Activator Protein-1 (AP-1) DNA Binding Activity Is Induced by Hydroxyurea in Organogenesis Stage Mouse Embryos

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Hydroxyurea is a potent teratogen; free radical scavengers or antioxidants reduce its teratogenicity. Activator Protein-1 (AP-1) and NF-κB are redox-sensitive transcription factors with important roles in normal development and the stress response. This study was designed to determine if exposure to teratogenic doses of hydroxyurea induces oxidative stress and alters gene expression by activating these transcription factors. Pregnant mice were treated with saline or hydroxyurea (400, 500, or 600 mg/kg) on gestation day 9 (GD 9) and killed either on GD 9, 0.5, 3, or 6 h after treatment, to assess oxidative stress and transcription factor activities, or on GD 18, to assess fetal development. Exposure to 400 mg/kg hydroxyurea did not affect the progeny, whereas exposure to 500 or 600 mg/kg resulted in dose-dependent increases in fetal resorptions and malformations, including curly tails, abnormal limbs (oligodactyly, hemimelia, and amelia), and short ribs. Hydroxyurea did not induce oxidative stress, as assessed by the ratio of oxidized to reduced glutathione, nor did it alter NF-kB DNA binding activity in the GD 9 conceptus. In contrast, exposure to hydroxyurea at any dose increased AP-1 DNA binding activity in embryos and yolk sacs 0.5 or 3 h after treatment. Hydroxyurea-induced c-Fos heterodimer activity in the embryo peaked 3–4-fold above control at 3 h and remained elevated by 6 h; in contrast, the activity of c-Jun dimers was not altered by drug exposure. A dramatic and region-specific increase in c-Fos immunoreactivity was found in hydroxyurea-treated embryos. The induction of AP-1 DNA binding activity by hydroxyurea represents an early, sensitive marker of the embryonic response to insult.

Key Words: c-Fos; c-Jun; teratogen; oxidative stress; glutathione; redox-sensitive transcription factor.

INTRODUCTION

Hydroxyurea is a potent teratogen, inducing growth retardation, mortality, and malformations in many experimental species (Aliverti et al., 1980; Chaube and Murphy, 1966; Wilson et al., 1975). Maternal treatment with hydroxyurea blocks DNA synthesis and induces cell death in the embryo (Herken et al., 1978; Scott et al., 1971). Hydroxyurea inhibits ribonucleotide diphosphatase reductase, the enzyme that catalyzes the reduction of ribonucleotides to the corresponding deoxyribonucleotides that are required for de novo DNA synthesis. However, inhibition of DNA synthesis cannot explain the rapid cell death that is induced by hydroxyurea. The hydroxylamine (–NHOH) group in the hydroxyurea molecule is able to react with oxygen, producing hydrogen peroxide (H2O2) that is, in turn, converted to the hydroxyl radical (·OH) (Freese et al., 1967). Pretreatment of rabbits with antioxidants (propyl gallate, ethoxyquin, nordihydroguaiaretic acid) or a free radical scavenger (d-mannitol) delays the onset of embryonic cell death and lowers the incidence of malformations caused by hydroxyurea (DeSesso, 1981; DeSesso and Goeringer, 1990; DeSesso et al., 1994), thus suggesting that the oxidative stress induced by reactive oxygen species (H2O2, ·OH) contributes to the developmental toxicity of hydroxyurea.

Oxidative damage to embryonic macromolecules has been observed after maternal exposure to various embryotoxic chemicals (Wells et al., 1997). Oxidative stress disturbs the cellular redox status, inducing oxidative damage to cellular macromolecules (DNA, lipid, and protein) and altering gene expression, possibly primarily by post-transcriptional modification of redox-sensitive transcription factors. Activator Protein-1 (AP-1) is a redox-sensitive early response transcription factor composed of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra2) families of nuclear proteins. The AP-1 members form heterodimers (Fos-Jun) or homodimers (Jun-Jun) that recognize the DNA consensus sequence 5′-TGAG/CTCA-3′ (Angel and Karin, 1991). Oxidative stress regulates the activation of AP-1 through a variety of mechanisms, including the phosphorylation of c-Fos or c-Jun by mitogen-activated protein kinase (MAPK), or oxidative/reductive modification of the cysteine residues present in the DNA binding sites of both c-Fos and c-Jun (Abate et al., 1990; Hirotta et al., 1997). Increased AP-1 DNA binding activity in response to oxidative stress regulates the transcription of genes associated with antioxidant defense, cell cycle control, and apoptosis, all processes that are important in protecting...
embryos from oxidative damage or disrupting normal development. Previous in vitro studies with organogenesis stage whole embryos reported that culture induced an increase in the ratio of oxidized to reduced glutathione (GSSG/GSH) and AP-1 DNA binding activity (Ozolins and Hales, 1997, 1999); depletion of GSH with L-buthionine-S,R-sulfoximine induced embryotoxicity and prolonged AP-1 binding activity (Ozolins et al., 2002).

NF-κB, also a redox-sensitive transcription factor, is involved in development, positional signaling, and programmed cell death. NF-κB is a ubiquitous, pleiotropic, multisubunit transcription factor, made up of five subunits (p50, p52, p65 or RelA, c-Rel, and Rel-B) that form homodimers and heterodimers (Verma et al., 1995). The predominant forms, p50 and p65 (RelA), are sequestered in the cytoplasm by association with I-κB; dissociation of the NF-κB:I-κB complexes, followed by translocation of the released NF-κB into the nucleus, activates NF-κB post-translationally. Exposures that induce oxidative stress, such as H₂O₂, tumor necrosis factor, phorbol esters, glutathione depletion, and ultraviolet (UV) or ionizing radiation, induce NF-κB DNA binding activity (Gius et al., 1999; Haddad et al., 2000; Li and Karin, 1998, 1999; Marshall et al., 2000). The regulation of NF-κB is particularly important during development. NF-κB may act in the developing limb to disturb fibroblast growth factor (FGF) signals between the apical ectodermal ridge and the underlying mesenchyme (Bushdid et al., 1998; Kanegae et al., 1998). Inhibition of NF-κB/Rel activity results in a dysmorphic apical ectodermal ridge, loss of digit formation, and reversal of the direction of limb outgrowth. Interestingly, there is evidence that NF-κB may be involved in the teratogenesis of thalidomide (Hansen et al., 2002) and phentoin (Kennedy et al., 2004).

Glutathione, the major intracellular nonprotein thiol, exists mainly in the reduced form (GSH). Upon oxidative stress, GSH is oxidized to GSSG, protecting cellular macromolecules; GSSG can be reduced to GSH in the presence of glutathione reductase and NADPH. The GSSG/GSH ratio is tightly regulated, maintaining cellular redox status; this ratio has been used as a marker of oxidative stress (Bajt et al., 2004; Lauterburg et al., 1984; Ozolins and Hales, 1997; Suliman et al., 2004).

The goal of this study was to elucidate the impact of an exposure to hydroxyurea that is teratogenic during early organogenesis on oxidative stress and the activation of redox-sensitive transcription factors in the conceptus. Pregnant mice were treated with hydroxyurea on gestational day 9, during early organogenesis, the period of development most susceptible to oxidative stress and teratogenic insult.

**MATERIALS AND METHODS**

**Animals and treatments.** Timed-pregnant CD1 mice (20–25 g) were purchased from Charles River Canada Ltd. (St. Constant, QC, Canada) and housed in the McIntyre Animal Resource Centre (McGill University, Montreal, Canada). All animal protocols were conducted in accordance with the guidelines outlined in the Guide to the Care and Use of Experimental Animals, prepared by the Canadian Council on Animal Care. Female mice, mated between 8:00 AM and 10:00 AM (gestation day 0, GD 0), were treated with vehicle (saline) or hydroxyurea (Aldrich Chem. Co., Madison, WI) at 400, 500, or 600 mg/kg by intraperitoneal injection at 9:00 am on GD 9. Dams were killed on GD 9 (0.5, 3, or 6 h after treatment; 7–10 litters/treatment group) or GD 18 (8–10 litters/treatment group) by cervical dislocation. On GD 9, the maternal liver was excised; the uterus was removed and embryos and yolk sacs were dissected out in Hanks’ balanced salt solution (Gibco Laboratories, ON, Canada) for subsequent assessment of transcription factor activity or oxidative stress. On GD 18, the uteri were removed, and the numbers of implantations, resorption sites, and live and dead fetuses were recorded. All live fetuses were inspected for external malformations. Two malformed and two normal (without obvious external malformations) fetuses were randomly chosen from each litter for skeletal double staining and evaluation.

**Double staining for fetal skeletal analysis.** Ethanol-fixed fetuses were immersed in a water bath (70°C) for 7 s. The fetuses were skinned, eviscerated, and placed in 95% ethanol overnight. The ethanol was decanted and replaced with alcin blue solution (15 mg alcin blue; 80 ml 95% ethanol; 20 ml glacial acetic acid) for 24 h. The solution was then replaced with 95% ethanol. After 24 h, the ethanol was substituted with alizarin red S solution (25 mg l-1 alizarin red S in 1% potassium hydroxide) for 48 h. The dye was drained and replaced with 0.5% potassium hydroxide for 24 h. The skeletons were placed in a 2:2:1 solution (2 parts 70% ethanol; 2 parts glycerin; 1 part benzyl alcohol). After 24 h, stained skeletons were placed in 1:1 solution (1 part 70% ethanol:1 part glycerin) for subsequent evaluation and storage.

**Glutathione determinations.** At the time of collection, the embryos and yolk sacs from each litter were placed in 60 µl of RIPA buffer (15 mM NaCl; 1% NP-40; 0.5% deoxycholate; 0.1% SDS; 50 mM Tris, pH 7.5) containing 10 µl/ml protease inhibitor cocktail (Active Motif, Carlsbad, CA). The samples were homogenized with an ultrasonicator (Sonics & Materials Inc., Newtown, CT) and centrifuged at 10,000 × g for 10 min at 4°C. From each sample, 30 µl of supernatant was removed, added to 90 µl of 5% 5-sulfosalicylic acid, and centrifuged at 10,000 × g for 10 min at 4°C. Total glutathione (GSSG + GSH) and GSSG were measured spectrophotometrically, using an enzymatic recycling assay, as previously described (Ozolins and Hales, 1997). The remaining supernatant from each sample (prior to the addition of 5-sulfosalicylic acid) was aliquoted, flash frozen in liquid nitrogen, and stored at −80°C for protein assays (Bradford, 1976) (Bio-Rad Laboratories, ON, Canada) and the ELISA tests.

**Electrophoretic mobility shift assays (EMSA).** To prepare nuclear extracts, tissues were placed in 3 ml/g ice-cold complete hypotonic buffer (20 mM Hepes, pH 7.5; 5 mM NaF; 10 µM Na3VO4; 0.1 mM EDTA; 1 µl/ml 1M DTT; 1 µl/ml detergent) and homogenized on ice with a pellet pestle (Kontes, Vineland, NJ). After 15 min incubation on ice, the samples were centrifuged at 4°C for 10 min at 850 × g. The pellet was resuspended in 100 µl hypotonic buffer and incubated on ice for 15 min. After the addition of 5 µl of detergent, the samples were vortexed for 10 s at the highest setting. After centrifugation at 14,000 × g for 30 s, 5 µl complete lysis buffer was added to the pellet, which was then sonicated for a few seconds and rocked on ice for 15 min. The supernatant was obtained by centrifugation at 14,000 × g for 10 min. After determination of the protein content (Bradford, 1976; Bio-Rad Laboratories, ON, Canada), the extracts were adjusted to the same protein concentration by the addition of complete lysis buffer, flash frozen in liquid nitrogen, and stored at −80°C. Hypotonic buffer, lysis buffer, dithiothreitol, protease inhibitor cocktail, and detergent were supplied in a Nuclear Extract Kit (Active Motif).

DNA fragments containing the AP-1 DNA binding site (5'-CGCGTGG-TGACTCAGCCGGAAA-3') were end-labeled with 32P-ATP by T4 poly-nucleotide kinase (Promega Corporation, Madison, WI) and purified by chromatography on a MicroSpin G-25 column (Amersham Biosciences UK) for 10 min. After determination of the protein content (Bradford, 1976; Bio-Rad Laboratories, ON, Canada), the extracts were adjusted to the same protein concentration by the addition of complete lysis buffer, flash frozen in liquid nitrogen, and stored at −80°C. Hypotonic buffer, lysis buffer, dithiothreitol, protease inhibitor cocktail, and detergent were supplied in a Nuclear Extract Kit (Active Motif).
Limited, Little Chalfont, UK). Nuclear extracts (9 μg protein) were incubated for 20 min at 4°C with 4 μl binding buffer supplied in a GelShift kit (Active Motif). Samples were then reacted with 1 μl labeled AP-1 probe (about 200,000 cpm) for 20 min at 4°C and electrophoresed on 5% polyacrylamide gels in 1× TGE buffer at 200 V. Gels were dried, and radioactivity was detected by autoradiography at ~80°C. To confirm the specificity of binding, a 50-fold excess of the unlabeled oligonucleotide probes or mutated AP-1 oligonucleotides (5′-CGCTTGAGAGCTGCGCCGAA-3′) was preincubated with the sample for 20 min at 4°C.

**Enzyme-linked immunosorbent assays (ELISA).** Embryo and yolk sac extracts were prepared as described above for glutathione determinations. The DNA binding activity of the c-Fos heterodimer complex or the c-Jun homo/heterodimers was detected using ELISA transcription factor assay kits (Active Motif). Briefly, a 96-well plate was coated with oligonucleotide containing the AP-1 consensus site, 5′-TGGATGTC – 3′. Complete binding buffer (30 μl) (10 mM Hepes, pH 7.5; 8 mM NaCl; 12% glycerol; 0.2 mM ethylene diamine tetraacetic acid [EDTA]; 0.1% bovine serum albumin [BSA]; 0.88 mM dithiothreitol [DTT]; 0.17 mg/μl poly [d(I-C)]) was added to each well. To confirm the specificity of binding, 20 pmol AP-1 wild-type oligonucleotide or 20 pmol mutated oligonucleotide was added to two wells, respectively. As a positive control, K562 cell nuclear extract (2.5 μg) in 20 μl complete lysis buffer (20 mM Hepes, pH 7.5; 400 mM NaCl; 20% glycerol; 0.1 mM EDTA; 10 mM NaF; 10 μM NaMoO4; 1 mM NaVO3; 10 mM NaF; 10 mM PNP; 10 mM β-glycerophosphate; 0.89 mM DTT; 0.01% protease inhibitor cocktail) was added to two wells containing complete binding buffer. Embryo or yolk sac extracts were diluted with complete lysis buffer to 20 μl and then added to the remaining wells; embryo or yolk sac extracts containing 20 μg protein were used for the examination of c-Jun dimer binding activity and 40 μg protein for the c-Fos dimer assays. The plates were incubated for 1 h and washed with washing buffer (10 mM phosphate buffer, pH 7.5; 50 mM NaCl; 0.1% Tween 20; 2.7 mM KCl). One hour after the addition of the phospho-c-Jun or c-Fos antibody, the plates were washed with washing buffer. Horseradish peroxidase (HRP)-conjugated secondary antibody was added and incubated for 1 h. After washing, the plates were developed with developing solution (TMB substrate solution in 1% DMSO) for 5–10 min; the reaction was terminated by the addition of the stop solution (0.5 M H2SO4). The plates were read on a spectrophotometer (SPECTRAMax Plus 348, Molecular Devices Corporation, Sunnyvale, CA) at 450 nm with correction at 655 nm. The binding activity of samples was normalized by the relative absorbance of the positive standard.

**RESULTS**

**Developmental Toxicity Induced by Hydroxyurea**

To assess the embryotoxicity of hydroxyurea during early organogenesis, pregnant mice on GD 9 were treated with varying doses of hydroxyurea (400, 500, or 600 mg/kg) or vehicle and were killed on GD 18. The developmental toxicity induced by hydroxyurea is illustrated in Figure 1. The dead fetuses were observed as resorptions. The vehicle control group had a low rate of fetal death (Fig. 1A). Exposure to 400 mg/kg hydroxyurea did not alter the incidence of fetal death per litter. In contrast, a dramatic increase in fetal death rate was observed in both the 500 mg/kg and the 600 mg/kg hydroxyurea treatment groups; in the high-dose treatment group, more than half of the implantations per litter were resorbed (Fig. 1A).

There were no obvious external abnormalities in the control group, with the exception of one fetus with exencephaly. A few fetuses with curly tails were found in the litters exposed to 400 mg/kg hydroxyurea, but the rate of malformations per litter was not significantly different from control (Fig. 1B). However, exposure to 500 or 600 mg/kg of hydroxyurea induced a dose-dependent increase in malformations. In the 500 mg/kg treatment group, the mean number of malformed fetuses per litter was 22.2%, whereas in the high-dose treatment group, 87.7% of the surviving fetuses were malformed. Both curvy tail and limb malformations were observed; hindlimb malformations predominated, but some fetuses had forelimb abnormalities as well. The hindlimb malformations were characterized by oligodactyly (missing digits), hemimelia (total or partial absence of the distal half of a limb), and amelia (absence of a limb); interestingly, malformed limbs were usually found on only one side (either left or right), although there were fetuses in which the development of both hindlimbs was abnormal.

Double-stained skeletons of representative fetuses from each treatment group are shown in Figure 1D. The cartilage is stained blue and the bone is red. No skeletal abnormalities were apparent in the control and 400 mg/kg hydroxyurea treatment groups. In contrast, one third of the surviving fetuses in the 500 mg/kg treatment group had skeletal abnormalities; the rate of skeletal malformations reached 90.2% after exposure to 600 mg/kg hydroxyurea (Fig. 1C). The skeletal malformations observed included limb...
malformations (missing digits, hemimelia, amelia), curly tails, and short ribs (Fig. 1D).

The Effects of Hydroxyurea Exposure on AP-1 DNA Binding Activity

Electrophoretic mobility shift assays (EMSA) were done to test the hypothesis that teratogenic exposures of hydroxyurea induce AP-1 DNA binding activity (Fig. 2). In the absence of nuclear extract (free probe), the migration of the oligonucleotide was not impeded; addition of nuclear extract retarded the migration of the probe, indicated as the AP-1 DNA binding band. The addition of excess unlabeled wild-type oligonucleotide inhibited this binding, but excess mutated sequence did not influence the binding (Fig. 2A). An increase in AP-1 DNA binding activity (relative to control) was detected in the nuclear extracts prepared from the hydroxyurea-treated embryos and yolk sacs collected 3 h after treatment on GD9 (Fig. 2B and 2C). DNA binding activity increased in a dose-dependent manner with hydroxyurea dose (Fig. 2B).

Multiple AP-1 family members may contribute to AP-1 DNA binding activity; various combinations of AP-1 dimers may regulate specific sets of genes, influencing different cellular functions. The contributions of two of the principal AP-1 members, c-Jun (c-Jun homodimers or heterodimers) and c-Fos (heterodimers), to the AP-1 DNA binding activity were detected by ELISA and are reported in Figure 3. AP-1 c-Jun DNA binding activity was not influenced in either embryos or yolk sacs by hydroxyurea exposure at any dose (400, 500, or 600 mg/kg) or time (0.5, 3, or 6 h) after treatment (Fig. 3A and 3B). At 30 min after hydroxyurea treatment, there was also no effect on AP-1 c-Fos DNA binding activity in embryos.
In contrast, AP-1 c-Fos DNA binding activity was significantly increased in embryos 3 h after exposure to any dose of hydroxyurea (Fig. 3C). A threefold increase in c-Fos binding activity was observed in embryos exposed to 400 mg/kg hydroxyurea; a fourfold induction of c-Fos activity was seen in embryos exposed to 500 or 600 mg/kg of hydroxyurea (Fig. 3C). By 6 h, c-Fos activity in embryos exposed to 400 mg/kg hydroxyurea did not differ from control, but activity remained elevated in embryos exposed to 500 or 600 mg/kg hydroxyurea. A similar trend was observed in AP-1 c-Fos binding activity in the yolk sac after 3 h or 6 h exposure to hydroxyurea, but these increases were not statistically significant (Fig. 3D).

The Effects of Hydroxyurea Exposure on NF-κB DNA Binding Activity

NF-κB DNA binding activity consists of homodimeric or heterodimeric complexes of members of NF-κB families; of these, the p50/p65 heterodimers and p50 homodimers are major components. Thus, to determine if hydroxyurea exposure altered NF-κB DNA binding activity, we examined the binding activities of NF-κB p65 and p50 dimers by ELISA (Fig. 4). The binding activity of NF-κB p65 dimers in the embryo (Fig. 4A) was approximately half that in the yolk sac (Fig. 4B). Interestingly, NF-κB p50 binding activity was lower in embryos (Fig. 4C) on GD 9 at 3 h and 6 h post-injection than at 0.5 h; a similar trend was observed in yolk sacs (Fig. 4D), but the differences were not statistically significant in this tissue. However, hydroxyurea exposure had no effect on NF-κB p65 or p50 dimer activities in either the embryo or the yolk sac at any time after treatment (Fig. 4). Thus, hydroxyurea exposure did not “non-discriminately” induce the activity of all redox-sensitive transcription factors.

The Effects of Hydroxyurea Exposure on c-Fos Immunoreactivity

To further explore the impact of hydroxyurea exposure on c-Fos in the embryo, c-Fos immunoreactivity was localized in embryos 3 h after treatment with vehicle or hydroxyurea (Fig. 5), the time point at which c-Fos heterodimer DNA binding was maximal after drug exposure. C-Fos immunoreactivity was observed in the control embryo around the circumferences of the forebrain and hind brain, in the facial-acoustic neural crest complex, in somites, in the neural tube,
and in the heart (Fig. 5B). The expression of c-Fos in mice exposed to 400 mg/kg hydroxyurea (Fig. 5C) was similar to control. However, exposure to 600 mg/kg hydroxyurea dramatically increased the amount of c-Fos immunoreactivity in all the areas in which c-Fos activity was displayed in the control embryos (Fig. 5D). In the brain region, c-Fos immunoreactivity was clearly shown around the edge of the hindbrain in the control embryo (Fig. 5E); in the 600 mg/kg hydroxyurea–treated embryos, immunoreactivity was intense throughout the whole hindbrain region (Fig. 5H). In the caudal region of the tail of 600 mg/kg hydroxyurea–exposed embryos, c-Fos staining was dramatically increased in the neural tube and the area around the dorsal aorta (Fig. 5I), compared to control embryos (Fig. 5F). A dramatic increase in c-Fos immunoreactivity in hydroxyurea-treated embryos was also found in the areas around the blood cells, in the branchial arch (Fig. 5D), and in the atrial and ventricular walls of the heart (Fig. 5J).

The Effects of Hydroxyurea on Glutathione Homeostasis

To estimate the oxidative stress induced by hydroxyurea, the GSH concentrations and the ratios of GSSG:GSH were determined in the maternal liver, embryos, and yolk sacs. The GSH concentrations in the maternal liver were approximately twofold higher than those in the embryo or yolk sac (Table 1); this is consistent with a previous report that GSH content is relatively low in the conceptus (Serafini et al., 1991). Exposure to hydroxyurea did not change the GSH content in the maternal liver, embryo, or yolk sac at any one time point (0.5, 3, or 6 h). However, there was a decrease in GSH content
with time in the maternal liver and embryo after exposure to either 500 or 600 mg/kg hydroxyurea ($p \leq 0.05$, two-way ANOVA). The GSH concentrations were lower at 6 h than at 0.5 or 3 h in maternal liver after exposure to 600 mg/kg hydroxyurea (Table 1). In the embryo, exposure to 500 mg/kg hydroxyurea resulted in lower GSH concentrations at 3 h than at 0.5 h, whereas exposure to 600 mg/kg hydroxyurea resulted in a decrease in GSH content at both 3 and 6 h compared to 0.5 h (Table 1). Interestingly, the ratios of GSSG:GSH were about twofold higher in the maternal liver and yolk sac than in the embryos; variation was high in the yolk sac. However, GSSG:GSH ratios were not influenced by hydroxyurea exposure (400, 500, or 600 mg/kg) in the maternal liver, embryo, or yolk sac at any time (0.5, 3, or 6 h) after hydroxyurea treatment (Table 1). Thus, exposure to hydroxyurea did not induce oxidative stress, as assessed by the ratio of oxidized to reduced glutathione.

**DISCUSSION**

The goal of this study was to determine the relationship between hydroxyurea teratogenesis during early organogenesis, oxidative stress, and alterations in gene expression as a consequence of the activation of redox-sensitive transcription factors, AP-1 and NF-κB. The GSH concentrations measured in the embryo and yolk sac were less than half those in the maternal liver, suggesting that tissues in the conceptus may be less protected from oxidative stress than the maternal organs. Exposure to teratogenic doses of hydroxyurea did not alter the GSH content of the maternal livers, embryos, or yolk sacs within any one time point assessed; however, a significant decrease in GSH content was found from 0.5 h to 3 or 6 h in maternal livers and embryos exposed to high doses of hydroxyurea. These data suggest that there may be an interaction between hydroxyurea and time post-treatment. The
times post-treatment when this interaction occurred correspond to 12 noon (3 h after hydroxyurea administration at 9 AM) and 1500 h (6 h after hydroxyurea exposure). Hepatic reduced GSH concentrations have been reported to be at a nadir in mice under a normal lighting schedule at 1400 h (White et al., 1987). Decreased GSH content may be a consequence of either GSH depletion or a decrease in GSH synthesis. It is interesting that GSH content was not depleted in the yolk sac after exposure to high-dose hydroxyurea. One explanation for this finding may be that the yolk sac has a higher GSH synthesis capacity than the embryo (Hansen et al., 2004); indeed, GSH synthesis is differentially regulated in the embryo and yolk sac in response to insult. Alternatively, the high variability in GSH content measurements in this tissue may contribute to this tissue difference in response.

As the major cellular thiol-disulfide redox buffer, GSH is important in maintaining the cellular redox status. Changes in the intracellular thiol/disulfide status trigger signal transduction pathways which increase antioxidant defenses to maintain redox homeostasis, but at same time, influence normal cellular function, such as cell proliferation, differentiation, adhesion, or death. In the embryo, the ratio of GSSG/GSH was about twofold lower than in the yolk sac or maternal liver, suggesting that redox homeostasis in the embryo may be different from that in the maternal liver or yolk sac. Interestingly, after exposure of whole embryos in culture to diamide, more...
extensive S-thiolation of protein sulfhydryls by GSH was observed in the yolk sac compared to the embryo proper; thus, protein sulfhydryls in the yolk sac were more sensitive or accessible to oxidation (Hiranruengchok and Harris, 1995).

Exposure to teratogenic doses of hydroxyurea did not significantly induce oxidative stress in the maternal liver, embryo, or yolk sac, as assessed by the ratio of GSSG/GSH. Previous studies reported that antioxidants or a free radical scavenger delayed the cell death and partially rescued the development. The antioxidant defense system is not equally distributed throughout the embryo; interestingly, limbs and the neural tube may have lower antioxidant defenses than do other regions (Mackler et al., 1998).

Significantly, AP-1 DNA binding activity was dramatically increased, even after exposure to a dose of hydroxyurea (400 mg/kg), which did not induce developmental toxicity. Higher doses of hydroxyurea (500 and 600 mg/kg), which were teratogenic, further elevated AP-1 DNA binding activity. If, as seems likely, there are a number of components that contribute to hydroxyurea teratogenicity, the risk of having a malformation may be a continuous variable, with a threshold value (Fraser, 1976). Thus, it is possible that exposure to 400 mg/kg hydroxyurea induces AP-1 activity but is below the “threshold” for malformations. AP-1 regulates the transcription of genes that are important in cell differentiation, proliferation, and apoptosis, all basic processes during embryo development. AP-1 activity may be induced by various noxious stimuli, such as heat shock (Andrews et al., 1987), heavy metals (Jin and Ringertz, 1990; Ramesh et al., 1999), and alkylating chemicals (Futschek and Erickson, 1990), all of which can be teratogenic. Interestingly, c-Fos expression was elevated in the deciduas of embryos 6 h after exposure to ethanol on GD 8 (Poggi et al., 2003). Stable overexpression of Bcl-2, a gene that protects against cell death by apoptosis, enhanced AP-1 DNA binding activity in cell lines (Feng et al., 2004). In the presence of different stimuli and in various cell types, the activation of AP-1 may have different consequences, from protecting the cell to triggering cell death. It is not clear whether hydroxyurea induced activation of AP-1 indicates a role for AP-1 in protecting the embryo from the potential teratogenic insult or in disrupting normal embryonic development.

We have shown that c-Fos heterodimers contribute to the AP-1 DNA binding activity increase induced by hydroxyurea and that c-Jun homo-/hetero dimers do not. Different AP-1 members display multiple roles in regulating embryonic development. The targeting of individual AP-1 members in transgenic null mouse experiments has demonstrated that c-Jun, JunB, or Fra-1 are essential for embryo development; however, mice lacking c-Fos, JunD, and FosB are viable but have a variety of birth defects. C-Jun null mice died during the fetal period because of cardiac defects (Eferl et al., 1999); the knockout of Fra-1 or JunB induced embryolethality as a consequence of defects in the extra-embryonic tissues (Schorpp-Kistner et al., 1999; Schreiber et al., 2000). Mice lacking FosB or JunD had a nurturing defect, or male sterility, respectively (Gruda et al., 1996; Thepot et al., 2000). Knockout c-Fos mice were viable and fertile but lacked osteoclasts, and they produced progeny with osteopetrosis (Johnson et al., 1992; Wang et al., 1992). Overexpression of c-Fos induced the
transformation of osteoblasts leading to osteosarcomas (Wang et al., 1995).

The developmental toxicity of hydroxyurea was characterized by skeletal malformations, including curly tails, abnormal limbs, and short ribs. The evidence that c-Fos is involved in osteogenesis (Closs et al., 1990; Sakano et al., 1997) leads us to suggest that the effects of hydroxyurea on skeletal morphogenesis may be a consequence of disturbances in the regulation of c-Fos expression and activity. The relationship between where increased c-Fos immunostaining was found in the embryos exposed to hydroxyurea and the subsequent malformations is interesting. Increased c-Fos immunostaining was found in the somites of the hydroxyurea-exposed embryos, many of which are likely to develop shortened ribs. After exposure to 600 mg/kg hydroxyurea, c-Fos expression increased dramatically in the caudal region of the neural tube; neural tube defects at the caudal region of the tail result in curly tail malformations. Although we did not observe craniofacial defects in this study, hydroxyurea has been reported to induce craniofacial defects and cardiovascular anomalies in rats (Aliverti et al., 1980). In utero exposure of rabbits to hydroxyurea altered the embryonic cardiovascular system as early as 2 min post-treatment, followed by petechial hemorrhages and hematomas in the forebrain, postocular region, and the mandibular and nasal processes, as well as apparent collapse of the vasculature in the forelimb bud (Millicovsky and DeSesso, 1980). Our data show that exposure to doses of hydroxyurea that are teratogenic dramatically increased the immunoreactivity of c-Fos in the brain regions and in areas around the blood vessels. It is possible that the absence of cardiac and craniofacial defects among the fetuses in this study may be due to the protective effects of c-Fos in these regions or, alternatively, that the insult in these regions leads to an increase in embryolethality that is manifested in embryo resorptions. Elucidation of the different combinations of the c-Fos heterodimers activated by hydroxyurea in various regions of the embryo may help to explain specificity of the response to insult.

In contrast to the effect on AP-1 DNA binding activity, NF-κB DNA binding activity was not influenced by hydroxyurea treatment, although both NF-κB and AP-1 are redox-sensitive transcription factors, and NF-κB is important in limb development. This may be due to a difference in the sensitivity of these two transcription factors to insult. These data indicate that different mechanisms are involved in regulating the response of embryonic AP-1 and NF-κB DNA binding activity to stress.

AP-1 DNA binding activity may be triggered by genotoxicity or by oxidative stress. Hydroxyurea inhibits ribonucleotide reductase, resulting in inhibition of DNA synthesis, affecting the cell cycle at G0 to S phase. AP-1 family members are involved in promoting cell cycle progression; specifically, AP-1 has been linked to the transcriptional regulation of cyclin D1. Stress induces a complex program of c-Fos and Fra-1 chromatin trafficking that is required for cyclin D1 expression during cell cycle re-entry (Burch et al., 2004). The induction of AP-1 DNA binding activity in the conceptus by hydroxyurea may disturb cell cycle recovery in the conceptus. Whether increased AP-1 DNA binding activity mediates the response of the conceptus to insult or represents a failed attempt at self-protection, it is evident that it represents a sensitive and early indicator of insult.

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