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The Potential Role of Redox Cycling as a Mechanism for Chemical Teratogenesis

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A survey of the literature indicates that several chemicals whose reduced metabolites are capable of undergoing redox cycling in biological systems also possess significant teratogenic properties when tested *in vivo*. We have initiated investigations to determine whether the embryotoxic effects of such chemicals could result from their redox cycling properties and whether redox cycling could be an important mechanism in chemical teratogenesis. In order to obviate the potentially confounding influences of maternal factors, our initial studies have been performed with a whole embryo culture system with redox cycling agents added directly to the culture medium. Several representative redox cycling agents including doxorubicin, paraquat, a series of nitroheterocycles, nitrosofluorene, and diethylstilbestrol (converted metabolically to redox cycling quinone/semiquinone radicals) have been investigated thus far. The nitroheterocycles which bear nitro groups with comparatively high redox potentials produced a striking, asymmetric defect involving primarily the right half of the prosencephalic and mesencephalic regions. The effect was exacerbated under conditions of low O_2 tension. Accumulated data to date strongly suggest that reduction of the nitro group is an essential feature in the embryotoxic mechanism. Quinones (doxorubicin, paraquat) and compounds metabolically converted to quinones (diethylstilbestrol) appeared to produce embryotoxic effects via mechanisms not associated with redox cycling. Nitrosofluorene embryotoxicity was markedly exacerbated by changes in both intra- and extracellular glutathione levels, but definitive dependence on a radical-mediated effect or redox cycling was not demonstrated.

Introduction

The biochemistry of chemical teratogenesis is a relatively new area of research that remains largely unexplored (*1*). Because of the intricate nature of morphogenesis, the complexity of the problems associated with such research is enormous and undoubtedly discourages many potential investigators who otherwise could contribute to a better understanding of mechanistic aspects. Thus much of the existing information concerning chemically elicited developmental abnormalities is still of a more descriptive than mechanistic nature. Some recent, fundamental scientific advances, however, should encourage researchers to explore more seriously the biochemical mechanisms whereby chemicals can elicit developmental structural anomalies. The evolution of a successful whole embryo culturing system represents a highly significant technological advance and now permits investigators to study the effects of chemicals on mammalian prenatal development in the absence of potentially confounding maternal factors. The culture system has also enabled a full recognition of the importance of both maternal and embryonic biotransformation/bioactivation as critical determinants of the capacity of chemicals to elicit defects (*2*). Ascertainment of the identities of ultimate chemical dysmorphogenic agents and of regulatory factors governing their generation during organogenesis will undoubtedly assist in elucidating teratogenic mechanisms. Thus far, focus in this research area has been on the cytochrome P-450-dependent generation of reactive intermediates by both extraembryonic (*3–6*) and embryonic (*7–9*) enzyme sources. The purpose of this article is to discuss some initial and preliminary findings with respect to cytochrome P-450-independent embryonic systems that appear capable of converting potential prodysmorphogenic agents to their ultimate, biologically active forms. Most of the drugs and chemicals in this category that have been investigated thus far are those that can undergo reduction by cellular reducing agents followed by reoxidation by molecular oxygen (O_2). Reoxidation results in the generation of the original, parent compound plus superoxide anion (O_2) and, subsequently, highly reactive metabolic derivatives of oxygen. Such chemicals are also known as "redox cyclers" and may produce biological effects via a variety of consequences of the redox cycling process (*10*). Agents to be discussed will include doxorubicin (Adriamycin), a series of heterocyclic nitro compounds, certain nitroarenes, paraquat, menadione, diethylstilbestrol (DES), nitrosofluorene,

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and 2-acetylaminofluorene. Each of these substances (or their respective metabolites) is known to be capable of undergoing redox cycling in biological systems and has also been studied in terms of its embryotoxicity in the whole embryo culture system in our laboratories. It should be re-emphasized that studies performed as of this writing have only very recently been initiated and many are thus of a preliminary nature. They represent the first reported attempts to investigate redox cycling as a potential mechanism of chemically induced terata. Therefore, any conclusions drawn therefrom must be regarded at present as strictly tentative and/or hypothetical.

Redox Cycling: The Phenomenon

Redox cycling agents are chemicals that are capable of undergoing single electron reduction reactions to yield radical species (*10*). Such agents, in accordance with their respective redox potentials, can accept electrons from a variety of biological reducing agents including reduced flavoproteins, reduced ferredoxin, NADPH, NADH, reduced glutathione (GSH) and other thiol-containing compounds, ascorbate, and probably others. Enzymes known to catalyze such reactions are frequently flavoproteins and include NADPH–cytochrome P-450 reductase, xanthine oxidase, mitochondrial flavoproteins of the electron transport chain and various other dehydrogenases. Frequently, redox cycling xenobiotics accept electrons directly from reduced flavoproteins.

In the presence of O_2, the reduced organic chemical can be reoxidized to the original parent compound by donating a single electron to O_2 thus converting it to $O_2^{\cdot-}$. As long as both O_2 and suitable reducing equivalents (e.g., NADH, NADPH, GSH) are available, the repetitive reduction and reoxidation reactions result in the occurrence of a redox cycle and the process is referred to as "futile cycling." Clearly, this process (Fig. 1) could conceivably elicit cytotoxicity via depletion of essential reducing equivalents, depletion of O_2 (particularly if involved cells are already living under somewhat hypoxic conditions) or via interactions of reactive organic radical forms with critical biological macromolecules. Interaction with small biomolecules is also a potential damaging factor. GSH, for example, can compete with O_2 for nitro anion radicals (*11*), and redox cycling can thus lead to depletion of the reduced form of cellular glutathione which constitutes one of the cell's most important defenses against cytotoxic agents and also subserves several highly important biological functions (DNA synthesis, etc.). Such organic radicals can also initiate lipid peroxidation reactions resulting in the disruption or disorganization of cellular membranes and ultimate cell death if sufficiently extensive.

In addition, generation of $O_2^{\cdot-}$ leads to the formation of hydrogen peroxide, both spontaneously and via the catalytic action of superoxide dismutase. In the presence of transition metals (Fe^{2+}, Cu^{+}) and under other appropriate conditions, hydrogen peroxide, in turn, can be converted to highly reactive and potentially very toxic hydroxyl radicals ($\cdot$ OH) and to singlet oxygen. Under conditions in which O_2 tensions are relatively high, toxicity resulting from the generation of hydroxyl radicals and/or from the depletion of important reducing equivalents may predominate whereas, under conditions of low O_2 tension, the major cause of toxicity may be an exacerbation of the hypoxic state and/or an increase in the steady-state levels of organic radicals. Radical species may bind covalently to critical macromolecules, initiate free radical-mediated reactions such as lipid peroxidation or act via less well-established mechanisms.

To what extent do redox cycling chemicals exist in the modern environment? Prototype redox cycling agents currently undergoing intensive investigation are aromatic nitro compounds (*12*) and quinone/quinonoids (*13*). The aromatic nitro group can undergo single-electron reduction to yield nitro anion radicals that readily reduce O_2 to $O_2^{\cdot-}$. Their capacity to undergo redox cycling under conditions of relative hypoxia has been implicated in their antimicrobial, radiosensitizing, carcinogenic, and mutagenic properties. Nitro compounds can be further reduced to the corresponding nitroso analogs

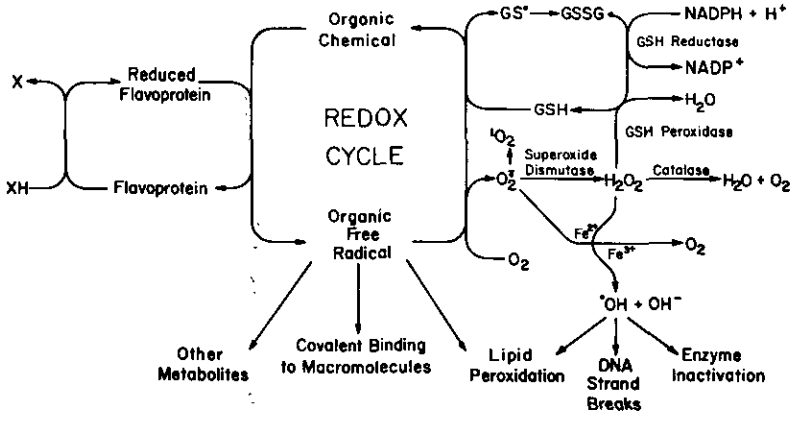

FIGURE 1. Metabolic pathways involved in the redox cycling of foreign organic chemicals (*10*).

that may undergo redox cycling via conversion to nitroso anion radicals. Other radical species feasibly may be generated during the ultimate reduction to primary amines via the hydroxylamine, itself a reactive, toxic intermediate.

Quinones may undergo either one- or two-electron reduction reactions. The former, resulting in the formation of radical semiquinones, represents a bioactivation reaction due primarily to the reactivity of the highly unstable semiquinone radical. The two-electron reduction reactions, catalyzed by diaphorases, result in the formation of fully reduced hydroquinones which appear to be virtually inactive biologically and are normally regarded as detoxication processes. One of the most notorious teratogenic chemicals, DES, appears capable of producing various deleterious biological effects by virtue of its conversion to quinones and semiquinones (*14–16*).

Other foreign organic chemicals that accept single electrons in a stepwise fashion and are thus converted to free radical intermediates potentially capable of redox cycling are azo derivatives, nitroso compounds, *N*-oxides, *S*-oxides, and polyhalogenated aliphatic hydrocarbons (*10*). Carbonyl compounds also undergo dehydrogenase-catalyzed reduction reactions but these are two-electron reduction reactions that do not involve the production of radical intermediates. Living cells have strongly negative redox potentials and are thus capable of readily reducing each of these classes of chemicals. A large number of factors are important determinants of the quantitative generation of biologically active, reactive intermediates from these chemicals. These include the redox potential of the reducible organic chemical and the availability and activity of enzymes that catalyze their respective reduction reactions. Also, the energy status and O_2 tension of potentially affected cells are clearly major factors. Under certain conditions, the availability of reducing equivalents and cellular concentrations of antioxidants (α-tocopherol, ascorbate, retinoids, GSH, uric acid, etc.) can also be of considerable importance. Clearly, the manner in which the conceptus handles such chemicals and the embryotoxicity caused by these agents should be a matter of highest importance and interest.

Redox Cyclers as Teratogens *in Vivo*

Which chemicals known or reputed to produce biological effects via redox cycling are also reasonably well established teratogens or embryotoxins? Some of the more well known redox cyclers with reported teratogenic properties are listed in Table 1. Currently, there is no evident relationship between redox cycling and the qualitative nature of malformations produced *in vivo*. Clearly, many of the qualitative differences observed thus far might be accounted for in terms of dispositional factors. For example, a number of the compounds listed in Table 1 do not cross membranes readily, whereas others are distributed rather evenly throughout biological systems. Trypan blue (actively excluded from most cells) is an azo dye reputed to produce teratogenic effects by virtue of a specific action on the visceral yolk sac (*17*). It would be of interest to determine whether other highly polar redox cyclers might produce teratogenic effects similar in nature to those elicited by trypan blue. Additionally, the toxic effects of some redox cyclers (e.g., bleomycin, streptonigrin) are exacerbated (*10*) by high O_2 tension (tending to implicate reactive oxygen metabolites) whereas the most profound effects of other redox cyclers (e.g., misonidazole, mitomycin c) are observed under conditions of low O_2 tension. Thus, even if it were to be presumed that the chemicals listed in Table 1 produced their teratogenic effects exclusively via redox cycling, one would nonetheless expect these agents to elicit a variety of different abnormalities as a result of differences in disposition and chemical properties. It must be borne in mind, of course, that the teratogenic effects produced may result from actions entirely independent of redox cycling.

Effects of Redox Cyclers on Cultured Whole Embryos

In order to eliminate the potentially confounding influences of maternal factors, we have elected to utilize the whole embryo culture system to initiate investigations of biochemical mechanisms of chemically elicited embryotoxicity including dysmorphogenesis. Advantages of this system also include a greater capacity to control the concentrations of chemicals and/or their metabolites that actually reach the various tissues of the conceptus and the ability to stage the embryos accurately prior to administration of the chemicals. Disadvantages include a lack of capacity to determine the extent to which observed toxicities would persist beyond the period of culturing and the effort involved in the performance of rigorous embryo-culture experiments.

With respect to redox cyclers and chemicals that may be converted to such, we have initiated investigations on several prototypic examples of various classes of these agents. These include doxorubicin (adriamycin), menadione, and paraquat as representative of quinones, diethylstilbestrol as representative of a proquinone or chemical which must be first converted metabolically to a quinone, nifuroxime, furazolidone, nitrofurazone, nitrofurantoin, niridazole, 2-nitroimidazole, ronidazole, metronidazole, 4-nitroimidazole, and 4-methylniridazole as representative of nitroheterocycles, chloramphenicol as a nitroarene, nitrosofluorene as an aromatic nitroso compound and 2-acetylaminofluorene which is capable of undergoing conversion to several different types of reactive intermediates including redox cyclers. Some of the results obtained have been quite surprising, others enigmatic, and still others have been those expected.

Table 1. Examples of foreign organic chemicals which behave as a redox cyclers in biological systems and their reported teratogenicity.

Chemical	Teratogenic effects	Species	References
Quinones			
Doxorubicin	Multiple	Rats (specific)	(*18*)
Mitomycin C	Skeletal	Rats, mice	(*17*)
Streptonigrin	Eye, CNS, trunk, tail, limbs, etc.	Rats	(*17*)
Paraquat	Costal cartilage defects	Rats, hens	(*17*)
Aromatic nitro compounds			
Nitrofurazone	Limbs, digits, tail	Mice	(*19*)
Nitrofurantoin	Not defined	Mice	(*20*)
Misonidazole	Limbs, eye, brain, palate	Mice	(*21*)
Nitrofen	Eye, kidney, diaphragm	Rats, mice	(*18*)
Nitrobenzene	Multiple	Rats	(*18*)
Azo compounds			
Trypan Blue	Hydrocephaly, spina bifida	Several	(*17*)
Evans Blue	Brain, others	Rats	(*17*)
Congo Red	Hydronephrosis, microophthalmia, hydrocephalus	Rats	(*17*)
Aminoazobenzenes	Skeletal, cleft palate	Mice	(*18*)
Miscellaneous			
Bleomycin-Fe complex	Multiple	Rats, mice	(*18*)

Quinones

Initial investigations of the effects of redox cyclers on embryonic development *in vitro* began with a study of doxorubicin (*22*). Somewhat surprisingly, the data gathered in the studies argued against a role for redox cycling in the observed embryotoxic effects produced by the anthracycline antibiotic. Failure of several radical scavenging agents and antioxidants to ameliorate the embryotoxic effects and a lack of an observable effect of changes in O_2 tension of the culture medium provided the principal arguments against the participation of redox cycling or of radical species. Interestingly, inclusion of an S-9 fraction from methylcholanthrene-induced rats in the culture system markedly exacerbated the embryotoxicity and led us to speculate that a methylcholanthrene-inducible, P-450-dependent *O*-demethylase catalyzed the formation of the 4-hydroxy derivative which is known to exhibit greater antitumor activity than the parent 4-methoxy compound. Seemingly, the embryotoxic effects produced by doxorubicin were more closely akin to its antitumor properties (which do not appear to depend on redox cycling) than to its reputedly redox-dependent cardiotoxic effects. Menadione, a quinone which undergoes reduction catalyzed by the methylcholanthrene-inducible DT-diaphorase, did not affect the embryotoxicity of doxorubicin (*22*) nor did it exhibit embryotoxicity by itself. However, the highest concentration added to the culture medium was only 2 μM. Therefore, the embryotoxicity *in vitro* of this quinone will require further evaluation.

Paraquat is a prototype redox cycler that is capable of producing severe toxicity in the lungs. Like doxorubicin, it is a quinone but is far more water-soluble and thus diffuses across cell membranes much less readily. At 5 μM concentrations, this agent produced 100% embryolethality in 12 embryos (unpublished), but at 1 μM concentrations no effect on 10 embryos could be detected. At concentrations above 10 μM it was apparent that paraquat could produce severe effects on the visceral yolk sac and, in view of its high polarity, it is tempting to speculate that the embryotoxic effects observed were due to a specific effect on yolk sac integrity. Such an effect might be analogous to that produced by trypan blue (*17*), an azo dye and also a potential redox cycler. Further studies on the mechanism of the *in vitro* embryo toxicity of paraquat will be of high interest.

Proquinones

Diethylstilbestrol, a synthetic diphenolic estrogen, undergoes oxidative conversion to *p*-quinone and *p*-semiquinone metabolites via direct (presumably peroxidative) oxidation of the *p*-phenolic groups (*14*). Conversion to *o*-quinone and *o*-semiquinone intermediates can occur via P-450-dependent oxidation at carbons adjacent to phenolic groups to yield catechols. The catechols, in turn, are then peroxidatively converted to the *o*-quinone and (presumably) *o*-semiquinone radical intermediates (*15*). Both of these pathways have been implicated in the genotoxic and carcinogenic effects produced by DES (*14,15*). However, evidence obtained to date in our laboratory indicates that the embryotoxic effects of DES on cultured whole embryos are direct and are neither a function of redox cycling nor bioactivation via other metabolic pathways (*23*). Preliminary studies with the estrogen antagonist, tamoxifen, also indicated that the effects are independent of interactions with estrogen receptors. Since Z,Z-dienestrol, a nonestrogenic metabolite, produced effects similar to those observed with DES, the argument for an estrogen receptor-independent mechanism is strengthened. Also, the similar effect of hexestrol, a synthetic nonsteroidal estrogen differing from DES only in lack of the stilbene double bond, argues against the participation of *p*-quinone/semiquinone intermediates. All results to date tend to foster the concept that the observed

effects of DES on whole cultured embryos are direct and totally independent of metabolic activation. In addition, supplementation of the culture medium with various metabolic systems has failed thus far to influence the embryotoxic effect of DES. Therefore, as with doxorubicin, effects on the morphologic development of cultured embryos appears independent of redox cycling at this stage of the research.

Nitroheterocycles

The discovery of a highly unusual asymmetric malformation produced by niridazole (*24,25*), an antischistosomal nitroheterocycle, led us to investigate a series of structurally related chemicals in order to gain insights into the possible mechanism whereby the defect is elicited. Studies thus far (*26,27*) have shown that the only chemicals capable of producing the striking asymmetrical malformation are those bearing a reducible nitro group. All heterocyclic nitro compounds with redox potentials higher than -0.480 (with the enigmatic exception of nitrofurantoin) produced the axial asymmetry when cultured with whole rat embryos. A very large number of other chemicals that have undergone studies in the whole embryo culture system have not exhibited the capacity to produce the asymmetric defect. A good correlation between single electron redox potentials of nitro groups and capacity to elicit the defect was also observed. The effect was also exacerbated by reducing the O_2 tension of the culture medium (*28*). The results were highly compatible with the idea that the cultured tissues of the conceptus are capable of reducing the aromatic nitro groups to one or more reactive intermediates which were more directly responsible for the observed asymmetric defect. No exogenous enzyme source was required for elicitation of the abnormality and S-9 fractions in fact, tended to reduce the embryotoxicity (*26*). Interestingly, carbon monoxide tended to ameliorate the effect, suggesting that a heme-dependent reduction or reoxidation reaction was required for generation of the reactive species. Previous investigations have shown (*29,30*) that flavoproteins or even free, reduced flavins can reduce aromatic nitro groups (e.g., *p*-nitrobenzoate) to hydroxylamines but that reduction to the fully reduced primary amine (*p*-aminobenzoate) required either a hemoprotein or free hemin as a catalyst. Addition of *N*-acetylcysteine to the culture medium did not appear to affect the incidence or severity of defects produced (*21*) but lowering of intracellular levels of GSH in tissues of the conceptus with buthionine-S, R-sulfoximine (unpublished) markedly exacerbated the effect. Nevertheless, experiments designed to rigorously test the possibility that redox cycling of various potential nitroheterocycle intermediates is responsible for embryotoxic effects of nitroheterocycles have not yet been performed. Exacerbation of the effect under conditions of lowered O_2 tension tends to argue against a mechanism involving the exhaustion of intracellular reducing equivalents because higher O_2 tension should exhaust these equivalents more effectively. It also argues against a mechanism involving the generation of hydroxyl radicals or other reactive oxygen species (singlet oxygen, hydrogen peroxide, etc.) since levels of these species should be increased with increasing O_2 tension. A mechanism involving localized depletion of tissue O_2 levels remains a possibility as does a more direct effect of intermediate organic radicals. At present, the nitroheterocyclic compounds appear to be the most probable candidates as examples of chemicals capable of producing embryotoxic effects via redox cycling.

Aromatic Nitroso

Several metabolites of 2-acetylaminofluorene (AAF) have been investigated as embryotoxins in whole embryo cultures (*6,31–33*). A number of metabolites of AAF have the potential to behave as redox cyclers although this mechanism has not been seriously considered in terms of its carcinogenicity or mutagenicity. Nitrosofluorene, an AAF metabolite, is a relatively potent, direct-acting dysmorphogenic agent *in vitro* and the possibility of its conversion to redox cycling radical intermediates is conceivable. Alterations of extracellular as well as intracellular GSH levels also markedly influences its dysmorphogenic effects (*34*). Again, however, much more investigation will be required to determine whether redox cycling can be implicated even partially. Certainly many other potential mechanisms are conceivable.

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