Biochemical Pharmacology, Vol. 57, pp. 1165–1175, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00020-9

Tissue-Specific Regulation of Glutathione Homeostasis and the Activator Protein-1 (AP-1) Response in the Rat Conceptus

*Terence R. S. Ozolinš and Barbara F. Hales**

Department of Pharmacology and Therapeutics, McGill University, Montreal, Québec, Canada H3G 1Y6

ABSTRACT. Oxidative stress in the conceptus is characterized by an increased oxidized to reduced glutathione (GSSG:GSH) ratio and the induction of fos and jun mRNAs, transcripts for components of the activator protein-1 (AP-1) transcription factor. We investigated the role of glutathione homeostasis in the rat conceptus in the regulation of: (1) AP-1 expression and activity, and (2) the activities of glutathione-dependent cytoprotective enzymes. Glutathione content was enhanced with the addition of l-2-oxothiazolidine-4-carboxylate (OTC), a precursor of cysteine, a rate-limiting substrate in glutathione biosynthesis. Day 10 rat conceptuses were cultured for 44 hr with 0, 5, 10, or 20 mM OTC. High concentrations (10 and 20 mM) of OTC were embryotoxic. Incubation of the conceptus in 5 mM OTC caused mild (not statistically significant) embryotoxicity, increased significantly the embryonic glutathione content, prevented culture-induced oxidative stress, and inhibited the induction of AP-1 transcripts and DNA binding activity in the embryo. In contrast, in the yolk sac, 5 mM OTC failed to increase glutathione content or to prevent oxidative stress or AP-1 induction. Thus, regulation of glutathione status in the conceptus is tissue-specific. Glutathione *S*-transferase and glutathione peroxidase activities were increased approximately 50% in cultured embryos and yolk sacs. OTC treatment (5 mM) prevented this induction in the embryo, but not in the yolk sac, suggesting a role for glutathione homeostasis in the regulation of these enzymes. Tissue-specific regulation of glutathione status and of cytoprotective enzymes in the conceptus during organogenesis may impact on the consequences of insult with oxidative stress. BIOCHEM PHARMACOL **57**;10:1165–1175, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. embryo; yolk sac; organogenesis; activator protein-1 (AP-1); oxidative stress; glutathione

The embryotoxicity of agents that induce oxidative stress or deplete glutathione is well documented (reviewed in Ref. 1), although the associated gene changes are poorly understood. In many models, exposure to reactive oxygen species induces the mRNA expression and activity of AP-1†, a family of immediate-early transcription factors. The AP-1 transcription factors comprise the cellular proto-oncogene products Fos–Jun hetero- or Jun–Jun homodimers (reviewed in Ref. 2). AP-1 members play important roles in normal embryogenesis (reviewed in Ref. 2). In addition, AP-1 may serve to activate the transcription of genes whose products protect the embryo against insult. Short-term changes in AP-1 expression and activity lead to long-term cellular changes [3] that promote cell survival [4].

In a number of cell types, the steady-state concentrations of fos and jun mRNAs increase significantly after treatment with oxidizing agents [5–10], or chemicals that deplete glutathione [11]. AP-1 is induced in response to signals that include carbon tetrachloride-induced hepatic metabolic oxidative stress [12] and pro-oxidants, such as hydrogen peroxide or diamide [13].

The whole rat embryo culture system is used widely to test the effect of putative teratogens on embryos during organogenesis [14]. There is a transient oxidative stress response in the mid-organogenesis stage rat conceptus in culture, as assessed by the presence of increased protein [15] and DNA [16] oxidation products. Within the embryo, a number of factors protect against reactive oxygen species, including the tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine) (reviewed in Ref. 1). Glutathione exists in oxidized (GSSG) and reduced (GSH) forms, with GSH predominating intracellularly as the most abundant non-protein thiol. The GSSG:GSH ratio indicates, to a large extent, cellular redox balance and serves as an important measure of oxidative stress [17, 18]. The oxidative stress response in the 8–10 somite rat embryo in culture is characterized by a transient peak in the GSSG:GSH ratio and by the induction of fos and jun mRNAs and AP-1 DNA binding activity [15]. Importantly, the addition of antioxidants to the medium protects against the rise in the GSSG:GSH ratio and inhibits the induction of both AP-1 mRNAs and DNA binding activity.

* Corresponding author: Barbara F. Hales, Ph.D., Department of Pharmacology and Therapeutics, McGill University, 3655 Drummond St., Montréal, Québec, Canada H3G 1Y6. Tel. (514) 398-3610; FAX (514) 398-7120; E-mail: bhales@pharma.mcgill.ca

† *Abbreviations*: AP-1, activator protein-1; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; MAPK, mitogen-activated protein kinases; OTC, l-2-oxothiazolidine-4-carboxylate; PBS-T, phosphate-buffered saline-Tween®; and TBS-T, Tris-buffered saline-Tween®.

Received 13 June 1998; accepted 13 October 1998.

The profile of the induction of AP-1, at the message and protein levels, in response to pro-oxidant treatments, parallels very closely that for the induction of cytoprotective genes such as glutathione *S*-transferases Y_a [11] and Y_p [19]. Because the genes for a number of these cytoprotective enzymes contain upstream AP-1 or AP-1-like response elements, increased AP-1 DNA binding activity and the resultant gene transactivation may be involved in their induction in the embryo. Significant increases in the activities of the glutathione *S*-transferases and glutathione peroxidases were reported in organogenesis-stage embryos in culture [20]. An increase in the transcript for glutathione *S*-transferase Y_p was observed in the yolk sacs of cultured embryos [21]. We hypothesize that these enzymes may be induced by oxidative stress in the embryo culture system, and that this induction may be dependent, at least partially, on AP-1.

MATERIALS AND METHODS

Embryo Culture

Timed-gestation pregnant Sprague–Dawley rats (200–225 g) (Charles River Canada Ltd.) were housed in plastic cages with hardwood chip bedding and maintained in a temperature-controlled environment with a 12-hr light/dark cycle. Food (Purina Rat Chow, PMI Feeds) and tap water were provided *ad lib*. On the morning of day 10 of gestation (the morning of sperm-positive vaginal smears was defined as gestational day zero), embryos were explanted from the dams and cultured according to the method of New [14], as previously described [15]. The 6–10 somite stage conceptuses, surrounded by an intact yolk sac and ectoplacental cone, were cultured for up to 44 hr at 37°. OTC (Sigma Chemical Co.) was added to enhance glutathione biosynthesis [22]. The ability of OTC to increase glutathione content in the conceptus [23] was verified by culturing embryos for 44 hr in medium containing 0, 5, 10, or 20 mM OTC. Development was assessed using the Brown and Fabro [24] morphological score. Exposure of embryos to 5 mM OTC did not significantly decrease the morphological score compared with controls, and subsequent studies were done with this concentration of OTC.

The embryos that were photographed were incubated with acridine orange (ICN Pharmaceuticals Inc.) at the end of the culture period. The protocol was similar to that described by Zucker *et al.* [25] with the following modifications: fixation was for 3 hr in 2.5% glutaraldehyde, incubation with acridine orange was for 4 min, and specimens were washed six times with PBS to remove excess dye. Photographs were taken using fluorescence microscopy (Leitz Laborlux with an I_3 Filter, Leica Canada Inc.). The remaining conceptuses were rinsed three times in Hanks' solution and separated for further analysis into embryo proper and yolk sac; the yolk sac samples included the ectoplacental cone.

Glutathione Determinations

At the time of collection, embryos and yolk sacs were sonicated with an ultrasonic processor (Sonics & Materials Inc.) in 5-sulfosalicylic acid (5%, w/v), divided into two aliquots, flash frozen in liquid N_2, and stored at −80°. Glutathione determinations were performed exactly as previously described [15].

Western Blot Analysis

Samples were boiled for 5 min in sample loading buffer (62.5 mM Tris–HCl, pH 6.8, 12% glycerol, 2% SDS, 5% β-mercaptoethanol) and fractionated with 7.5% polyacrylamide gel electrophoresis as previously described [15]. The proteins were transferred as previously described [15] to Hybond-C Super supported nitrocellulose membranes, and detected using enhanced chemiluminescence (both from Amersham Canada Ltd.). All washes were performed exactly as described by the manufacturer. To detect 5-oxoprolinase, blocking was performed for several hours on an orbital shaker at room temperature with 5% skim milk powder in PBS with 0.5% Tween 20 (PBS-T) at pH 7.5 (disodium hydrogen orthophosphate anhydrous, 80 mM; sodium dihydrogen orthophosphate, 20 mM; NaCl, 100 mM). Then the membranes were incubated for 3 hr in 5% blocker (as above) at room temperature with affinity purified rabbit anti-rat, anti-5-oxoprolinase antibody (1:50,000; a gift from G. Batist, Lady Davis Research Institute [26]). For the detection of α-actin, membranes were blocked first with 5% skim milk powder and 1% BSA fraction V (Sigma) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at pH 8 (NaCl, 137 mM; Tris base, 20 mM). Employing similar blocking conditions as just described, the membrane was incubated for 2 hr at room temperature with a rabbit antibody specific for α-actin (1:2000; Zymed Inc.). Incubation with the secondary antibody (1:2500) was for 1 hr in 5% blocker in either PBS-T (5-oxoprolinase) or TBS-T (α-actin). The membranes were stripped as described by the manufacturer.

Northern Blot Analysis

Northern blot analysis and signal quantitation were done exactly as previously described [15].

EMSA

Crude nuclear extracts were obtained as follows: tissues were placed in buffer A [Tris, 10 mM, pH 8; $MgCl_2$, 1.5 mM; KCl, 5 mM; DTT, 0.5 mM; Nonidet 40, 0.5%; aprotinin, 1.5 μg/mL; bestatin, 40 μg/mL; leupeptin, 1 μg/mL; pepstatin, 1 μg/mL; phenylmethylsulfonyl fluoride (PMSF), 0.5 mM; β-glycerophosphate, 8 mM; NaF, 0.4 mM; $NaVO_4$, 0.4 mM] and disrupted on ice with an ultrasonicator (3-mm probe at 30% output for 2 sec (Sonics & Materials Inc.). Samples were left on ice for 10 min, then

centrifuged for 15 min at 2000 *g* (Beckman Microfuge 12, Beckman Instruments Canada Inc.). The pellet was gently resuspended in buffer A and centrifuged for 10 min at 2000 *g*. The supernatant was removed, and 20 μL of buffer B (Tris, 20 mM, pH 8; glycerol, 25%; $MgCl_2$, 1.5 mM; NaCl, 0.4 M; EDTA, 0.2 mM; DTT, 0.5 mM; aprotinin, 1.5 μg/mL; bestatin, 40 μg/mL; leupeptin, 1 μg/mL; pepstatin, 1 μg/mL; PMSF, 0.5 mM; β-glycerophosphate, 8 mM; NaF, 0.4 mM; $NaVO_4$, 0.4 mM) was added to the pellet, which was gently resuspended and left on ice for 15 min. This was followed by centrifugation at 14,000 *g* for 30 min. The supernatant was removed and retained, and the pellet was resuspended once more in buffer B. The solution was left on ice for 15 min, centrifuged at 14,000 *g*, and this second supernatant was pooled with the first. Protein concentrations were determined (Bio-Rad Laboratories, Inc.), and the samples were normalized for protein content by the addition of buffer B. They were then flash frozen in liquid nitrogen and stored at −80°. EMSA was carried out as previously described, except that the concentration of DTT used in the binding mixture was the minimum amount required to achieve detectable binding after overnight autoradiography [15]. In all cases, the DTT concentration was less than 0.5 mM. Quantitation of EMSA experiments was as previously described [15].

Enzyme Activity Assays

The activities of the glutathione peroxidases [27] and glutathione *S*-transferases [21] were determined as previously described, except that the activities were determined from individual embryos and yolk sacs homogenized in 5× volume of 0.25 M RNase-free sucrose (Pharmacia, Biotech. Inc.). Glutathione peroxidase activities were determined with two substrates: cumene hydroperoxide as a substrate to assess total peroxidase activity (contributions by non-selenium-dependent glutathione peroxidases or glutathione *S*-transferases, and selenium-dependent glutathione peroxidase) and hydrogen peroxide to detect only the selenium-dependent peroxidase activity (reviewed in Ref. 28). Glutathione *S*-transferase activity was assessed using 1-chloro-2,4-dinitrobenzene as the substrate (Sigma).

Statistical Analysis

Statistical analyses were done by *t*-test or one-way ANOVA using the CSS (Complete Statistics System) computer program (Statsoft), followed by a *post hoc* Duncan or Tukey's test. The *a priori* level of significance was $P < 0.05$.

RESULTS

Effects of OTC on Embryonic Development

The treatment of embryos with OTC for a period of 44 hr caused a concentration-dependent impairment in a number of parameters of embryonic growth and development. Significant reductions in both the total morphological scores and the yolk sac scores were apparent after treatment with 10 or 20 mM OTC (Fig. 1). The most striking effect of 44 hr of culture with 20 mM OTC was the complete absence of yolk sac blood islands and vasculature. Interestingly, at 24 hr, the yolk sac development of all four groups was indistinguishable (data not shown).

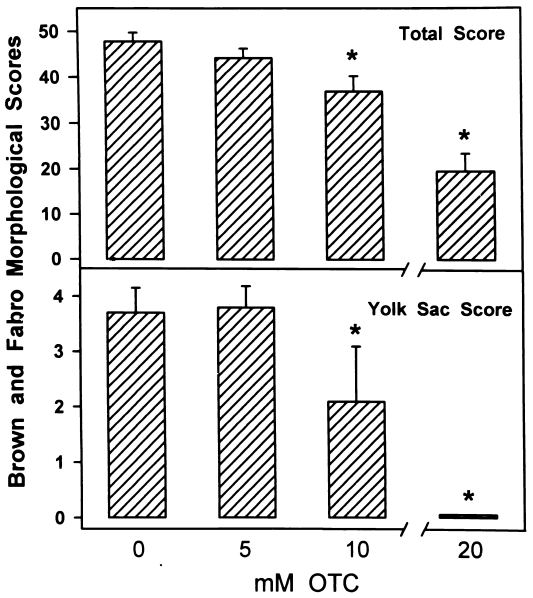

FIG. 1. Brown and Fabro total morphological scores and the yolk sac scores after 44 hr of culture in the presence of 0, 5, 10, or 20 mM OTC. The data are expressed as means ± SD, N = 9–10. Asterisks denote a significant difference from control (0 mM OTC) as determined by ANOVA ($P < 0.05$).

Treatment with 10 or 20 mM OTC significantly inhibited the growth of conceptuses as assessed by the number of somites, crown-rump length, head length, and yolk sac diameter (Table 1). After treatment with 5 mM OTC, yolk sac diameter was reduced significantly with respect to controls. Although some embryos treated with 5 mM OTC did exhibit retarded head development (Fig. 2), the effects on head growth were not statistically significant. Dorsoventral turning was adversely affected by OTC treatment in a concentration-related manner. The incidence of turning after treatment with 5, 10, and 20 mM OTC was 10/10, 9/10, and 3/10, respectively (Table 1).

Embryos from the various treatment groups are shown in Fig. 2. Distinguishing characteristics of the control embryo shown in panel A include the well elevated telencephalic hemispheres, the maxillary process that is about to fuse with the nasal process, and the distinct evagination of the Wolffian crest at somites 26–30, indicative of early hind limb development. Some embryos cultured in 5 mM OTC (Fig. 2B) displayed less growth and development of the head region compared with the control, as evidenced by a relative absence of the elevation of the telencephalic hemispheres, although the telencephalic invaginations were apparent. In addition, the maxillary process and nasal ridge were more distant. The occurrence of such mild developmental delays in some embryos was not sufficient to

TABLE 1. Developmental and growth parameters in the conceptus after 44 hr of exposure to OTC

OTC	N	Number of somites	Crown-rump length (mm)	Head length (mm)	Yolk sac diameter (mm)
0 mM	9	32.0 ± 2.0	4.3 ± 0.2	2.5 ± 0.1	4.5 ± 0.2
5 mM	10	29.5 ± 1.6	4.1 ± 0.1	2.2 ± 0.2	4.2 ± 0.3*
10 mM	10	28.6 ± 1.6*	3.8 ± 0.2*†	2.0 ± 0.3*	4.0 ± 0.1*
20 mM	10	18.5 ± 3.9*	1.8 ± 0.2*‡	0.7 ± 0.1*	3.2 ± 0.5*

Data are means ± SEM.
*Significantly different from controls (0 mM OTC) as determined by ANOVA, $P < 0.05$.
†N = 9, only 9 embryos had undergone turning.
‡N = 3, only 3 embryos had undergone turning.

cause a decrease in the Brown and Fabro score, despite the heavy weighting of the head region in this scoring system (10 of 17 parameters). The embryo cultured in 10 mM OTC, depicted in Fig. 2C, was retarded significantly with respect to growth and development. The telencephalic invagination, nasal ridge, and prominent maxillary process were absent; furthermore, there was little evidence of hind limb development. Embryos exposed to 20 mM OTC displayed severely inhibited growth and development (Fig. 2D). Characteristic features included only partial completion of dorso-ventral turning, an absence of both front and hind limb buds, and an unfused gut. Despite a virtual absence of yolk sac vasculature, the hearts of the embryos exposed to 20 mM OTC were still beating. Although some embryos treated with 5 mM OTC exhibited mild retardation of head growth (head length) and development (distant nasal and maxillary process), there were no head region-specific malformations. This, in addition to the absence of change in the morphological score, was the basis for the decision to use 5 mM OTC in all subsequent studies.

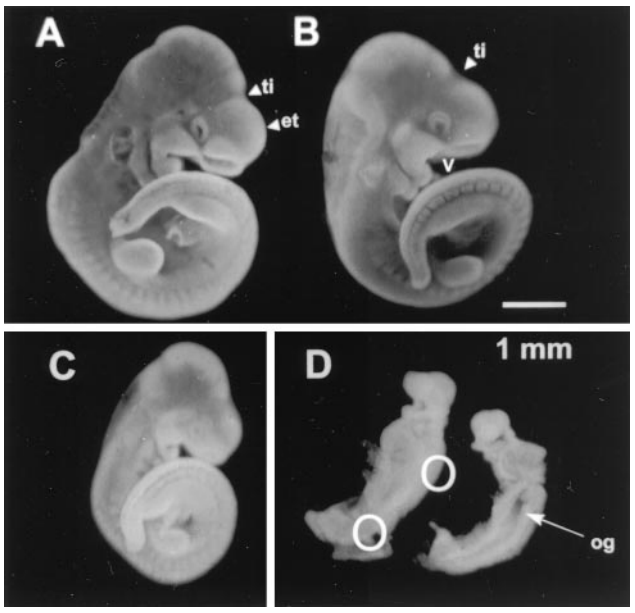

FIG. 2. Embryos cultured for 44 hr in the absence of OTC (A), or in the presence of 5 mM OTC, (B), 10 mM OTC (C), or 20 mM OTC (D). Distinguishing features included elevated telencephalic hemispheres (et), telencephalic invaginations (ti), distant nasal ridge and maxillary process (V), absence of limb buds (O), and open gut (og).

Effects of OTC on GSH Content and Culture-Induced Oxidative Stress

The concentration of GSH in the untreated embryos remained relatively constant throughout the 44-hr culture period (Fig. 3). Within 15 min, there was a significant increase in the GSSG:GSH ratio from less than 0.035 at the beginning of culture (0 hr) to 0.09 at 0.25 hr; this ratio returned to baseline by 1.5 hr (Fig. 3). The addition of 5 mM OTC to the culture medium transiently increased the GSH content of the embryo, presumably due to increased *de novo* biosynthesis of GSH [22, 23]. Peak GSH accumulation occurred at 0.5 hr (49.8 nmol/mg protein); GSH concentrations were elevated significantly above control between 0.5 and 1.5 hr (Fig. 3). Exposure to 5 mM OTC prevented the rise in the GSSG:GSH ratio in the embryo.

In the yolk sacs of the untreated conceptuses, the GSH content was similar to that of the embryo; GSH concentrations remained unchanged throughout culture (Fig. 3). The GSSG:GSH ratio increased dramatically in the yolk sac after the initiation of culture (Fig. 3). Exposure to 5 mM OTC did not result in an increase in GSH content in the yolk sac, in contrast to the embryo. Treatment with 5 mM OTC did not protect the yolk sac from the culture-induced rise in the GSSG:GSH ratio.

To determine whether the tissue-specific effect of OTC on glutathione homeostasis was due to limiting substrate, the cellular content of glutathione and the GSSG:GSH ratio were examined 30 min after the initiation of culture in the presence of 10 or 20 mM OTC. In the embryo proper, these higher concentrations of OTC failed to increase the glutathione content beyond that achieved by 5 mM OTC (Table 2); the GSSG:GSH ratio was also unaffected by 10 or 20 mM OTC, suggesting that 5 mM OTC maximally stimulated glutathione biosynthesis in the embryo. However, in the yolk sac, 20 mM OTC did increase the cellular content of glutathione and reduce the GSSG:GSH ratio (Table 2). Thus, up to a 4-fold higher OTC concentration was required to stimulate glutathione biosynthesis in the yolk sac relative to the embryo.

Since OTC may fail to enhance glutathione synthesis in

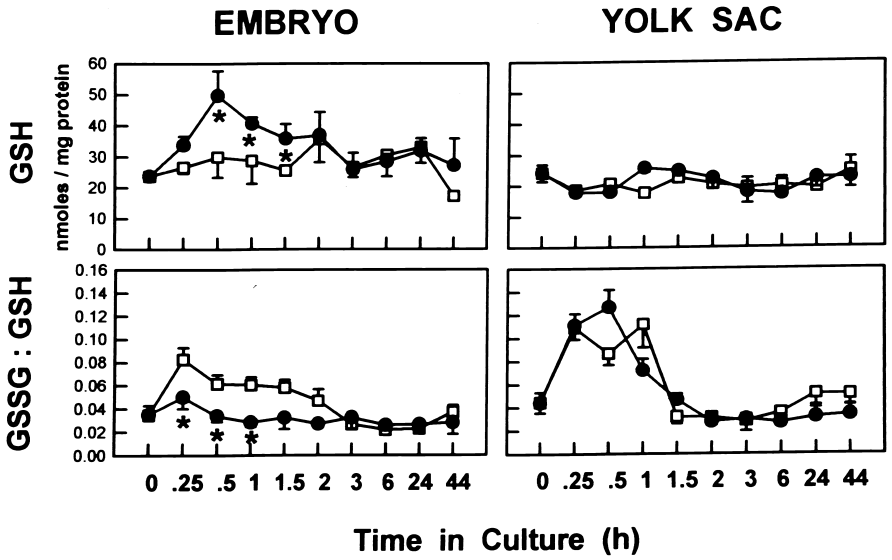

FIG. 3. GSH and the GSSG:GSH ratio in embryos and yolk sacs at various times after the initiation of culture. Day 10 whole rat embryos were cultured *in vitro* for up to 44 hr in either the absence (□) or the presence of 5 mM OTC (●). Oxidative stress, as reflected by increased GSSG production with no compensatory increase in GSH synthesis, is represented by the GSSG:GSH ratio. Each data point (mean ± SEM) represents at least 4 separate embryo or yolk sac samples assayed in triplicate. Asterisks indicate a significant difference ($P < 0.05$) from control, as determined by *t*-test.

tissues in which 5-oxoprolinase content is low, western blot analysis was done to compare 5-oxoprolinase protein content between the gestational day 10.5 (0 hr) embryo and the yolk sac (Fig. 4). α-Actin served as a control for the amount of protein loaded. Unexpectedly, 5-oxoprolinase immunoreactivity was higher in the yolk sac than in the embryo.

Effects of Oxidative Stress on AP-1 mRNA Induction in the Conceptus

The effects of OTC on glutathione content and its protection against oxidative stress in the embryo occurred within the first 90 min of culture. Subsequent comparisons of AP-1 regulation between the oxidatively stressed (untreated) and the OTC-treated conceptuses were done within this time period.

Northern blot analysis showed single molecular size transcripts for c-fos (2.2 kb), junB (2.1 kb) and junD (1.7 kb), while two bands (3.1 and 2.6 kb) were observed for c-jun in the embryo and yolk sac (Fig. 5). In both the embryo and the yolk sac there were marked increases in the steady-state concentrations of the messages for c-fos, c-jun, junB, and junD 30 min after the initiation of culture in the absence of OTC (−). In the presence of OTC (+), this increase was attenuated in the embryo, but not in the yolk sac.

Autoradiographic signals from three to five separate experiments were normalized for the amount of RNA loaded (18S rRNA; [29]), and expressed relative to 0 min or the initiation of culture. The induction of transcripts for AP-1 constituents in the untreated (oxidatively stressed) embryos was maximal at 30 min for all four messages (Fig. 6). In the embryo, the steady-state concentrations of mRNAs for fos and jun returned to baseline by 60 min. Protection of the embryo from oxidative stress by OTC significantly attenuated the induction of all four AP-1 mRNAs.

The induction of transcripts for AP-1 constituents in the untreated (oxidatively stressed) yolk sac was also maximal at 30 min for all four messages (Fig. 6). In the yolk sac, all fos and jun messages remained elevated 90 min after the

TABLE 2. Effect of different concentrations of OTC on glutathione homeostasis after 30 min of culture

Treatment	N	Total glutathione (nmol/mg protein)	GSSG:GSH ratio
Embryo			
No OTC	6	28.7 ± 3.6	0.085 ± 0.018
5 mM OTC	5	49.8 ± 5.7*	0.034 ± 0.008*
10 mM OTC	5	51.4 ± 5.4*	0.038 ± 0.009*
20 mM OTC	5	53.3 ± 6.1*	0.044 ± 0.011*
Yolk sac			
No OTC	6	25.4 ± 3.1	0.114 ± 0.020
5 mM OTC	5	27.1 ± 2.9	0.098 ± 0.015
10 mM OTC	5	31.7 ± 4.5	0.104 ± 0.018
20 mM OTC	5	45.5 ± 5.2*	0.061 ± 0.012*

Data are means ± SEM.
*Significantly different from no OTC, as determined by ANOVA, $P < 0.05$.

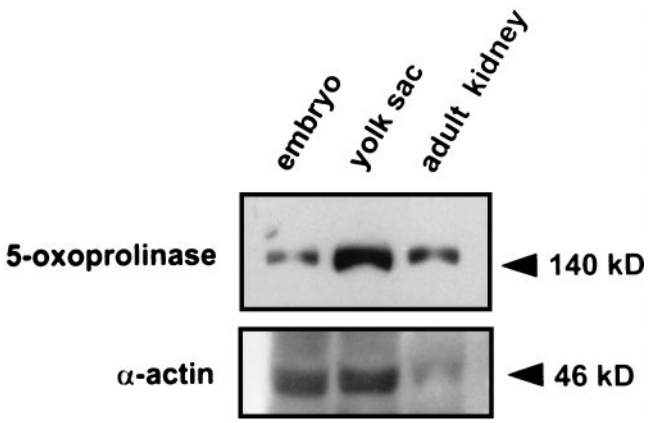

FIG. 4. Immunoblot analysis of the 5-oxoprolinase protein content in gestational day 10.5 embryos (400 μg of total protein), yolk sacs (400 μg of protein), and adult kidney (50 μg of protein). The membrane was reprobed for α-actin as a measure of equal protein loading.

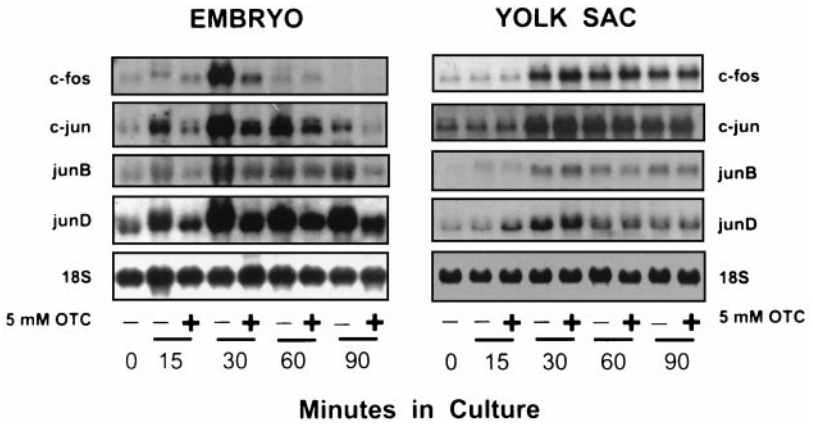

FIG. 5. Representative northern blots of embryo and yolk sac samples probed with radiolabeled cDNAs for c-fos, c-jun, junB, and junD. Each lane contains total RNA, 10 or 15 μg per lane, from embryos or yolk sacs at various times (minutes) after the initiation of culture in the absence or presence of 5 mM OTC. Membranes were sequentially probed and stripped with radiolabeled cDNAs against c-fos, c-jun, junB, and junD. A synthetic probe against 18S rRNA was used as an internal control for RNA loading.

initiation of culture, suggesting increased oxidative stress in this tissue compared with that in the embryo. As anticipated, because 5 mM OTC did not protect against oxidative stress in the yolk sac, steady-state concentrations of the fos and jun messages in the yolk sac remained elevated after OTC treatment (Fig. 6).

Effects of Oxidative Stress on AP-1 DNA Binding Activity

EMSA was done to assess the ability of AP-1 proteins to bind to a double-stranded synthetic oligonucleotide containing the AP-1 consensus site. The authenticity of AP-1 binding was established in several ways. First, in the absence of nuclear extract (free), the migration of the oligonucleotide was not impeded (Fig. 7). Furthermore, when excess unlabeled oligonucleotide was added to the buffer, binding was inhibited (Fig. 7; embryo); however, the addition of excess mutated sequence to which AP-1 does not bind (mAP-1) had no effect (Fig. 7; yolk sac). Finally, the addition of polyclonal rabbit antibodies recognizing specific AP-1 proteins to the binding buffer further retarded migration of the protein–oligonucleotide complex, whereas control rabbit IgG did not (data not shown). Thus, the binding activity is sequence-specific AP-1.

Within 30 min of the initiation of culture, nuclear extracts obtained from untreated embryos displayed increased DNA binding activity (relative to 0 min), which returned to baseline by 90 min; protection from oxidative stress with OTC treatment prevented AP-1 induction (Fig. 7). AP-1 DNA binding activity in the yolk sac was also increased at 30 min and remained elevated up to 90 min; treatment with 5 mM OTC had no effect on the AP-1 DNA binding activity in the yolk sac (Fig. 7).

A quantitative analysis of the autoradiographic signals for AP-1 DNA binding activity from three to five separate experiments is presented in Fig. 8. AP-1 DNA binding activity increased 4-fold (relative to 0 min) in the embryo within 30 min of the initiation of culture; OTC protection

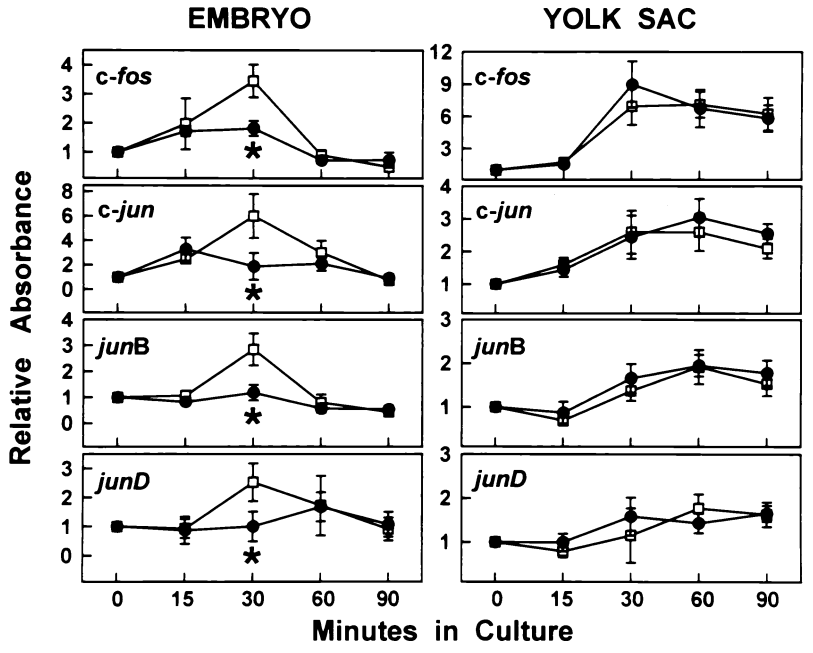

FIG. 6. Quantification of steady-state concentrations of the mRNA transcripts for c-fos, c-jun, junB, and junD in control (open squares) and 5 mM OTC-treated (solid circles) embryos and yolk sacs. Autoradiograms were scanned using laser densitometry, normalized for the amount of RNA loaded (18S rRNA), and expressed relative to 0 hr. The bars represent the means ± SEM from 3 to 5 separate experiments. Asterisks indicate a significant difference between the OTC-treated samples and the controls at the same time point, as determined by *t*-test ($P < 0.05$).

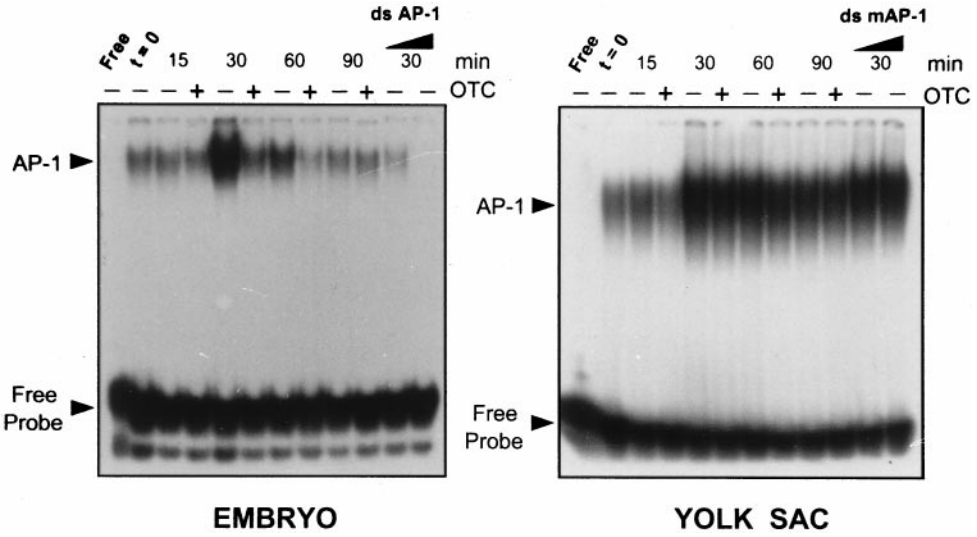

FIG. 7. Representative EMSA of the AP-1 DNA binding activity in embryo and yolk sac samples. Nuclear extracts (10 μg) were prepared from control or OTC-treated samples collected at the specified times (minutes) after the initiation of culture. The retarded AP-1 complex, and the free radiolabeled oligonucleotide, containing the human collagenase AP-1 response element, are indicated. The binding of extracts obtained from control embryos at 30 min was competed out by incubation in the presence of up to a 200-fold molar excess of unlabeled oligonucleotide (ds AP-1). Up to a 100-fold molar excess of an unlabeled mutated sequence (ds mAP-1) to which AP-1 does not bind was unable to inhibit the binding of extracts prepared from 30-min control yolk sacs.

from oxidative stress prevented the induction of AP-1 binding activity at both 30 and 60 min (Fig. 8). In the yolk sac, there was a 4-fold increase in the AP-1 DNA binding activity by 30 min, which fell to 3-fold by 90 min; treatment with 5 mM OTC had no significant effect on AP-1 DNA binding activity in the yolk sac (Fig. 8).

Effects of OTC Exposure on the Culture-Induced Increases in Cytoprotective Enzyme Activities

In the embryo *in utero*, the activity of the glutathione S-transferases approximately doubled from gestational day 10 (0 hr) to gestational day 12 (Table 3). Culture of the embryos *in vitro* for an equivalent period of time resulted in a further 50% increase in this activity. The glutathione S-transferase activity of embryos cultured in the absence of OTC (untreated) was significantly higher than that of embryos cultured in the presence of 5 mM OTC (i.e. protected from oxidative stress). Thus, OTC treatment prevented the culture-related increase in glutathione S-transferase activity in the embryo. *In vivo*, the activity of the glutathione S-transferases tripled in the yolk sac between gestational days 10 and 12. Again, culture induced a further increase in yolk sac glutathione S-transferase activity; however, unlike in the embryo, treatment with 5 mM OTC did not attenuate this increase.

There were significant increases in glutathione peroxidase activities with both cumene hydroperoxide and hydrogen peroxide as substrates between gestational days 10 and 12 *in vivo* in the embryo (Table 4). Culture of the embryos for 44 hr *in vitro* resulted in a further induction of these

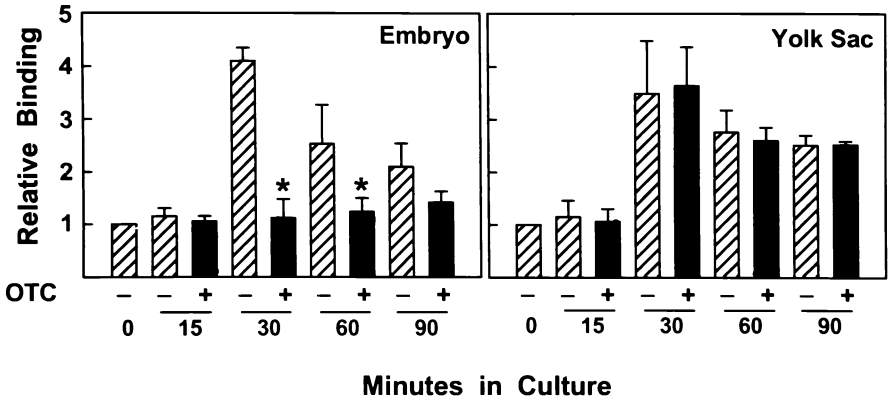

FIG. 8. Quantitation of the EMSA of AP-1 DNA binding activity in embryos and yolk sacs at the specified times (minutes) following the initiation of culture. Autoradiograms were quantified using laser densitometry and expressed relative to 0 hr. The control (hatched bars) and 5 mM OTC-treated samples (solid bars) are the means (± SEM) of at least 3 separate experiments. Asterisks indicate a significant difference ($P < 0.05$) from the control at the same time point, as determined by *t*-test.

TABLE 3. Glutathione *S*-transferase activity in the conceptus after 44 hr of treatment with 5 mM OTC

	Sample	Glutathione *S*-transferase activity (nmol/min/mg protein)	N
Embryo			
In vivo	gd 10	5.58 ± 2.37	4
	gd 12	13.12 ± 1.89*	7
In vitro	Untreated	19.46 ± 3.42†	6
	OTC (5 mM)	15.37 ± 2.01‡	6
Yolk sac			
In vivo	gd 10	8.65 ± 3.32	4
	gd 12	23.42 ± 2.33*	8
In vitro	Untreated	36.34 ± 4.95†	7
	OTC (5 mM)	33.79 ± 6.19	7

Data are means ± SD.

*Developmental changes; significantly different from *in vivo* gestational day (gd) 10 by ANOVA, $P < 0.05$

†Culture-induced increase; significantly different from *in vivo* gd 12 by ANOVA, $P < 0.05$.

‡Effect of OTC treatment; significantly different from untreated *in vitro* samples by ANOVA, $P < 0.05$.

activities with both substrates. In the embryo, protection from oxidative stress by OTC treatment prevented the culture-induced increase in glutathione peroxidase activities. Glutathione peroxidase activities were higher in the yolk sac than in the embryo and further increased between gestational days 10 and 12. Culture of the conceptus further induced these activities in the yolk sac; exposure to 5 mM OTC did not modulate glutathione peroxidase activities.

DISCUSSION

When mid-organogenesis rat embryos were placed in culture, there was a transient increase in the GSSG:GSH ratio [15], indicative of oxidative stress [18]. The present studies determined the consequences of increasing the cellular content of glutathione with a cysteine pro-drug, OTC, on AP-1 gene expression and AP-1 DNA binding activity in the conceptus; furthermore, the role of oxidative stress and of AP-1 in the induction of these activities of the glutathione *S*-transferases and glutathione peroxidases in the conceptus *in vitro* were evaluated.

High (10 and 20 mM) concentrations of OTC were embryotoxic in the rat embryo culture system. *In vivo* studies have shown that another cysteine pro-drug, *N*-acetylcysteine, may be embryotoxic also. The co-administration of a high dose of *N*-acetylcysteine (275 mg/kg, i.p.) and phenytoin in CD-1 mice significantly increased the incidence of cleft palate and fetal resorptions [30]. This was attributed to the formation of GSH or cysteinyl free radicals as a result of *N*-acetylcysteine-induced elevations in cellular GSH or cysteine content. Overproduction of GSH may also cause dysmorphogenesis via alterations in prostanoid production. The incubation of ram seminal vesicles *in vitro* with 5 mM GSH produces teratogenic prostaglandin E_2 exclusively [31, 32], whereas concentrations of less than 1 mM GSH produce a broad spectrum of prostaglandins [33]. Thus, the embryotoxicity of 10 or 20 mM OTC may be due to the formation of GSH or cysteinyl free radicals or to other disturbances in metabolism.

Exposure to 5 mM OTC increased embryonic GSH levels (likely due to increased GSH biosynthesis [22, 23]) and prevented the culture-induced increase in the GSSG:GSH ratio in the embryo; however, in the yolk sac, only a higher concentration of OTC (20 mM) increased GSH content. The failure of 5 mM OTC to increase glutathione content in the yolk sac in these studies is in contrast to a previous report in which the GSH content of the yolk sac was increased when the rat conceptus was incubated for 24 hr in the presence of 5 mM OTC and 41 μM 2-nitrosofluorene [23]. After oxidative stress, the total glutathione pool remains intact; by depleting total glutathione, nitrosofluorene may be a more potent stimulus of glutathione biosynthesis in the yolk sac than is oxidative stress. Alternatively, since the degree of oxygen saturation in the

TABLE 4. Glutathione peroxidase activity in the conceptus after 44 hr of treatment with 5 mM OTC

	Sample	Glutathione peroxidase activity (nmol NADPH oxidized/min/mg protein)			
		CmOOH	N	H_2O_2	N
Embryo					
In vivo	gd 10	17.52 ± 7.31	3	19.17 ± 4.62	3
	gd 12	58.89 ± 4.78*	7	54.46 ± 6.81*	4
In vitro	Untreated	84.70 ± 9.79†	6	77.84 ± 12.04†	5
	OTC (5 mM)	62.75 ± 4.72‡	6	58.74 ± 6.21‡	4
Yolk sac					
In vivo	gd 10	50.74 ± 1.09	3	48.23 ± 14.63	3
	gd 12	84.63 ± 7.70*	5	78.11 ± 3.41*	3
In vitro	Untreated	119.34 ± 34.06†	5	114.69 ± 14.97†	3
	OTC (5 mM)	108.31 ± 19.48	4	99.60 ± 12.12	4

Data are means ± SD.

*Developmental changes; significantly different from *in vivo* gd 10 by ANOVA, $P < 0.05$.

†Culture-induced changes; significantly different from *in vivo* gd 12 ANOVA, $P < 0.05$.

‡Effect of OTC treatment; significantly different from untreated *in vitro* samples by ANOVA, $P < 0.05$.

medium is proportional to its protein content, the use of 90% rat serum as a culture medium may expose the conceptus to a higher oxygen tension than that in previous studies in which the medium was 33% serum [23].

The basis for the observation that, in the yolk sac, only the highest (20 mM) OTC concentration increased glutathione content and prevented glutathione oxidation remains enigmatic. This is due to the unexpected observation that immunoreactivity to 5-oxoprolinase, the enzyme responsible for the conversion of OTC to cysteine, was present at higher concentrations in the yolk sac than in the embryo. Thus, when conceptuses are cultured in the presence of 90% serum, cysteine may not be rate limiting, rendering OTC less effective. While cysteine availability may determine the rate of glutathione biosynthesis in the embryo, glutamate may be rate limiting in the yolk sac. Interestingly, in the embryo, the K_m of γ-glutamylcysteine synthetase for glutamate is higher than that for cysteine [34]. More information is needed on the nature of the tissue-specific regulation of glutathione biosynthesis in the conceptus, and its role in the response of the conceptus to insult.

Inhibition of oxidative stress in the embryo by OTC (this report) or with antioxidant enzymes [15] prevented the induction of increased steady-state concentrations of the c-fos, c-jun, junB, and junD mRNAs. Taken together, these data strongly suggest that changes in cellular redox balance may play a significant role in modulating the expression of AP-1 in the conceptus; however, OTC may also alter AP-1 regulation via other cellular mechanisms. The pathway by which oxidative stress signals an increase in AP-1 expression and activity in the conceptus is unknown. In HeLa cells, increased c-fos expression after H_2O_2 treatment was initiated by Ras stimulation of $p42^{MAPK}$ activity [10]. Another MAPK family member, p38, was important for induced jun expression in an *in vivo* carbon tetrachloride model of radical induced hepatic damage [12]. The H_2O_2-mediated induction of c-jun in the NIH3T3 cell line was dependent upon non-receptor protein tyrosine kinase activity [35]. Therefore, both the MAP kinases and non-receptor protein tyrosine kinases may be important in the oxidative stress signaling cascade in the conceptus.

In addition to effects on transcription, both OTC (this report) and antioxidants [15] were capable of inhibiting the induction of AP-1 DNA binding activity in the conceptus. In cell culture models, post-translational modification of the AP-1 proteins by $p42^{MAPK}$, $p44^{MAPK}$, $p46^{MAPK}$, and $p54^{MAPK}$, all members of the MAPK superfamily, regulated AP-1 activity (reviewed in Ref. 36). Treatment with antioxidants inhibited the response of some of these kinases to a variety of pro-oxidant stimuli [37]; thus, post-translational mechanisms may be involved also in the signal transduction cascade leading to the AP-1 oxidative stress response in the conceptus.

The activities of two families of glutathione-dependent detoxification enzymes, the glutathione *S*-transferases and glutathione peroxidases, were induced in the conceptus in culture (this report; [20]). We hypothesized that the oxidative stress-induced increase in the GSSG:GSH ratio in the conceptus in culture, associated with increased AP-1 expression and activity, was involved in the induction of these cytoprotective enzymes. In support of this hypothesis was the observation that OTC treatment protected the embryo against an increase in the GSSG:GSH ratio, prevented the induction of AP-1 expression and activity, and prevented the increase in the activities of the glutathione *S*-transferases and glutathione peroxidases in the embryo. While the glutathione and AP-1 status of the conceptus may be important in regulating the activities of these enzymes, it is likely that other factors are involved also, particularly since OTC may do more than just alter glutathione homeostasis. The role of AP-1 in mediating the induction of glutathione *S*-transferases Y_a and Y_p (which also possess a glutathione peroxidase activity; reviewed in Refs. 28 and 38, respectively) is not clear. This is due to the controversy surrounding the ability of *bona fide* AP-1 to bind to the "AP-1-like" elements upstream of glutathione *S*-transferases Y_a [39] and Y_p [19]. Studies have demonstrated that Fos and Jun do not bind to the Y_a enhancer element [40], and that Y_a induction through its "AP-1-like" response element is dependent upon an unidentified transcription factor [41]. In contrast, others have demonstrated that a reporter construct containing elements of the Y_a promoter is driven by the heterodimers c-Fos/c-Jun or c-Fos/JunB, but not the homodimer c-Jun/c-Jun [42]. However, the AP-1 DNA binding activity of both the embryo and the yolk sac in the mid-organogenesis rat conceptus is void of detectable JunB and contains very little c-Fos and c-Jun (unpublished results). Therefore, the conceptus may not possess the AP-1 constituents required to transactivate the expression of glutathione *S*-transferase Y_a. This would provide an explanation for our findings in a previous report that the induction of the transcript for glutathione *S*-transferase Y_p in the conceptus was refractory to phorbol esters, potent activators of AP-1 activity [21].

In summary, these data demonstrated that glutathione homeostasis in the conceptus is regulated differentially in the embryo and yolk sac. AP-1 expression and DNA binding activity, as well as the activities of glutathione-dependent detoxification enzymes, are responsive to changes in glutathione status. Oxidative stress-induced AP-1 may play a role in the response of the embryo to insult during organogenesis.

This research was supported by the Medical Research Council of Canada. We are grateful to Gerald Batist for the gift of the anti-5-oxoprolinase antibody, and Bernard Robaire for valuable suggestions concerning the manuscript.

References

1. Winn LM and Wells PG, Free radical-mediated mechanisms of anticonvulsant teratogenicity. *Eur J Neurol* **2** (Suppl 4): 5–29, 1995.

2. Angel P and Herrlich PA, *The Fos and Jun Families of Transcription Factors*. CRC Press, Boca Raton, 1994.
3. Curran T and Morgan JI, Fos: An immediate-early transcription factor in neurons. *J Neurobiol* **26:** 403–412, 1995.
4. Devary Y, Gottlieb RA, Smeal T and Karin M, The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. *Cell* **71:** 1081–1091, 1992.
5. Amstad PA, Krupitza G and Cerutti PA, Mechanism of *c-fos* induction by active oxygen. *Cancer Res* **52:** 3952–3960, 1992.
6. Maki A, Berezesky IK, Fargnoli J, Holbrook NJ and Trump BF, Role of $[Ca^{2+}]_i$ in induction of *c-fos*, *c-jun* and *c-myc* mRNA in rat PTE after oxidative stress. *FASEB J* **6:** 919–924, 1992.
7. Meyer M, Schreck R and Baeuerle PA, H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-response factor. *EMBO J* **12:** 2005–2015, 1993.
8. Rao GN, Lasségue B, Griendling KK, Alexander RW and Berk BC, Hydrogen peroxide-induced c-fos expression is mediated by arachidonic acid release: Role of protein kinase C. *Nucleic Acids Res* **21***:* 1259–1263, 1993.
9. Galter D, Mihm S and Dröge W, Distinct effects of glutathione disulphide on nuclear transcription factor κB and activator protein-1. *Eur J Biochem* **221:** 639–648, 1994.
10. Müller JM, Cahill MA, Rupec RA, Baeuerle PA and Nordheim A, Antioxidants as well as oxidants activate c-*fos* via Ras-dependent activation of extracellular-signal-regulated kinase-2 and Elk-1. *Eur J Biochem* **244:** 45–52, 1997.
11. Bergelson S, Pinkus R and Daniel V, Intracellular glutathione levels regulate Fos/Jun induction and activation of glutathione *S*-transferase gene expression. *Cancer Res* **54:** 36–40, 1994.
12. Mendelson KG, Contois L-R, Tevosian S, Davis RJ and Paulson KE, Independent regulation of JNK/p38 mitogen-activated protein kinases by metabolic oxidative stress in the liver. *Proc Natl Acad Sci USA* **93:** 12908–12913, 1996.
13. Wemmie JA, Steggerda SM and Moye-Rowley WS, The *Saccharomyces cerevisiae* AP-1 protein discriminates between oxidative stress elicited by the antioxidants H_2O_2 and diamide. *J Biol Chem* **272:** 7908–7914, 1997.
14. New DAT, Whole embryo culture and the study of mammalian embryos during organogenesis. *Biol Rev* **53:** 81–122, 1978.
15. Ozolinš TRS and Hales BF, Oxidative stress regulates the expression and activity of transcription factor activator protein-1 in rat conceptus. *J Pharmacol Exp Ther* **280:** 1085–1093, 1997.
16. Winn L and Wells PG, Phenytoin-initiated DNA oxidation in murine embryo culture, and embryo protection by the antioxidative enzymes superoxide dismutase and catalase: Evidence for reactive oxygen species-mediated DNA oxidation in the molecular mechanism of phenytoin teratogenesis. *Mol Pharmacol* **48:** 112–120, 1995.
17. Meister A, Glutathione and the γ-glutamyl cycle. In: *Glutathione: Metabolism and Function* (Eds. Arias IM and Jackoby WB), pp. 35–44. Raven Press, New York, 1976.
18. Halliwell B and Gutteridge JM, Oxygen is a poison—An introduction to oxygen toxicity and free radicals. In: *Free Radicals in Biology and Medicine* (Eds. Halliwell B and Gutteridge JM), 2nd Edn, pp. 1–21. Clarendon Press, Oxford, 1989.
19. Morrow CS, Goldsmith ME and Cowan KH, Regulation of human glutathione *S*-transferase π gene transcription: Influence of 5′-flanking sequences and *trans*-activating factors which recognize AP-1-binding sites. *Gene* **88:** 215–225, 1990.
20. Ishibashi M, Akazawa S, Sakamaki H, Matsumoto K, Yamasaki H, Yamaguchi Y, Goto S, Urata Y, Kondo T and Nagataki S, Oxygen-induced embryopathy and the significance of glutathione-dependent antioxidant system in the rat embryo during early organogenesis. *Free Radic Biol Med* **22:** 447–454, 1997.
21. Hales BF and Huang C, Regulation of the Y_p subunit of glutathione *S*-transferase P in rat embryos and yolk sacs during organogenesis. *Biochem Pharmacol* **47:** 2029–2037, 1994.
22. Williamson JM and Meister A, Stimulation of hepatic glutathione formation by administration of L-2-oxothiazolidine-4-carboxylate, a 5-oxo-L-prolinase substrate. *Proc Natl Acad Sci USA* **78:** 936–939, 1981.
23. Harris C, Namkung MJ and Juchau MR, Regulation of intracellular glutathione in rat embryos and visceral yolk sacs and its effects on 2-nitrosofluorene-induced malformations in the whole embryo culture system. *Toxicol Appl Pharmacol* **88:** 141–152, 1987.
24. Brown NA and Fabro S, Quantitation of rat embryonic development *in vitro*: A morphological scoring system. *Teratology* **24:** 65–78, 1981.
25. Zucker RM, Elstein KH, Shuey DL, Ebron-McCoy M and Rogers JM, Utility of fluorescence microscopy in embryonic/fetal topographical analysis. *Teratology* **51:** 430–434, 1995.
26. Chen X, Schecter RL, Griffith OW, Hayward MA, Alpert LC and Batist G, Characterization of 5-oxoprolinase in normal and tumor tissues of human and rat: A potential new target for biochemical modulation of glutathione. *Clin Cancer Res* **4:** 131–138, 1998.
27. Ozolinš TRS, Siksay DLA and Wells PG, Modulation of embryonic glutathione peroxidase activity and phenytoin teratogenicity by dietary deprivation of selenium in CD-1 mice. *J Pharmacol Exp Ther* **277:** 945–953, 1996.
28. Mannervik B and Danielson UH, Glutathione transferases—Structure and catalytic activity. *CRC Crit Rev Biochem* **23:** 283–337, 1988.
29. Szyf M, Milstone DS, Shimmer BP, Parker KL and Seidman JG, *Cis* modification of the steroid 21-hydroxylase gene prevents its expression in the Y1 mouse adrenocortical tumor cell line. *Mol Endocrinol* **4:** 1144–1152, 1990.
30. Wong M and Wells PG, Effects of N-acetylcysteine on fetal development and on phenytoin teratogenicity in mice. *Teratog Carcinog Mutagen* **8***:* 65–79, 1988.
31. Hibelink DR and Persaud TVN, Teratogenic effects of prostaglandin E_2 in hamsters. In: *Progress in Lipid Research* (Ed. Holman RT), Vol. 20, pp. 241–242. Pergamon Press, London, 1982.
32. Liebgott B and Wiley MJ, Prenatal hamster development following maternal administration of PGE_2 at mid-term. *Prostaglandins Leukot Med* **17:** 309–318, 1985.
33. Eling TE, Curtis JF, Harman LS and Mason RP, Oxidation of glutathione to its thiyl free radical metabolite by prostaglandin H synthase: A potential endogenous substrate for the hydroperoxidase. *J Biol Chem* **261:** 5023–5028, 1986.
34. Harris C and Lee E, Determination of γ-glutamylcysteine synthetase activity and apparent kinetic parameters in the post implantation rat embryo and visceral yolk sac. *Teratology* **55:** 44, 1997.
35. Lee SF, Huang YT, Wu WS and Lin JK, Induction of c-jun protooncogene expression by hydrogen peroxide through hydroxyl radical generation and $p60^{SRC}$ tyrosine kinase activation. *Free Radic Biol Med* **21:** 437–448, 1996.
36. Whitmarsh AJ and Davis RJ, Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J Mol Med* **74:** 589–607, 1996.
37. Lo YYC, Wong JMS and Cruz TF, Reactive oxygen species mediate cytokine activation of c-Jun NH_2-terminal kinases. *J Biol Chem* **271:** 15703–15707, 1996.
38. Scott TR and Kirsch RE, The isolation of a fetal rat liver glutathione *S*-transferase isoenzyme with high glutathione peroxidase activity. *Biochim Biophys Acta* **926:** 264–269, 1987.

39. Frilling RS, Bergelson S and Daniel V, Two adjacent AP-1-like binding sites from the electrophile responsive element of murine glutathione *S*-transferase Ya subunit gene. *Proc Natl Acad Sci USA* **89:** 668–672, 1992.
40. Nguyen T, Rushmore TH and Pickett CB, Transcriptional regulation of a rat liver glutathione *S*-transferase Ya subunit gene. Analysis of the antioxidant response element and its activation by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate. *J Biol Chem* **269:** 13656–13662, 1994.
41. Liu S and Pickett CB, The rat liver glutathione *S*-transferase Ya subunit gene: Characterization of binding properties of a nuclear protein from HepG2 cells that has high affinity for the antioxidant response element. *Biochemistry* **35:** 11517–11521, 1996.
42. Bergelson S and Daniel V, Cooperative interaction between Ets and AP-1 transcription factors regulates induction of glutathione S-transferase Ya gene expression. *Biochem Biophys Res Commun* **200:** 290–297, 1994.