fetus, and these enzymes have been shown in animal models to contribute to the bioactivation and teratogenicity of several drugs including benzo[a]pyrene, thalidomide, phenytoin, and structurally related antiepileptic drugs.

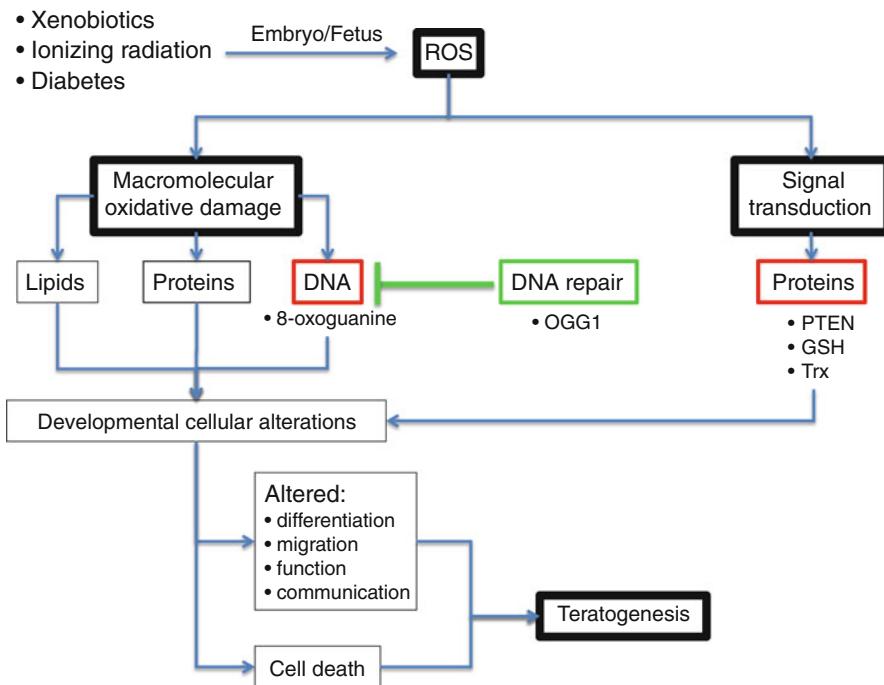
The nature of ROS is discussed elsewhere in this book. Briefly, ROS include hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide anion (O<sub>2</sub><sup>•-</sup>), and hydroxyl radical (HO•). Unlike O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub>, which are relatively stable and diffusible forms of ROS, HO• is highly unstable and nondiffusible, reacting rapidly with nearby molecular targets within the subcellular compartment in which it is formed. ROS are an important component of many physiological signal transduction pathways; however, if levels of ROS exceed the capacity for detoxification, this results in “oxidative stress,” including the oxidation of



**Fig. 1.4** Bioactivation of xenobiotics catalyzed by prostaglandin H synthases (PHSs) and lipoxygenases (LPOs). The hydroperoxidase component of PHSs, and hydroperoxidases associated with LPOs, can oxidize xenobiotics to free radical intermediates that can initiate ROS formation, oxidative stress, and teratogenesis. Arachidonic acid is released from membrane phospholipids by the action of phospholipase A<sub>2</sub> and can then serve as a substrate in both the cyclooxygenase- and lipoxygenase-dependent eicosanoid pathways generating the corresponding hydroperoxides, which are then reduced by hydroperoxidases to the corresponding alcohols. In this pathway, xenobiotic may serve as a reducing co-substrate, itself being oxidized to a reactive free radical intermediate. If not detoxified, this free radical can initiate oxidative stress resulting in ROS-mediated oxidative macromolecular damage and teratogenesis (Modified from Yu and Wells [115])

proteins, lipids, DNA, and other cellular macromolecules and/or alterations in signal transduction pathways, which can play a role in disease, aging, and xenobiotic toxicity, including developmental abnormalities (Fig. 1.5) [24, 25, 99].

The instability of ROS, and particularly hydroxyl radicals, means that proximate formation of ROS within the conceptus, rather than distal maternal formation, plays a critical role in teratogenesis. Embryo culture studies have shown that the embryo has the necessary enzymes for bioactivating “proteratogens” to free radical reactive intermediates, as well as ROS-producing NOXs, balanced by variable but critically important activities of protective antioxidative and DNA repair enzymes. As discussed in the next section on mechanisms, this has important implications for the



**Fig. 1.5** Alternative biochemical effects of reactive oxygen species (ROS): oxidative damage to cellular macromolecules and altered signal transduction. Physiological or environmentally enhanced levels of embryonic and fetal ROS can adversely affect development via one or both of the following two mechanisms: (1) Irreversible oxidative damage to cellular macromolecules including DNA, proteins, peptides, and lipids. 8-Oxoguanine is the most prevalent of over 20 DNA lesions initiated by ROS, and this lesion is repaired by oxoguanine glycosylase 1 (OGG1), one of several DNA repair proteins known to protect the developing embryo and fetus (see Fig. 1.3). (2) Reversible oxidative modification of signaling molecules, exemplified by phosphatase and tensin homolog (PTEN), glutathione (GSH), and thioredoxin (Trx). The degree to which either or both of these two alternative pathways contribute to the pathogenic mechanism may vary with the particular nature and timing of ROS initiation and the conceptual target tissue, among other factors. These biochemical changes can lead to non-apoptotic alterations in cellular function including differentiation, migration, function, and communication or may result in cell death. If these cellular alterations occur during critical windows of development, they may cause gross morphological birth defects or postnatal functional abnormalities such as neurodevelopmental deficits.

determinants of teratological risk as well as for the analysis of data when teratogenesis is initiated by the formation of ROS, as distinct from receptor-mediated mechanisms. Evidence for a role of these pathways in embryonic and fetal ROS formation, as well as the involvement of ROS in mechanisms of teratogenesis, is derived primarily if not exclusively from animal models, with no human studies to provide corroboration.

## Mechanisms of ROS-Initiated Teratogenesis

### *ROS-Dependent Mechanism*

ROS can initiate developmental toxicity via oxidative damage to embryonic or fetal cellular macromolecules (lipids, proteins, DNA, etc.) and/or by altering signal transduction (Fig. 1.5) [14, 25, 99]. It is important to remember that studies implicating macromolecular damage in the mechanism of teratogenesis do not preclude a contribution from altered signal transduction nor a contribution from mechanisms that do not involve ROS, as discussed later for thalidomide. It seems likely that particular types of developmental abnormalities can be initiated via more than one mechanism.

Representative xenobiotics, agents, and diseases for which a ROS-mediated mechanism of teratogenesis has been implicated are listed in Table 1.1, although other mechanisms including receptor-mediated actions may also contribute [98]. It is also possible that different mechanisms may lead to a similar developmental abnormality and that the relative contribution of a particular mechanism may vary with the nature of the ROS-initiating agent, the exposure concentration, the gestational time of exposure, and the target tissue or cell type.

Evidence of a ROS-dependent mechanism of teratogenesis has been obtained by a variety of approaches involving the use of pharmacological probes and genetically modified animal models (Table 1.2). Pharmacological probes all have actions in addition to their intended activity, so the degree of certainty is enhanced by the use of multiple probes that modulate complementary pathways and by corroborating studies using animal models in which the pathway in question has been genetically modified. Even results from studies using genetically modified animals such as knockout mice can be confounded by unappreciated biochemical changes in these mice in response to the loss of an important gene, so corroborating approaches improve the confidence of an interpretation. Studies supporting ROS-dependent mechanisms of developmental toxicity include: (1) Blocking xenobiotic bioactivation by enzymes like prostaglandin H synthases (PHSs) to a free radical intermediate, using PHS inhibitors such as acetylsalicylic acid (ASA, aspirin) or eicosatetraenoic acid (ETYA), which reduce the teratogenicity of drugs like phenytoin, benzo[a]pyrene, and thalidomide in vivo and/or in embryo culture [47, 99]. These studies have been corroborated by the use of knockout mice lacking a PHS isozyme, which exhibit reduced embryonic bioactivation of xenobiotics like phenytoin and benzo[a]pyrene, and are protected from the resulting increase in oxidatively damaged cellular macromolecules and developmental toxicity in vivo and/or in embryo culture [99]. In the case of drugs like ethanol and methanol, which induce the expression of ROS-producing NADPH oxidases (NOXs), pretreatment with a NOX inhibitor reduces embryonic DNA oxidation and teratogenesis [18, 100]. (2) Altering antioxidative pathways, as discussed below in section “Antioxidative Pathways,” where increases in antioxidants and antioxidative pathways protect the embryo and fetus from xenobiotic-initiated oxidative damage to cellular macromolecules and from the associated



**Table 1.1** Representative drugs and environmental chemicals (xenobiotics) and other agents and diseases for which ROS have been implicated in the mechanism of teratogenesis

Alcohol (ethanol)*
Cocaine*
Cyclophosphamide*
Benzo[a]pyrene
Diethylstilbestrol*
Hydroxyurea
Methamphetamine
Methanol
Methylmercury*
Phenytoin and structurally related antiepileptic drugs*
Thalidomide*
Tobacco smoke*
Valproic acid*
Ionizing radiation*
Diabetes*

From studies in animal models. Asterisks indicate known human teratogens [100], although xenobiotics like benzo [a]pyrene, a component of tobacco smoke, are presumed human teratogens despite the absence of human studies evaluating them in isolation. Evidence for ROS involvement does not preclude a contribution from other mechanisms, including receptor-mediated initiation, which has been implicated for many of the above xenobiotics. Teratological outcomes include structural birth defects (e.g., ethanol, thalidomide) and postnatal functional abnormalities including neurodevelopmental deficits (e.g., ethanol, valproic acid, and methamphetamine) and cancer (e.g., diethylstilbestrol) (Based upon data from papers and recent reviews [15, 25, 85, 94, 99])

developmental abnormalities in vivo and/or in embryo culture. A converse increase in conceptual macromolecular damage and developmental toxicity is observed when antioxidants and antioxidative pathways are reduced. (3) Pretreatment with free radical spin trapping agents like phenylbutylnitrone (PBN), which block oxidative damage to cellular macromolecules and the associated developmental toxicity of drugs like phenytoin, thalidomide, methanol and ethanol in vivo and in embryo culture [6, 47, 99, 100], and lipopolysaccharide-initiated toxicity in vivo [113]. In addition to implicating ROS in the teratological mechanism, such approaches provide insights into the relative contribution of ROS, as distinct from other mechanisms, such as the covalent binding of xenobiotic electrophilic reactive intermediates to embryonic and fetal cellular macromolecules or receptor-mediated effects of the parent compound or a stable metabolite.

**Table 1.2** Representative approaches implicating reactive oxygen species (ROS) in the mechanism of developmental toxicity

Process	Drug	Probe/outcome	Citation
Xenobiotic free radical formation	Phenytion and structurally related drugs	ESR/EPR spectrometry	[43, 73]
	Ethanol (EtOH)	ESR/EPR spectrometry	[4, 40]
	Methanol	ESR/EPR spectrometry	[32, 91]
	Methamphetamine	ESR/EPR spectrometry	[30]
<i>ROS production</i>			
Fluorescent probes	Valproic acid	DCF fluorescence	[94]
Hydroxyl radical formation	Phenytion	Salicylate hydroxylation	[36]
<i>Oxidatively damaged macromolecules</i>			
Lipids	See Table 1.3	MDA formation	
		HNE formation	
		Isoprostane formation	
		TBARS formation	
Proteins and peptides	See Table 1.3	Carbonyl formation	
		Protein thiol and GSH oxidation	
<i>DNA</i>			
<i>Oxidation</i>	EtOH	8-oxoguanine formation	[18, 53–56]
	Thalidomide and two hydrolysis products	8-oxoguanine formation	[47, 75]
<i>Strand brakes</i>	Ionizing irradiation	Comet assay	[50]
<i>Double-strand breaks</i>	Methylmercury	H2AX phosphorylation	[70]
	Valproic acid	Gamma-H2AX foci	[87]
<i>Reduced xenobiotic bioactivation to a free radical intermediate</i>			
PHS, LPOS	Phenytion	Enzyme inhibitors (ASA, ETYA, and others)	[43, 58, 115]
	Thalidomide	Enzyme inhibitor (ASA, ETYA)	[5, 47]
	Benzo[a]pyrene	PHS knockout mice	[74]

Reduced activity of ROS-producing NOX enzymes	EtOH	Enzyme inhibitor (DPI)	[18]
Reduced antioxidative protection	See Table 1.5	Genetically deficient mice Dietary depletion GSH depletion	
Enhanced antioxidative protection	See Table 1.5	Antioxidants GSH precursors Antioxidative enzymes Genetically enhanced mice	
Free radical spin trapping agents	EtOH	PBN	[54]
	Phenytoln	PBN	[43, 101]
	Thalidomide	PBN	[47, 75]
	Cocaine	PBN	[119]
	Lipopolysaccharide	PBN	[117]

Abbreviations: ASA acetylsalicylic acid, DCF dichlorofluorescein, DPI diphenylene iodonium, EPR electron paramagnetic resonance, ESR electron spin resonance, ETYA 5,8,11,14-eicosatetraynoic acid, EtOH ethanol, gamma-H2AX phosphorylated histone 2AX protein, GSH glutathione, HNE 4-hydroxy-2-nonenal, LPO lipoxygenase, MDA malondialdehyde, NOX NADPH oxidase, PBN phenylbutylnitron, PHS prostaglandin H synthase, ROS reactive oxygen species, TBARS thiobarbituric acid reactive substances

The high reactivity of ROS, and particularly hydroxyl radicals, means that ROS cannot readily travel from the mother to the embryo. Therefore, proximal embryonic and fetal pathways for ROS formation, as distinct from distal maternal pathways, are major determinants of risk. An illustration of the importance of proximate pathways can be seen for ROS formation in a mouse knockout model for PHS2, in which progeny from the same dam and litter may be wild type, or heterozygous or homozygous deficient for the *phs2* gene. In this example, among all littermates, the PHS2-deficient progeny, lacking bioactivating activity, are protected from benzo[a]pyrene teratogenicity, at least at lower doses, while the wild-type littermates are susceptible, although all are littermates from the same dam and are exposed to the same teratogen concentration [74]. Even strains of mice that are not genetically modified exhibit substantial littermate variability in embryonic determinants of risk, as exemplified with CD-1 mice, in which embryonic catalase activity can vary up to fourfold among embryos of the same litter [3]. Accordingly, for mechanisms involving the formation of ROS, the conceptual genotype is the fundamental determinant of risk rather than the litter or maternal genotype. Traditionally, teratological data are analyzed by litter, grouping all fetuses in the litter as equivalent, largely to compensate for the occasionally confounding occurrence of exceptional developmental anomalies in an isolated litter, often termed the “litter effect.” This approach usually works well for teratogens acting through a receptor-mediated mechanism, as long as there is no genetic modification or substantial spontaneous littermate variability in the receptor gene. However, in the case of a ROS-mediated mechanism, although it is important to evaluate a sufficient number of litters to dilute the impact of an anomalous litter response, the individual conceptus rather than the litter is the essential parameter. As discussed below in sections “[Antioxidative Pathways](#)” and “[DNA Repair](#),” the conceptual activity for antioxidative and DNA repair pathways is similarly critical. Accordingly, the balance among the conceptual pathways of ROS formation and detoxification, and DNA repair, within a single embryo or fetus is the fundamental determinant of developmental risk for that conceptus.

### ***ROS-Initiated Macromolecular Damage***

ROS oxidize or oxidatively damage all embryonic and fetal cellular macromolecules, including lipids, proteins, peptides (e.g., glutathione [GSH]), RNA, and DNA, as well as alter signal transduction (Fig. 1.5), so it is difficult to know which molecular events are contributing to developmental toxicity and to what degree. Proteins and lipids in particular are oxidatively damaged by enhanced conceptual ROS formation (Table 1.3) and would be expected to contribute to mechanisms of ROS-mediated teratogenesis, in which case the repair or removal of such macromolecular lesions would likely modulate the developmental toxicity of a ROS-initiating agent. However, approaches to definitively test this hypothesis have yet to be developed, and studies providing evidence of a causal role for oxidatively damaged lipids and proteins in the pathogenic mechanism are lacking. On the other hand, a number of genetically modified animal models have been developed that lack key proteins and

**Table 1.3** Representative studies of reactive oxygen species (ROS) formation, lipid peroxidation, and protein and peptide oxidation in embryonic and fetal tissues exposed to ROS-initiating teratogens

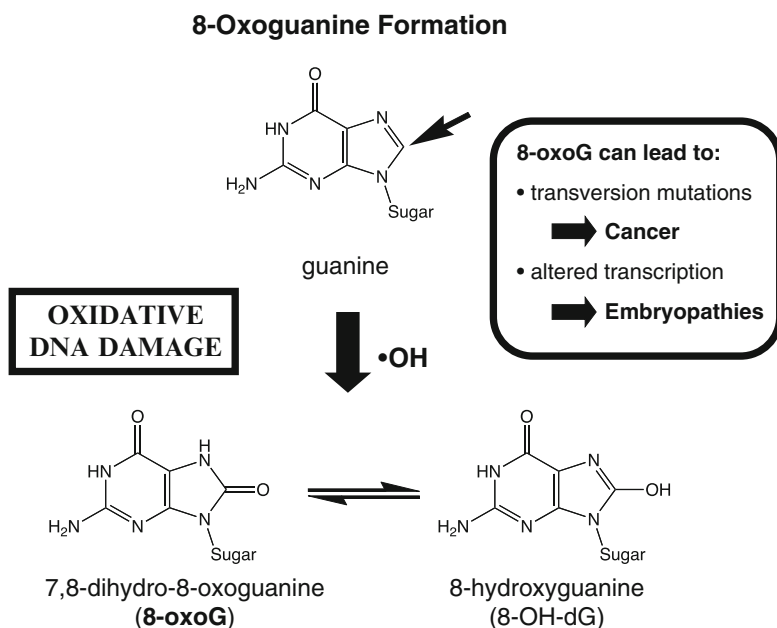
Species	Drug	Exposure duration	Probe	Evidence for oxidative stress and damage	Reference
<b>Lipid peroxidation</b>					
<i>Cell culture</i>					
Fetal rat astrocytes (GD 21)	25, 50 mM EtOH	7–10 days	L-cysteine, L-cystine, anti-CYP2E1 antibody	↑ ROS (DCF), lipid peroxidation (MDA and 4-HNE), ↓ GSH; protection with GSH addition	[60]
Sprague-Dawley fetal rat hepatocytes (GD 20)	2 mg/ml EtOH	24 h	None	↑ MDA (TBARS)	[16]
<i>Whole embryo culture</i>					
C57BL/6 mouse	5 mg/ml EtOH in ECM	6 h drug, 30 h medium	SOD co-treatment	EtOH ↑ superoxide anion production (NBT) lipid peroxidation (MDA), ↓ by SOD co-exposure	[42]
Rat (strain not specified)	WEC: 17–171 mM EtOH; <i>Midbrain cell culture</i> : 6–856 mM EtOH	WEC: 48 h Cell culture: 96 h	Catalase co-treatment, vitamin E (no protection)	WEC: ↑ 8-OHdG (ELISA), ROS (DCF); <i>Cell culture</i> : ↓ cytotoxicity with probes	[48]
<i>In vivo</i>					
C57BL/6 mouse	5–20 % EtOH in drinking water (p.o.)	2 weeks prior to gestation + throughout gestation to GD 18	Transgenic SOD mice	↓ Fetal hepatic 8-isoprostanes in mice with enhanced SOD expression	[102]
Wistar rat	2 g/kg EtOH (i.p.)	15 days prior to mating + entire gestation period	None	↑ Lipid peroxidation (TBARS) in fetal liver, brain, kidneys, testes	[64]
CD-1 mouse	65 mg/kg phenytoin (i.p.)	GD 12	Pretreatment with PBN or ASA	Phenytoin ↑ TBARS; ↓ by PBN and ASA	[49]
Wistar rat	1.5, 3 g/kg methanol (i.p.)	N/A	Acute	↑ Lipid peroxidation (TBARS), ↓ GSH-Px, SOD, GSSG reductase activities, ↓ ascorbate levels	[92]
CD-1 mouse	400, 600 mg/kg hydroxyurea (i.p.)	GD 9	None	Hydroxyurea ↑ HNE-protein adduct formation (western blot)	[85]

(continued)

**Table 1.3** (continued)

Species	Drug	Exposure duration	Probe	Evidence for oxidative stress and damage	Reference
<b>Protein and peptide oxidation</b>					
<i>Whole embryo culture</i>					
CD-1 mouse	80 uM phenytoin; 10 uM benzo[a]pyrene	4 h (biochemistry);	PBN	Phenytoin and benzo[a]pyrene ↑ protein oxidation (western) and phenytoin ↑ the oxidation of GSH to GSSG (HPLC)	[105]
		24 h embryopathies			
Sprague-Dawley rat	50–500 uM diamide	2 h	BCNU, BSO	Diamide ↑ embryotoxicity and the oxidation of GSH to GSSG; potentiated by BCNU and BSO	[27]
<i>In vivo</i>					
CD-1 mouse	65 mg/kg phenytoin (i.p.)	GD 12	Pretreatment with PBN or ASA	Phenytoin ↑ protein oxidation (carbonyl formation) and degradation (release of primary amines); blocked by PBN and ASA	[49]
CFT Wistar rat	400, 800 uM/kg t-butyl hydroperoxide (i.p.)	GD 5–7 or 8–10; sacrificed GD 13	None	t-Butyl hydroperoxide ↑ embryonic protein carbonyls (hydrazine derivatives), ROS formation (DCF), and depletion of GSH and protein thiols (fluorometry)	[89]
Wistar rat	1.5, 3 g/kg methanol (i.p.)	N/A	None	↓ Soluble and protein sulfhydryl groups	[92]

Abbreviations: 8-OHdG 8-hydroxy-2'-deoxyguanosine, ASA acetylsalicylic acid, CYP cytochrome P450, DCF dichlorofluorescein, ELISA enzyme-linked immunosorbent assay, EtOH ethanol, GD gestational day, GSH reduced glutathione, GSH-Px glutathione peroxidase, GSSG glutathione disulfide, HNE hydroxynonenal, HPLC high-performance liquid chromatography, MDA malondialdehyde, NBT nitroblue tetrazolium, PBN phenylbutyl nitron, ROS reactive oxygen species, SOD superoxide dismutase, TBARS thiobarbituric acid reactive substances, WEC whole embryo culture



**Fig. 1.6** Reaction of hydroxyl radicals ( $\text{HO}\bullet$ ) with guanine residues of DNA to form the molecular lesion 7,8-dihydro-8-oxoguanine (8-oxoguanine, 8-oxoG). If not repaired, this DNA lesion can cause mutagenic changes in gene sequences, and/or altered gene transcription, which respectively may lead to the development of postnatal cancer in the progeny, and/or structural or functional teratogenesis (From Wells et al. [99])

enzymes involved in the recognition of oxidatively damaged DNA and its repair, as discussed below in section “**DNA Repair.**” Knockout or conditional knockout mice lacking these DNA repair components exhibit an increase in oxidatively damaged DNA in embryos and in fetal brain, and in the associated embryopathies and postnatal neurodevelopmental deficits, caused by agents like benzo[a]pyrene, methamphetamine, ethanol, and ionizing radiation [80, 99, 100]. These studies show that DNA oxidation, as distinct from oxidative damage to other types of cellular macromolecules, is a pathogenic molecular event. They also provide insight into the relative teratogenic contribution of oxidatively damaged DNA as distinct from ROS-mediated alterations in signal transduction. The next two subsections accordingly focus upon DNA as a macromolecular target of ROS, which can cause both mutagenic changes in gene sequence and direct and indirect epigenetic changes with no change in gene sequence.

### Mutagenic Mechanisms

ROS initiate over 20 different lesions in DNA, with 8-oxoguanine (Fig. 1.6) being the most prevalent [31]. This lesion is mutagenic, leading to a change in the DNA structure, and likely is involved in the mechanism of transplacental carcinogenesis,

perhaps including postnatal cancer in the children of mothers treated with the synthetic estrogen diethylstilbestrol [82]. However, aside from cancers, other forms of developmental toxicity, including structural birth defects and postnatal neurodevelopmental deficits, likely result not from mutagenesis but rather from the direct effects of 8-oxoG and possibly other ROS-initiated DNA lesions on embryonic and fetal gene expression, as discussed below.

## DNA Oxidation and Altered Gene Expression: Epigenetic Mechanisms

### Direct Effects of ROS

Unlike the ROS-initiated genetic damage leading to mutagenesis, ROS-initiated lesions in DNA, and particularly the oxidation of DNA by hydroxyl radicals, may directly alter gene expression without changes to the DNA sequence, by interfering with transcriptional machinery [35, 37–39, 76]. At least for the 8-oxoG lesion, some of its developmental effects may occur via a mechanism that could be viewed as epigenetic.

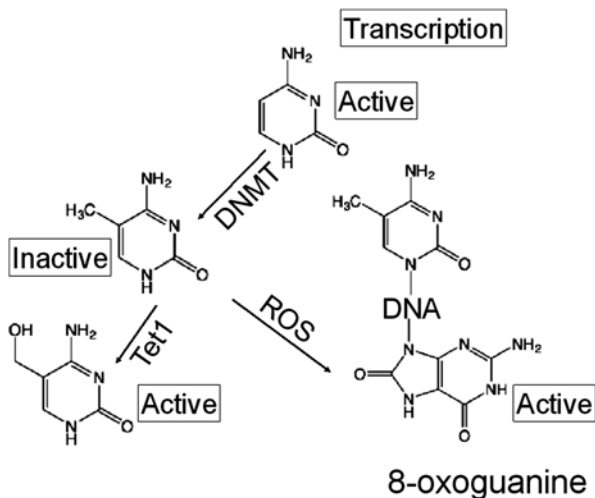
Epigenetic modulation of transcription involves the regulation of gene expression in a sequence-independent manner. One key form of epigenetic regulation is DNA methylation of cytosine residues within CpG islands, which either renders DNA available as euchromatin to promote transcription or sequesters DNA into heterochromatin to hinder transcription. The periods during gametogenesis and immediately following fertilization are marked by the global demethylation of genomic DNA with subsequent remethylation, catalyzed by DNA methyltransferases (DNMTs), in a process known as epigenetic reprogramming [77]. This comprehensive reprogramming provides an enhanced window of sensitivity to modulation by epigenetic regulation [10]. Experimentally, this has been shown in mice using the DNMT inhibitor 5-azacytidine shortly after fertilization. Inhibition of the remethylation of DNA by 5-azacytidine at this critical stage in development, in the days prior to the start of organ development, resulted in neural tube and ocular defects [83], suggesting that loss of epigenetic control during reprogramming can lead to teratogenesis.

DNA silencing by methylation involves the binding of methyl-CpG-binding domain proteins (MBDs), which leads to the incorporation of histones and subsequent conversion to heterochromatin [52]. Methylation of DNA can be reversed either enzymatically or by ROS and particularly the 8-oxoG lesion (Fig. 1.7). Enzymatic hydroxylation of the 5-methyl group of cytosine by Tet methylcytosine dioxygenase 1 (Tet1) forms 5-hydroxymethylcytosine (5-hmC) [93], which prevents MBD binding [52].

Similar to the inhibition of MBD binding by 5-hmC formation, oxidized guanine, in the form of 8-oxoguanine, when adjacent to 5-mC can block MBD binding, thereby maintaining DNA as euchromatin [95] (Fig. 1.7). Oxidative stress during development may therefore initiate teratogenesis via the interruption of epigenetic sequestering of DNA to heterochromatin.



**Fig. 1.7** Oxidatively “damaged” DNA in the activation of gene transcription. Hydroxylation of guanine residues by hydroxyl radicals forms 8-oxoguanine, which when adjacent to 5-methylcytosine in CpG islands can block the binding of methyl-CpG binding domain proteins (MBDs) to DNA, thereby maintaining DNA as euchromatin and genes in their active state. This could be viewed as an epigenetic effect of DNA oxidation



### Indirect Epigenetic Effects

Xenobiotics also may inhibit or induce DNMTs, resulting in gene activation or silencing. Similarly, xenobiotic-initiated alterations in the acetylation, methylation, or phosphorylation of histone proteins may alter chromatin structure and the binding of transcription factors to DNA. Finally, xenobiotics may alter the expression of related components like MBDs that are necessary gene transcription. It is not yet clear the extent to which, if any, ROS may contribute to the modulation of these epigenetic regulators, but if so this contribution would likely vary with the nature of the developmental toxicant and possibly the stage of gestation and conceptual target tissue. For example, the ROS-initiating teratogen ethanol in rodent embryos or fetuses alters the methylation status of several genes in more severely affected embryos, as well as gene expression of several DNMTs and at least one MBD, and DNMT activity in fetal brains; however, the involvement of ROS was not investigated ([121], [122]).

### Macromolecular Oxidative Damage Versus Signal Transduction

ROS-initiated changes in signal transduction have been suggested by some investigators to play a primary role in developmental toxicity, with ROS-initiated macromolecular damage contributing only at higher xenobiotic exposures. There appears to be little evidence to support this hierarchy, and oxidative damage to cellular macromolecules including DNA is typically evident at minimally developmentally toxic doses and concentrations of numerous ROS-initiating teratogens. For example, DNA oxidation in fetal brain is measurable in the absence of ROS-enhancing conditions and is elevated by a dose of ethanol that does not cause structural birth defects

when administered during embryogenesis [55]. Both oxidative damage and altered signal transduction are likely to play important roles in teratogenesis (Fig. 1.5). While macromolecular damage to lipids, proteins, and DNA are all potentially important mechanisms of teratogenesis, approaches for proving the selective mechanistic contributions of lipid and protein damage, as distinct from DNA damage, to teratogenesis have yet to be developed. An increase in conceptual lipid peroxidation and/or protein oxidation has been reported for embryopathies or birth defects caused by numerous teratogens (Table 1.4); however, while such results are biomarkers for oxidative stress, this association is not evidence for a causal role of these macromolecular lesions in the pathogenic mechanism. Accordingly, it is unclear in which cases the oxidative damage to lipids and proteins is causally involved in or merely associated with increased teratogenesis. In the case of oxidatively damaged DNA, on the other hand, the developmentally pathogenic role of 8-oxoG and possibly other DNA lesions is particularly demonstrable in genetically modified rodent models with deficient DNA repair. Indeed, physiological oxidative stress alone in the developing embryo and fetus, in the absence of xenobiotic exposure, can result in postnatal neurodevelopmental deficits in progeny with genetic deficiencies in DNA repair enzymes like ataxia telangiectasia mutated (ATM) [9] or oxoguanine glycosylase 1 (OGG1) [100]. However, ROS-initiated mechanisms of developmental toxicity involving macromolecular damage and alterations in signal transduction are not mutually exclusive, and the relative teratological contributions of each mechanism could vary with the teratogen, time of gestation, and conceptual target tissue.

There also is some cross-talk between ROS-mediated macromolecular damage and altered signal transduction, which further complicates the potential mechanism of teratogenesis, as exemplified by phosphatase and tensin homolog (PTEN). PTEN is a protein tyrosine phosphatase that controls cellular proliferation through the phosphatidylinositol 3-kinases (PI3K)/protein kinase B (PKB/Akt)/mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR) pathway [46]. PTEN regulates the PI3K/Akt/mTOR pathway by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate (PIP3) to phosphatidylinositol (4,5)-diphosphate (PIP2), which leads to the inhibition of cell growth and promotion of apoptosis [46].

In addition to the dephosphorylation of PIP3, a number of additional signaling pathways have been identified as PTEN dependent, including DNA damage repair. PTEN is important for chromosomal stability through its association with centromere-specific binding protein C (CENPB) and induction of Rad51, which is involved in homologous recombination repair (HRR) [78]. The loss of PTEN results in an inability to repair DNA as indicated by the presence of p53 binding protein 1 (53BP1) foci through a mechanism that is independent of nuclear phosphatase signaling. PTEN contains a phosphorylation site for ATM kinase activity, which promotes the SUMOylation of PTEN. This SUMOylation is thought to promote nuclear translocation and is necessary for double-strand break repair by HRR [7].

In the presence of ROS, PTEN can become reversibly inactivated through the generation of a disulfide bridge at Cys<sup>71</sup> and Cys<sup>124</sup> [44]. PTEN inhibition has been extensively studied in the context of neurodevelopment. Clinically, a number of nonsense mutations in the PTEN gene have been identified and are associated with Bannayan–Riley–Ruvalcaba syndrome, which is characterized by macrocephaly,

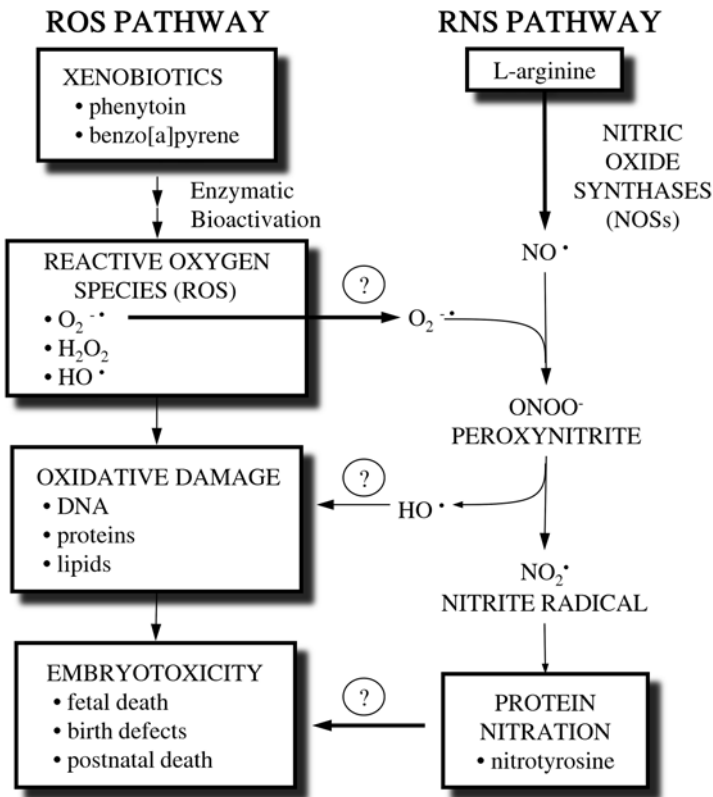
**Table 1.4** Redox-regulated transcription factors involved in development

Transcription factor	Regulation and developmental role
HIF-1	Activated in hypoxia
	Induced by reducing agents
	Controls:
	Vascular genes
	Iron regulatory elements
NF- $\kappa$ B	Activated in oxidative stress
	Developmental expression
	Controls:
	Apoptosis
AP-1	Activated in oxidative stress
	Developmental expression
	Controls:
	DNA repair (Ref-1)
Nrf1	Activated in oxidative stress
	Developmental expression
	Controls:
	Apoptosis
Nrf2	Activated in oxidative stress
	Developmental expression
	Controls:
	Redox balance
Wnt	Activated by hydrogen peroxide
	Developmental expression
	Controls:
	Body patterning
	Organogenesis

Abbreviations: *AP-1* activator protein-1, *HIF-1* hypoxia-inducible factor-1, *NF- $\kappa$ B* nuclear factor kappa B, *Nrf1* nuclear factor erythroid 2-related factor 1, *Nrf2* nuclear factor erythroid 2-related factor 2, *Wnt* wingless and integration site for mouse mammary tumor virus (From Dennery [14])

developmental delay, and a number of neoplastic lesions [86]. In PTEN knockout mice, the inhibition of mTOR complex 1 (mTORC1), a downstream effector of the PI3K/Akt/mTOR pathway, prevented macrocephaly, the incidence of seizures, and behavioral issues including anxiety and social interaction [118]. Mice deficient in the DNA repair protein ATM, which promotes nuclear translocation of PTEN, are at greater risk of xenobiotic-initiated embryopathies [8]. Taken together, these results suggest that the inhibition of PTEN can lead to adverse developmental outcomes by both alterations in signal transduction and loss of DNA repair.

## REACTIVE OXYGEN SPECIES (ROS) AND REACTIVE NITROGEN SPECIES (RNS)



**Fig. 1.8** Molecular damage by reactive nitrogen species (RNS) and potential interactions between the RNS and ROS pathways leading to enhanced damage to embryonic and fetal cellular macromolecules (From Kasapinovic et al. [33])

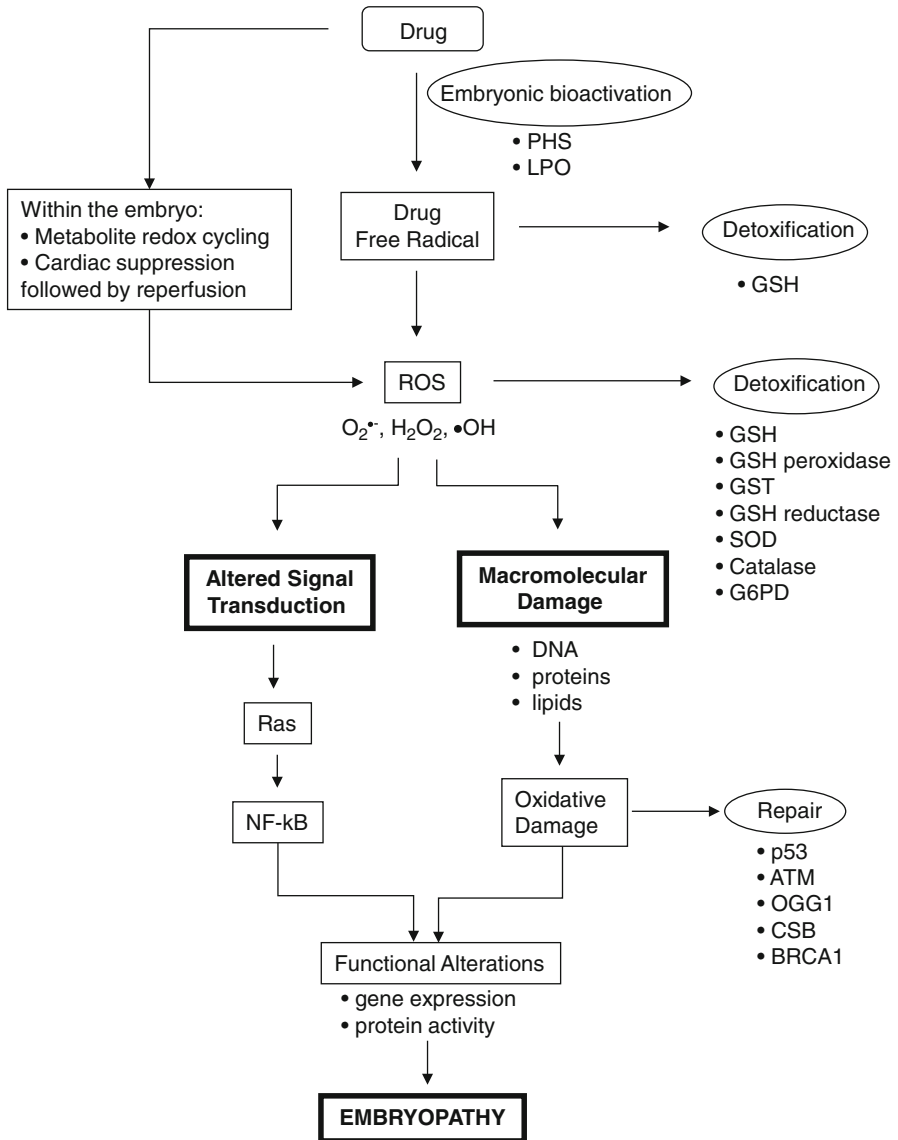
### *Reactive Nitrogen Species (RNS) in Teratogenesis*

Little is known about the role of RNS, produced via embryonic and fetal nitric oxide synthases (NOSs), in errors of development. However, it seems likely that RNS alone and/or in combination with ROS can adversely affect development (Fig. 1.8) via altered signal transduction and/or macromolecular damage. RNS have been implicated in the mechanism of teratogenesis for several xenobiotics [20], including phenytoin [33], although in the latter case using NOS knockout mice, RNS cannot fully account for the teratogenic effects observed. However, the contribution of RNS in phenytoin embryopathies revealed in NOS knockout mice was carried out in embryo culture, demonstrating that proximate embryonic NOS, as distinct from maternal activity, was the source of RNS.

## ***ROS-Mediated Signal Transduction***

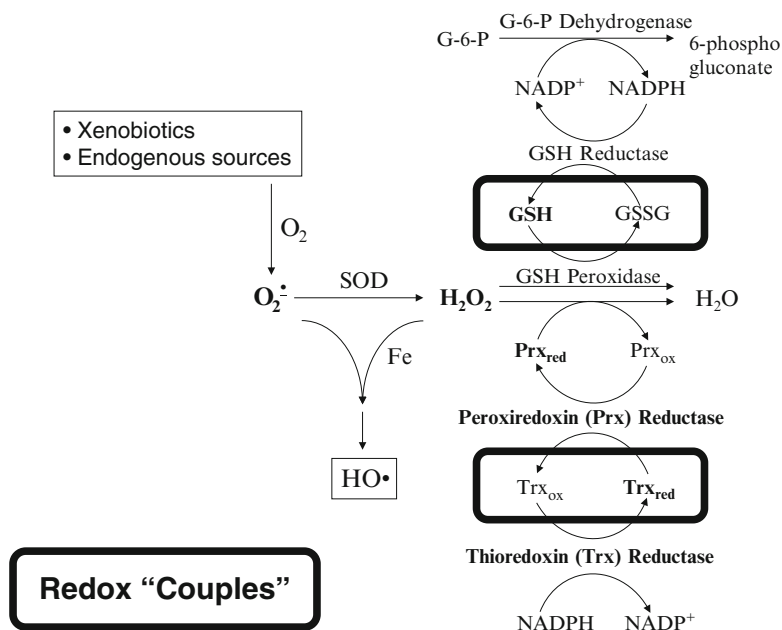
The potential for dual ROS mechanisms contributing to ROS-mediated teratogenesis is exemplified by the antiepileptic drug phenytoin. Although ROS-initiated macromolecular damage has been implicated in the mechanism of phenytoin teratogenesis, this drug also increases the embryonic levels of signal transduction proteins Ras and NF- $\kappa$ B (Fig. 1.9) [34, 107], as is typically observed during oxidative stress. The embryopathic effects of phenytoin were blocked by pretreatment with either a farnesyltransferase that prevents posttranslational Ras activation or an antisense oligonucleotide inhibitor of NF- $\kappa$ B, implicating this signal transduction pathway in the mechanism of teratogenesis. Thalidomide is another example of a drug for which multiple mechanisms of teratogenesis have been implicated, including some potentially unrelated to oxidative stress [47]. In regard to oxidative stress, thalidomide and at least two of its metabolites can enhance embryonic ROS formation, at least in part via bioactivation by embryonic prostaglandin H synthases (PHSs) to a reactive intermediate, which can alter embryonic signal transduction pathways (see below) and/or oxidatively damage embryonic DNA [25, 47, 99].

Mechanisms of ROS-initiated signal transduction in development have been reviewed in detail elsewhere [14, 25, 99]. Signal transduction pathways involving ROS and RNS are tightly and temporally regulated within cell types, subcellular organelles, and microenvironments within individual proteins and lipids [29], which likely accounts at least in part for the distinctive patterns of abnormal structural and/or functional development caused by different teratogens. Among the various forms of ROS, H<sub>2</sub>O<sub>2</sub> is relatively stable and diffusible and hence is commonly implicated in signal transduction via the selective oxidation of sulfhydryl groups of specific cysteine residues, which is reversible at lower H<sub>2</sub>O<sub>2</sub> concentrations, although higher concentrations can result in irreversible changes [24]. This usually reversible oxidation is determined by the redox state of the cell, which is regulated by the ratios of several redox “couples” (Fig. 1.10), including cysteine/cystine, GSH/glutathione disulfide, and, for both forms of thioredoxin (TRX1, TRX2), TRX<sub>reduced</sub>/TRX<sub>oxidized</sub>, all of which are localized in different cellular compartments. These redox couples are believed to regulate developmental pathways for cellular proliferation, differentiation, apoptosis, and necrosis in a fashion that is selective for the species, strain, time of gestation, target tissue and cell, and cellular compartment, thereby accounting at least in part for the distinctive teratological profiles that are characteristic of different teratogens [25]. As the cellular redox state of the developing embryo moves from an anaerobic reducing environment, which favors cellular proliferation, to an oxidizing environment, which favors differentiation, the contributions of these redox couples change according to the distinctive redox potentials of their components, contributing to the variably distinctive consequences of in utero exposure to different ROS-initiating teratogens [25]. ROS-mediated oxidation of selective cysteine residues is implicated in signaling by several redox-sensitive endogenous ligands (Table 1.5) [14] and by tumor necrosis factor alpha and epidermal growth factor, both of which stimulate ROS formation [25], and for teratogens like thalidomide, which is postulated to disrupt NF- $\kappa$ B signaling leading to changes in multiple genes involved in limb growth (Fig. 1.11) [25].



**Fig. 1.9** Dual effects of ROS in initiating signal transduction and oxidatively damaging structural macromolecules, which are not mutually exclusive. Potential contribution of Ras and NF-κB proteins in signal transduction pathways initiated by drug-enhanced formation of ROS (Modified from Kennedy et al. [34])

Oxidative stress can affect signaling in other ways, including, as examples, (1) reducing DNA and histone methylation reactions indirectly via the diversion of homocysteine to GSH synthesis, (2) altered gene transcription inactivation via reduced stability of hypoxia-inducible factor (HIF), and (3) altered gene transcription via an increase in sirtuin-dependent deacetylation of some transcription factors (increased transcription) or histone proteins (decreased transcription) [99].



**Fig. 1.10** Redox couples modulating the cellular effects of endogenous or xenobiotic-enhanced oxidative stress. Redox couples in addition to those circled include NADPH/NADP<sup>+</sup> and Prx<sub>red</sub>/Prx<sub>ox</sub> shown above and cysteine/cystine (not shown). Abbreviations: *ox* oxidized, *red* reduced (From Wells et al. [99])

## Antioxidative Pathways

In the absence of adequate antioxidative protection within the embryo and fetus, even physiological levels of conceptual ROS production can be developmentally toxic (Table 1.6). Untreated progeny with genetic deficiencies in glucose-6-phosphate dehydrogenase (G6PD) [67] or catalase [2, 3] exhibit increased embryopathies compared to wild-type littermates with normal activities of these antioxidative enzymes, even though embryonic and fetal activities of enzymes like catalase are less than 10 % of maternal activity. A role for endogenous ROS has been similarly implicated in the in utero initiation of postnatal carcinogenesis in cancer-prone p53 knockout mice: dietary supplementation with low-dose vitamin E reduced conceptual DNA oxidation and postnatal tumorigenicity [12], whereas high-dose vitamin had an opposite, pro-oxidant effect, enhancing conceptual DNA oxidation and postnatal tumorigenicity [13].

As discussed earlier for ROS formation and the predominant importance of individual embryonic or fetal activity over maternal activity or the average activity for a litter, due to the instability of ROS, proximate antioxidative protection within the conceptus, as distinct from maternal activities, is critical. The same will be reiterated for conceptual DNA repair in the following section. This observation is evident in genetically altered mice with either deficient or enhanced activities of catalase, in

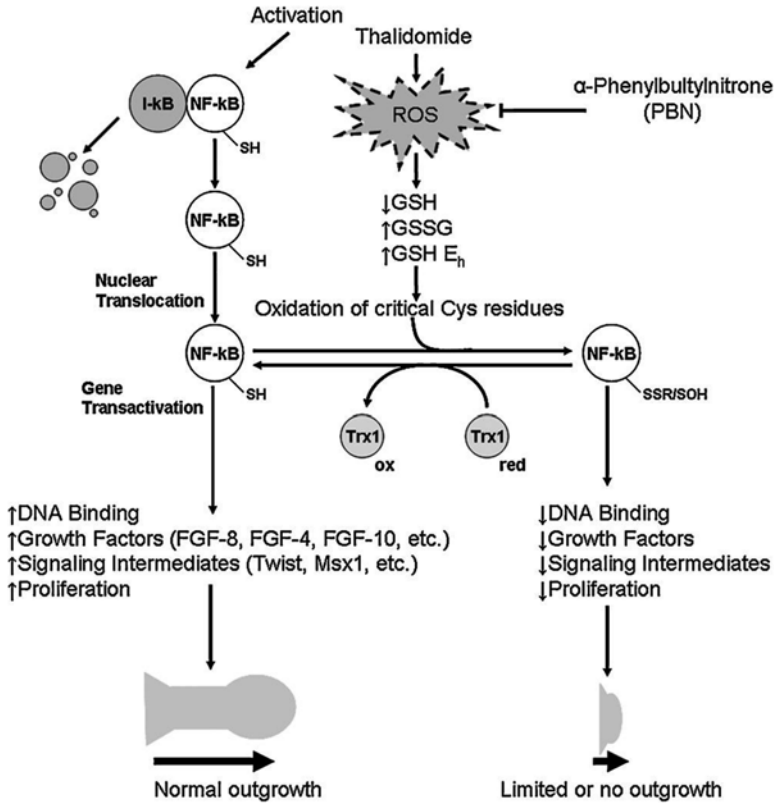
**Table 1.5** Modulation of representative antioxidants and antioxidative enzymes implicating endogenous and/or xenobiotic-enhanced reactive oxygen species (ROS) in the mechanism of developmental toxicity

Component	Drug/model	Modulation and outcome	Citation
<i>Antioxidants</i>			
Vitamin E	Phenytoln	Maternal pretreatment with lower doses of vitamin E reduces phenytoln teratogenicity	[12, 97]
	Phenytoln	Paradoxically, higher vitamin E doses have a pro-oxidant effect, enhancing phenytoln teratogenicity	[13, 96]
	p53 knockout mice	Low-dose dietary supplementation with vitamin E reduces oxidatively damaged DNA in fetal tissues and reduces spontaneous postnatal tumorigenicity in cancer-prone p53 mice	[12, 97]
	p53 knockout mice	High-dose dietary supplementation with vitamin E has a paradoxical pro-oxidant effect, enhancing DNA oxidation in fetal tissues of p53 knockout mice and increasing spontaneous tumorigenicity	[13]
	Ethanol (EtOH)	Maternal dietary supplementation with vitamin E decreased fetal malformations and fetal hepatic 8-iso-PGF(2alpha) levels	[103]
Caffeic acid	Maternal pretreatment with caffeic acid reduces phenytoln teratogenicity	[101]	
Propyl gallate	Maternal treatment with propyl gallate reduces hydroxyurea teratogenicity	[15]	
Glutathione (GSH)	DEM	Depletion with diethyl maleate (DEM) enhances the teratogenicity of thalidomide, 5-fluorouracil, cadmium hydrochloride, and phenytoln	[41, 63, 109]
	BSO	Inhibition of GSH synthesis with buthionine sulfoximine (BSO) decreases baseline growth parameters in embryo culture and enhances the teratogenic effects of methanol, phenytoln, 5-fluorouracil, and diabetes	[23, 26, 58, 62, 84, 109]
	GSH precursors	Pretreatment with GSH precursors including N-acetylcysteine and GSH esters reduces the teratogenic effects of cyclophosphamide, thalidomide, phenytoln, and diabetes	[22, 41, 84, 110]
<i>Antioxidative enzymes</i>			
GSH peroxidase	Phenytoln	Reduction in GSH peroxidase activity by dietary restriction of selenium cofactor enhances phenytoln teratogenicity	[72]
	High glucose	Addition of GSH peroxidase to embryo culture medium reduces glucose-initiated embryopathies	[19]
GSH reductase	Phenytoln	Inhibition of GSH reductase by a low, non-teratogenic dose of bis-chloroethyl-nitrosourea (BCNU) enhances phenytoln teratogenicity	[111]



Superoxide dismutase (SOD)	Phenytol, benzo[a]pyrene	Addition of polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) to embryo culture medium reduces oxidative macromolecular damage and embryopathies caused by phenytol and benzo[a]pyrene (this protection is not observed <i>in vivo</i> , where maternal pretreatment with PEG-SOD paradoxically enhances phenytol teratogenicity)	[105, 106]
	High glucose	Addition to SOD to embryo culture medium reduces glucose-initiated embryopathies	[19]
	Transgenic SOD expression with EtOH, diabetes	Transgenic mice expressing increased SOD were protected against malformations caused by EtOH and streptozotocin-induced diabetes, while SOD wild-type mice exhibited malformations and fetal loss	[21, 102]
	SOD knockout mice with EtOH	SOD knockout mice were more susceptible to EtOH-induced teratogenesis and fetal hepatic isoprostane formation	[102]
Catalase	Phenytol, benzo[a]pyrene	Addition of PEG-catalase to embryo culture medium reduces oxidative macromolecular damage and embryopathies caused by phenytol and benzo[a]pyrene	[2, 105]
	Phenytol, EtOH	<i>In vivo</i> maternal pretreatment with PEG-catalase reduces phenytol and EtOH teratogenicity	[3, 56]
	Genetic catalase-deficiency untreated and with phenytol, EtOH, methanol	Untreated mutant mice deficient in catalase exhibit greater embryopathies than wild-type controls and are more susceptible to macromolecular damage and embryopathies caused by phenytol, EtOH, and methanol <i>in embryo culture and/or in vivo</i>	[2, 56, 57]
	Transgenic catalase expression untreated and with phenytol, EtOH, methanol	Untreated transgenic mice with enhanced catalase activity exhibit fewer embryopathies than wild-type controls and are protected from macromolecular damage and embryopathies caused by phenytol, EtOH, and methanol <i>in embryo culture and/or in vivo</i>	[2, 56, 57]
	High glucose	Addition of catalase to embryo culture medium reduces glucose-initiated embryopathies	[19]
Glucose-6-phosphate dehydrogenase (G6PD)	Genetic G6PD-deficiency	Untreated mutant mice deficient in G6PD exhibit enhanced <i>in utero</i> and postnatal death compared to wild-type littermates	[67]
	Genetic G6PD-deficiency with phenytol	G6PD-deficient progeny are more susceptible to phenytol-initiated DNA oxidation and teratogenicity	[67]
Thioredoxin (Trx2)	<i>trx2</i> knockout mice	Untreated homozygous <i>trx2</i> knockout mice exhibit open neural tubes and apoptosis at embryonic day (E) 10.5 and die by E 12.5	[68]

Abbreviations: *BCNU* bis-chloroethyl-nitrosourea, *BSO* buthionine sulfoximine, *DEM* diethyl maleate, *E* embryonic day, *EtOH* ethanol, *G6PD* glucose-6-phosphate dehydrogenase, *GSH* glutathione, *p53* protein 53, *PEG* polyethylene glycol-conjugated, *ROS* reactive oxygen species, *Trx2* thioredoxin 2



**Fig. 1.11** A postulated role for ROS signaling in thalidomide teratogenicity. Normal limb outgrowth requires both the activation of NF- $\kappa$ B in the cytosol via oxidative and non-oxidative mechanisms, followed by translocation of NF- $\kappa$ B to the nucleus and binding to DNA. Thalidomide enhances ROS, which disrupt NF- $\kappa$ B activation and/or DNA binding, causing a loss of gene expression, with limited or absent limb growth. This effect is blocked by the free radical spin trapping agent phenylbutylnitron (PBN). Abbreviations: *cys* cysteine, *FGF* fibroblast growth factor, *GSH* reduced glutathione, *GSSG* oxidized glutathione, *I- $\kappa$ B* inhibitor of NF- $\kappa$ B, *NF- $\kappa$ B* nuclear factor kappa-light-chain-enhancer of activated B cells, *ox* oxidized, *PBN* phenylbutylnitron, *red* reduced, *ROS* reactive oxygen species, *SH* sulfhydryl group, *SOH* oxidized sulfhydryl group, *SSR* reduced sulfhydryl group, *Trx* thioredoxin, *Eh* redox potential (From Hansen and Harris [25])

which embryopathic susceptibility to phenytoin [2, 3], methanol [57], or ethanol [56] is dependent upon the embryonic genotype, with increasing effect of the genetic alteration going from wild-type to heterozygous to homozygous gene modification (knockout deficiency or transgenic enhancement), even though these embryos are littermates and exposed to the same drug concentrations and uterine environment. A similar embryonic gene determinant of susceptibility, as distinct from a maternal or litter determinant, has been observed for G6PD in the developmental toxicity of phenytoin.

**Table 1.6** Modulation of DNA damage response and repair proteins and enzymes implicating oxidatively damaged DNA, and particularly the 8-oxoguanine (8-oxoG) lesion, in the pathogenic mechanism of physiological and xenobiotic-enhanced oxidative stress and developmental toxicity

Protein	Modulation and outcome	Reference
ATM	Untreated <i>atm</i> knockout mice exhibit enhanced embryopathies compared to wild-type controls	[9]
	<i>atm</i> knockout mice are more susceptible than wild-type littermates to developmental toxicity caused by ionizing radiation and phenytoin <i>in vivo</i> and/or <i>in embryo</i> culture	[8, 45]
p53	<i>p53</i> knockout mice are more susceptible to benzo[a]pyrene teratogenicity	[66]
	Cultured embryonic limb buds from <i>p53</i> knockout mice exhibited increased malformations caused by 4-hydroperoxycyclophosphamide, the activated metabolite of cyclophosphamide	[59]
	<i>p53</i> knockout mice are more susceptible to malformations caused by ionizing radiation	[69]
OGG1	<i>In vitro</i> studies using <i>ogg1</i> knockout cells, which cannot repair the 8-oxoG lesion, show a higher level of DNA double-strand breaks (gamma-H2AX) and cytotoxicity when exposed to methylmercury	[70]
	<i>In vitro</i> studies using transgenic cells overexpressing OGG1 show a lower level of 8-oxoG, gamma-H2AX, and cytotoxicity compared to wild-type control cells when exposed to H <sub>2</sub> O <sub>2</sub> , menadione, cisplatin, oxaliplatin, phenytoin, and/or methylmercury	[71, 79]
	<i>In vivo</i> , untreated <i>ogg1</i> knockout mice exhibit postnatal neurodevelopmental deficits compared to wild-type controls	[53]
	<i>ogg1</i> knockout mice exhibit higher levels of oxidatively damaged DNA than wild-type controls and are more susceptible to birth defects and postnatal neurodevelopmental deficits caused by <i>in utero</i> exposure to methamphetamine and ethanol	[53, 108]
FPG	<i>In vitro</i> studies using transgenic cells expressing high levels of FPG, the bacterial homolog of OGG1, show a lower level of cytotoxicity compared to wild-type control cells when exposed to H <sub>2</sub> O <sub>2</sub> , menadione, cisplatin, oxaliplatin, and/or phenytoin	[79]
CSB	<i>csb</i> knockout mice exhibit higher levels of oxidatively damaged DNA than wild-type controls and are more susceptible to birth defects and postnatal neurodevelopmental deficits caused by <i>in utero</i> exposure to methamphetamine	[51]
BRCA1	<i>brca1</i> conditional knockout embryos exposed in culture to methamphetamine and alcohol exhibit higher levels of 8-oxoG, gamma H2AX, and embryopathies compared to wild-type controls	[88]

Abbreviations: 8-oxoG 8-oxoguanine, ATM ataxia telangiectasia mutated, BRCA1 breast cancer susceptibility protein 1, CSB Cockayne syndrome B, FPG formamidopyrimidine glycosylase, gamma-H2AX phosphorylated histone 2 AX protein, OGG1 oxoguanine glycosylase 1, p53 protein 53

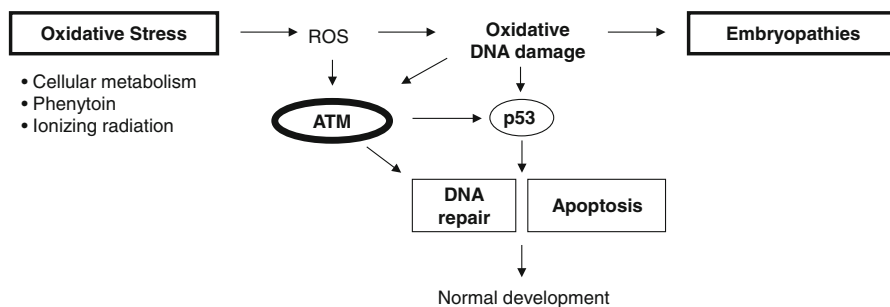
Pretreatment with lipid-soluble vitamin E or water-soluble caffeic acid reduces the teratogenicity of drugs like phenytoin (Table 1.6) [99]. Similarly, preincubation with exogenous polyethylene glycol (PEG)-conjugated (stabilized) forms of superoxide dismutase (SOD) and catalase reduces the oxidative macromolecular damage and embryopathies caused by xenobiotics like phenytoin and benzo[a]pyrene in embryo culture, as is also observed in genetically modified mice overexpressing endogenous catalase activity when exposed to phenytoin, ethanol, and methanol [1–3, 56, 57, 99, 100]. In vivo, maternal pretreatment with PEG-catalase increases embryonic catalase activity and reduces phenytoin teratogenicity, although in vivo pretreatment with PEG-SOD conversely enhances phenytoin teratogenicity, possibly due to the accumulation of H<sub>2</sub>O<sub>2</sub> [106]. Catalase overexpressing mice are similarly resistant to structural birth defects and/or postnatal neurodevelopmental deficits caused by in utero exposure to phenytoin and ethanol [1, 2, 56].

Conversely, depletion of cellular antioxidants like GSH, inhibition of antioxidative enzymes like GSH peroxidase and GSH reductase, or the use of genetically modified mice with a deficiency in catalase or G6PD enhance the macromolecular damage and teratogenicity caused by in utero exposure to xenobiotics like phenytoin, ethanol, and methanol in embryo culture and/or in vivo [1–3, 56, 57, 99]. While results from embryo culture and in vivo studies are similar for most teratogens, one exception is methanol, for which evidence of a ROS-dependent mechanism of developmental toxicity in embryo culture is comprehensive [57, 100], while in vivo results suggest an alternative mechanism [90].

## DNA Repair

The genetic engineering of cells and mouse models with altered DNA repair activity has provided the opportunity for evaluating the pathogenic role of oxidatively damaged DNA in developmental toxicity (Table 1.6), as distinct from the contributions of oxidative damage to other cellular macromolecules like proteins and lipids or receptor-mediated mechanisms. In addition, these models allow a selective evaluation of the relative contribution of ROS-initiated DNA oxidation as distinct from ROS-initiated alterations in signal transduction. The complementary insight provided by these models is the importance of embryonic and fetal DNA repair in protecting the conceptus from the developmental toxicity of endogenous and xenobiotic-enhanced conceptual ROS formation.

As with antioxidative pathways, studies in untreated genetically modified mice with deficient DNA repair proteins have revealed a potentially pathogenic role for oxidatively damaged DNA resulting from physiological levels of developmental ROS formation. For example, the ataxia telangiectasia-mutated (ATM) protein and p53 are critical components of the pathways for the detection of oxidatively damaged DNA and the transduction of the signal for DNA repair or apoptotic cell death



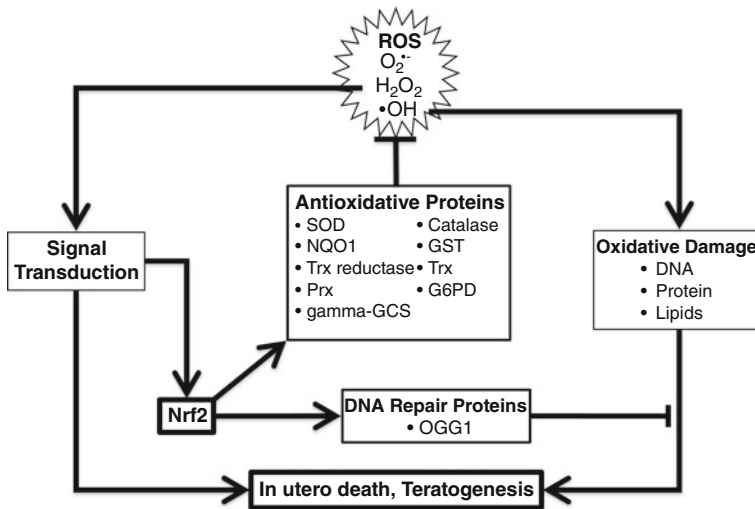
**Fig. 1.12** The relation of the ATM and p53 proteins in the cellular DNA repair process or apoptosis following DNA damage initiated by oxidative stress (From Bhuller and Wells [9])

(Fig. 1.12). The DNA repair-deficient progeny of untreated ATM knockout mice exhibit enhanced embryopathies in culture compared to wild-type littermates [9]. Similarly, untreated knockout mice lacking oxoguanine glycosylase 1 (OGG1), the major enzyme for repair of the 8-oxoG lesion in DNA, exhibit enhanced postnatal neurodevelopmental deficits compared to their wild-type littermates [100].

In genetically modified cells and/or mouse models with a deficiency in ATM, p53, OGG1, the DNA repair protein Cockayne syndrome B (CSB) or the DNA repair-coordinating protein breast cancer 1 (BRCA1), DNA oxidation and the cytotoxicity and/or embryopathic effects of xenobiotics like phenytoin, benzo[a]pyrene, methamphetamine, and ethanol, as well as ionizing radiation, in vivo and/or in embryo culture, are enhanced [51, 80, 88, 100]. Similarly in vitro, *ogg1* knockout cells are more sensitive to DNA damage and cytotoxicity caused by methylmercury [70, 71]. In some cases, the picture can be less straightforward. In the case of p53, which in response to DNA damage can transduce a signal either for DNA repair or apoptosis, the modulatory effect of this protein may vary with the drug and target tissue. Accordingly, while p53 knockout mice are more susceptible to the full spectrum of teratogenic effects of benzo[a]pyrene [66], they are protected from the ocular teratogenic effect of 2-chloro-2'-deoxyadenosine [112], in the latter case presumably due to a reduced signal for apoptosis.

Conversely, cells genetically engineered to express enhanced levels of OGG1 or its bacterial homolog, formamidopyrimidine glycosylase (FPG), exhibit reduced levels of oxidatively damaged DNA, DNA double-strand breaks, identified by the phosphorylation of histone protein 2AX (gamma H2AX), and cytotoxicity caused by H<sub>2</sub>O<sub>2</sub>, menadione, cisplatin, oxaliplatin, phenytoin, and/or methylmercury [71, 80].

The protective effects of the DNA damage detection and response proteins ATM, p53, and BRCA1 suggest that oxidatively damaged DNA plays an important pathogenic role in the developmental toxicity of endogenous ROS or xenobiotic-enhanced ROS formation, while the protective effects of OGG1, CSB, and FPG suggest that 8-oxoG specifically is a pathogenic lesion.



**Fig. 1.13** Central role of embryonic and fetal nuclear factor erythroid 2-related factor 2 (Nrf2) in protecting progeny from endogenous and xenobiotic-enhanced oxidative stress. ROS can enhance both the activation of conceptual Nrf2 in the cytoplasm and its binding to DNA in the nucleus, stimulate the comprehensive expression of a battery of proteins and enzymes that blocks redox cycling, detoxify reactive oxygen species, and repair oxidatively damaged DNA. Abbreviations: *gamma-GCS* gamma-glutamyl-cysteine synthase, *G6PD* glucose-6-phosphate dehydrogenase, *GST* glutathione S-transferase, *NQO1* NAD(P)H quinone oxidoreductase, *Prx* peroxiredoxin, *SOD* superoxide dismutase, *Trx* thioredoxin

## Regulation of Embryonic and Fetal ROS-Protective Pathways by Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) in Teratogenesis

Nrf2 is a transcription factor that regulates the transcription of numerous proteins, and particularly several important proteins and enzymes that protect against oxidative stress, including (1) NAD(P)H:quinone oxidoreductase (NQO1), which blocks the redox cycling of catechol metabolites; (2) antioxidative peptides and enzymes, including the GSH synthetic enzyme gamma-glutamyl-cysteine synthase, the glutamate/cystine transporter, TRX1, TRX reductase-1, peroxiredoxins, SOD, catalase, and G6PD; and (3) the DNA repair enzyme OGG1 (Fig. 1.13) [25, 81]. Nrf2 in the cytoplasm is activated by the ROS-initiated oxidation of sulfhydryl groups in cysteine residues of its repressor protein, Kelch-like ECH-associated protein 1 (Keap1), which releases Nrf2 from Keap1 and allows it to translocate to the nucleus, where it heterodimerizes with other proteins and binds to the antioxidant response element (ARE), thereby activating gene transcription. The DNA binding of Nrf2 also is redox-sensitive. Cytosolic activation of Nrf2 is primarily controlled by the GSH redox couple, while Nrf2 DNA binding is controlled by the nuclear Trx1 couple. Little is known about the role of Nrf2 in development. Although the viability and apparently normal

development of Nrf2 knockout mice suggests a negligible role, Nrf2 mRNA and protein are expressed during organogenesis [11]. Moreover, pretreatment of pregnant mice with 3H-1,2-dithiole-3-thione (D3T), a Nrf2 activator, protects their progeny from apoptosis and malformations caused by in utero exposure to the ROS-initiating teratogen ethanol [17], suggesting that Nrf2 serves an important embryoprotective role. More recently, the central role of Nrf2 in protecting the fetus from oxidative stress was corroborated in Nrf2 knockout mice, the Nrf2-deficient progeny of which when exposed in utero to the ROS-initiating teratogen methamphetamine exhibited increased oxidatively damaged DNA in fetal brain, and more severe postnatal neurodevelopmental deficits in activity and olfaction, compared to wild-type littermates [81]. Accordingly, as in adult tissues, Nrf2 appears to serve in the embryo and fetus as a master switch that, in response to either endogenous or xenobiotic-enhanced oxidative stress, activates the transcription of a comprehensive battery of enzymes and proteins that interrupt ROS formation, detoxify ROS, and repair DNA that has been oxidatively damaged by ROS (Fig. 1.12).

## Conclusions and Future Considerations

Oxidative stress in the developing embryo and fetus can adversely affect both gross morphological and functional development. This stress can occur with both physiological levels of ROS when conceptual ROS detoxification or macromolecular repair is deficient and with elevated conceptual ROS levels initiated by xenobiotics, ionizing radiation, and other ROS-initiating environmental factors. Mechanisms of teratogenesis include both alterations in ROS-mediated signal transduction pathways and ROS-initiated macromolecular damage, which are not necessarily mutually exclusive and in some cases may also involve ROS-independent mechanisms. At least in animal models (primarily rodents and rabbits), susceptibility is substantially determined by the conceptual balance among pathways for ROS formation, ROS detoxification, and repair of oxidative macromolecular damage, any of which can differ markedly among littermates.

Our understanding of the full role of ROS-mediated macromolecular damage will benefit from the development of more definitive approaches to establish a causal or pathogenic role for specific conceptual cases of lipid peroxidation and protein oxidation, similar to the use of knockout mice deficient in particular DNA repair proteins for revealing the embryopathic role of DNA oxidation and repair. This includes the role of lipid peroxidation products in subsequent reactions with proteins and DNA. More definitive studies of the cross-talk between ROS-mediated signal transduction and DNA oxidation, the latter of which could be considered a form of epigenetic signaling, will likely provide important new insights into the mechanisms of ROS-mediated teratogenesis.

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