Biochemical Pharmacology, Vol. 36, No. 12, pp. 2019–2025, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

ENHANCEMENT OF THE EMBRYOTOXICITY OF ACROLEIN, BUT NOT PHOSPHORAMIDE MUSTARD, BY GLUTATHIONE DEPLETION IN RAT EMBRYOS *IN VITRO*

VALERIE L. SLOTT and BARBARA F. HALES*
Department of Pharmacology and Therapeutics, Centre for the Study of Reproduction, McGill University, Montreal, Quebec, Canada H3G 1Y6

(*Received* 9 *September* 1986; *accepted* 2 *December* 1986)

Abstract—The intracellular thiol glutathione is known to protect cells against the toxicity of certain drugs and reactive intermediates. In this study, the role of glutathione in protecting the embryo against two embryolethal and teratogenic metabolites of cyclophosphamide, an anticancer drug, was assessed *in vitro* using the rat whole embryo culture system. Day 10.5 rat embryos were cultured in rat serum medium containing phosphoramide mustard (1, 10, or 25 μM) or acrolein (10, 25, 50 or 100 μM), with and without buthionine sulfoximine (10 or 100 μM), a compound which depletes glutathione by inhibiting its synthesis. After 45 hr, embryos were assessed for viability, malformations, growth and development, and the glutathione content of embryos exposed to buthionine sulfoximine alone was assayed. The glutathione levels of the embryos and their yolk sacs were decreased significantly by 100 μM buthionine sulfoximine, whereas 10 μM buthionine sulfoximine decreased glutathione levels in the yolk sacs only. Phosphoramide mustard alone, at concentrations of 10 and 25 μM, did not produce embryo deaths but did cause malformations and growth retardation in 100% of the exposed embryos. The addition of buthionine sulfoximine (100 μM) had no effect on the teratogenicity or growth-retarding effects of phosphoramide mustard. Acrolein alone produced a 25 and 48% incidence of embryo deaths at 50 and 100 μM, respectively, and a 46% incidence of embryo malformations, as well as significant growth retardation, among the surviving embryos at 100 μM. Buthionine sulfoximine (10 or 100 μM) significantly enhanced the embryotoxic effects of acrolein. The addition of 10 μM buthionine sulfoximine resulted in 100% embryolethality at 100 μM acrolein; this buthionine sulfoximine concentration decreased the EC_{50} values for embryo deaths and malformations to 50% of those for acrolein alone. The addition of 100 μM buthionine sulfoximine significantly potentiated the embryolethality of acrolein at 25, 50 and 100 μM; the combination of 100 μM acrolein plus 100 μM buthionine sulfoximine was 100% embryolethal. The incidence of embryo malformations was enhanced significantly at 10 and 25 μM acrolein by 100 μM buthionine sulfoximine. The EC_{50} values for embryo deaths and malformations were decreased to 50 and 20%, respectively, of those values for acrolein alone. Both buthionine sulfoximine concentrations produced significant growth retardation at all acrolein concentrations compared to either acrolein or buthionine sulfoximine alone. Thus, depletion of glutathione by buthionine sulfoximine dramatically enhanced the embryolethal, teratogenic and growth retarding effects of acrolein *in vitro*, but did not alter the embryotoxicity of phosphoramide mustard. Therefore, glutathione plays an important role in protecting the embryo against only one of the teratogenic metabolites of cyclophosphamide.

Many drugs and environmental contaminants are teratogenic to the developing embryo. Often, neither the mechanism of action of the chemical, nor the mechanisms by which embryonic cells might protect themselves and possibly detoxify the drug, are known. Adequate levels of intracellular free thiols are important in protecting many cells and organisms against drug toxicity [1, 2]. The major protective thiol in most organisms is the tripeptide glutathione; little is known about the concentrations or functions of glutathione in the embryo. However, it seems reasonable that glutathione is involved in protecting the embryo and fetus against damage from toxic chemicals and reactive intermediates.

Both Ashby *et al.* [3] and Hales [4] have found that pretreatment of pregnant rats with glutathione can protect markedly against the teratogenicity of the anticancer drug cyclophosphamide. Furthermore, Hales [4] has shown that pretreatment with diethylmaleate, a depleter of glutathione, exacerbates the *in vivo* teratogenicity of cyclophosphamide in rats. *In vitro*, Kitchin *et al.* [5] demonstrated that glutathione can protect against the embryolethality and growth-retarding effects, but not the teratogenicity, of mercuric chloride in cultured rat embryos. The effect of depletion of glutathione on the response of cultured embryos to teratogens has not, to the best of our knowledge, been reported.

Phosphoramide mustard and acrolein are toxic and reactive metabolites of the widely used anticancer drug and known teratogen, cyclophosphamide [6]. Both phosphoramide mustard and acrolein are teratogenic and embryolethal in rabbits and rats *in vivo* when administered intraamniotically [7–9] and *in vitro*, in whole rat embryo culture [10, 11]. At least one of these compounds, acrolein, is known to be very reactive toward thiols and can form conjugates

* Send correspondence to: Dr. Barbara F. Hales, Department of Pharmacology and Therapeutics, McGill University, 3655 Drummond St., Montreal, Quebec, Canada H3G 1Y6.

with glutathione both *in vivo* and *in vitro*; these reactions can occur nonenzymatically and enzymatically catalyzed by the glutathione-*S*-transferases [12–14]. Additionally, acrolein can deplete hepatic glutathione levels in rats *in vivo* [15] and in cultured hepatocytes *in vitro* [16].

Much can be learned about the functions of glutathione in the embryo and in other tissues by using depleters of endogenous glutathione to compare function in normal tissue to that in the depleted state. Buthionine sulfoximine was developed by Griffith and Meister [17] to block glutathione synthesis by specifically inhibiting one of the synthetic enzymes, γ-glutamylcysteine synthetase. Buthionine sulfoximine is less toxic and more specific in its action than either diethylmaleate or diamide, two widely used glutathione-depleting agents. In addition, when synthesis is the target for inhibition, inhibition should be sustained longer, since rebound synthesis is less likely to occur. Buthionine sulfoximine successfully depletes glutathione levels within many tissues in adult mice and rats when administered orally or subcutaneously, with no observable adverse effects [18–20]. *In vitro*, buthionine sulfoximine decreases glutathione levels in cultured macrophages [21], and in human lymphoid cells to less than 3% of control after 30 hr of culture [22].

In the present study, we investigated, using rat whole embryo cultures, the extent of glutathione depletion by buthionine sulfoximine within embryos and their yolk sacs and the effects of this depletion of glutathione on the embryolethality and teratogenicity of phosphoramide mustard and acrolein, the two teratogenic metabolites of cyclophosphamide.

METHODS

Chemicals. Acrolein (99% pure) was purchased from the Aldrich Chemical Co. (Montreal, Quebec). Phosphoramide mustard (ASTA-5317) was provided by Professor N. Brock (Asta-Werke, Bielefeld, Germany) and L-buthionine-*S,R*,-sulfoximine by Dr. A. Meister (Cornell University, New York, NY). Tyrode's saline, Hanks' balanced salt solution and penicillin/streptomycin (10,000 units/ml and 10,000 μg/ml respectively) were purchased from Gibco Laboratories (Burlington, Ontario). Glutathione reductase for the assay of glutathione was purchased from Boehringer Mannheim (Laval, Quebec).

Animals. Timed-gestation pregnant Sprague–Dawley rats (180–200 g) were purchased from Charles River Canada, Inc. (St. Constant, Quebec). The day on which spermatozoa were found in the vaginal smear was considered day zero of pregnancy. Rats were housed in the McIntyre Animal Centre (McGill University, Montreal, Quebec) and given Purina rat chow and water *ad lib*.

Embryo culture procedure. The embryo culture procedure used in this study was based on the system of New [23] as modified in our laboratory [11]. Pregnant rats were etherized on the morning of day 10 of gestation, and the embryos were dissected free of maternal tissue and the Reichert's membrane, leaving the ectoplacental cone and yolk sac intact. The embryos were placed in sterile 60 ml culture bottles containing medium consisting of 80% heat-inactivated rat serum, 20% Tyrode's saline and penicillin/streptomycin (final concentrations were 50 units/ml and 50 μg/ml respectively). Each bottle contained two to four embryos with 1.6 ml of medium per embryo. The bottles were gassed with a mixture of 20% O_2, 5% CO_2, 75% N_2, prior to the addition of phosphoramide mustard alone (final concentration of 1, 10 or 25 μM), acrolein alone (final concentration of 10, 25, 50 or 100 μM), or phosphoramide mustard or acrolein in combination with buthionine sulfoximine (10 or 100 μM). The bottles were placed in a rotator (New Brunswick Scientific Co., Edison, NJ) at 25 rpm and the embryos were cultured for 45 hr at 37°. After the first 24 hr, the embryos were regassed, with 95% O_2, 5% CO_2.

At the end of the culture period all embryos were removed and examined for viability. Only those embryos with yolk sac circulation (a score of 1 or greater by the scoring system of Brown and Fabro [24]) and a heart beat were evaluated further. The embryos were classified as normal or abnormal, and the abnormalities were documented. The yolk sac diameter, crown–rump length, and head length were measured, and the number of somites was counted. The embryo scoring system of Brown and Fabro [24] was used to determine a total morphological score for each embryo. Embryos and their yolk sacs were individually frozen at −80° for subsequent assay of glutathione and protein content. Total glutathione content of the homogenized samples was measured by the method of Tietze [25] as modified by Brehe and Burch [26] and expressed as nmol glutathione equivalents/mg protein (level of detection of 0.016 nmol glutathione/mg protein). Protein content was measured by the method of Lowry *et al.* [27].

Statistics. All data on embryo deaths and malformations were analyzed by the Fisher Exact Test [28]. In addition, comparisons of the dose–response curves for acrolein-induced embryo deaths and malformations in the absence and presence of buthionine sulfoximine were done by probit analysis with EC_{50} determinations [29]. Comparisons of the glutathione content, yolk sac diameter, crown–rump length, head length, number of somites, and morphological score were done by the one-way ANOVA with the F-Test used to isolate the differences between groups [30]. The level of significance used throughout was $P \leq 0.05$.

RESULTS

Effect of buthionine sulfoximine on embryo and yolk sac glutathione concentrations. The effects of the addition of buthionine sulfoximine on the glutathione levels within the cultured embryos and yolk sacs are shown in Fig. 1, A and B. Culture with 10 μM buthionine sulfoximine had no effect on the glutathione content of the embryo but significantly decreased the glutathione content within the yolk sacs. However, exposure to 100 μM buthionine sulfoximine markedly decreased the glutathione concentrations in both the embryos and yolk sacs to 16 and 17% of control respectively.