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INCREASED GLUTATHIONE IN CULTURED HEPATOCYTES ASSOCIATED WITH INDUCTION OF CYTOCHROME P-450

LACK OF EFFECT OF GLUTATHIONE DEPLETION ON INDUCTION OF CYTOCHROME P-450 AND δ-AMINOLEVULINATE SYNTHASE

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Abstract—Cellular glutathione concentrations in primary cultures of chick embryo hepatocytes were 15.3 ± 5.3 nmoles/mg protein (mean ± S.D.) and remained stable for up to 3 days in culture. The presence of insulin was not essential for the maintenance of glutathione concentrations. Induction of cytochrome P-450 by phenobarbital-like inducers (2-propyl-2-isopropylacetamide, 2-allyl-2-isopropylacetamide, and 2,4,5,2′,4′,5′-hexabromobiphenyl) was accompanied by 2- to 3-fold increases in glutathione concentrations and by increased glucuronidation of phenol red. The 3-methylcholanthrene-like inducers of cytochrome P-450 (β-naphthoflavone and 3,4,3′,4′-tetrachlorobiphenyl) did not have these effects. Glutathione was rapidly depleted to 15–30% of control levels in hepatocytes treated with buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthase. No toxicity was observed with glutathione depletion. Glutathione depletion did not affect the ability of 2-propyl-2-isopropylacetamide to induce cytochrome P-450, glucuronidation of phenol red, or δ-aminolevulinate synthase.

One of the recognized functions of GSH is its role in the detoxification of reactive intermediates formed during cytochrome P-450-mediated biotransformation of certain xenobiotics [1–4]. It is of interest to know what relationship might exist between induction of cytochrome(s) P-450 and GSH metabolism. In rats, phenobarbital and 3-methylcholanthrene, two prototypical inducers of different species of cytochrome P-450, are known to induce different species of glutathione-*S*-transferases [5–8] and, although both xenobiotics slightly increase hepatic concentrations of GSH [9, 10], only phenobarbital enhances biliary GSH efflux [10]. It has also been suggested that GSH is important in the regulation of ALA synthase† [11, 12], the first and rate-limiting enzyme for hepatic heme synthesis [13], and therefore, important in providing heme for cytochrome P-450.

Because hepatic GSH concentrations in whole animals can be affected by many factors, including nutritional and hormonal status of the animal and diurnal variations [1–3], we decided to study the relationship between GSH and cytochrome(s) P-450 induction in primary cultures of chick embryo hepatocytes. These cells are known to maintain basal levels of cytochrome P-450 and ALA synthase which can be induced by various xenobiotics [14, 15]. We recently found [16] that the porphyrogenic drug 2-allyl-2-isopropylacetamide, which induces both cytochrome P-450 and ALA synthase in the cultured chick hepatocytes [14, 15], also causes an increase in GSH concentrations in these cells. We now report that there is a parallel increase in GSH with induction of cytochrome(s) P-450 by other phenobarbital-like inducers but not by 3-methylcholanthrene-like inducers. The increase in GSH is also associated with induction of glucuronidation of phenol red. However, depletion of GSH using the inhibitor of γ-glutamylcysteine synthase, buthionine sulfoximine [17], does not inhibit induction either of ALA synthase, cytochrome P-450, or glucuronidation of phenol red. Portions of this work have appeared in abstract form [18].

MATERIALS AND METHODS

Hepatocyte cultures. Primary cultures from livers of 16 day White Leghorn chick embryos were prepared as previously described [19]. For the initial 18 hr in culture, cells were maintained in Williams E medium (Flow Laboratories, McLean, VA) to which we added 1 μg/ml insulin (Sigma Chemical Co., St. Louis, MO), 1 μg/ml triiodothyronine (Sigma), and 0.3 μg/ml dexamethasone (Elkins-Sinn, Cherry Hill, NJ). After 18 hr, cells were rinsed twice with, and

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†Abbreviations: ALA synthase, δ-aminolevulinate synthase; BSO, buthionine sulfoximine; GSH, reduced glutathione; PIA, 2-propyl-2-isopropylacetamide; and TCA, trichloroacetic acid.

then maintained in, Williams E medium containing triidothyronine and dexamethasone, but no insulin, unless otherwise indicated. When metabolic studies were extended beyond 42 hr, an additional medium change was made at this time.

Assays. GSH content was determined fluorometrically in chick embryo liver homogenates using the method of Hissin and Hilf [20]. In the cultured cells, we measured GSH in cellular extracts using a modification of a previous method [11]. Cells on 3.5 cm plates (0.3 to 0.6 mg protein) were rinsed with saline and scraped with a rubber policeman into 1 ml of 5% TCA–1 mM EDTA–0.1 N HCl. After vortexing and centrifuging (2000 *g*), 25 μl of the extract was mixed with 500 μl of 0.5 M NaH_2PO_4–5 mM EDTA (pH 8.0) and 50 μl of *o*-phthalaldehyde (1 mg/ml in methanol) and allowed to stand at 25° for 15 min; the fluorescence was measured on a Perkin–Elmer 512 spectrofluorometer with excitation wavelength 340 nm (slit width 10 nm) and emission wavelength 420 nm (slit width 20 nm). GSH standards were prepared in the same way. GSH was stable in the TCA solution for up to 3 days at 4°. The enzymatic assay for GSH of Tietze [21] was used in some experiments to corroborate the results of the fluorometric assay.

Cytochrome P-450 and glucuronidation of phenol red were determined as previously described [22, 23]. ALA synthase activity was assayed as previously described [24] measuring ALA formed in 1 hr. Proteins were assayed by the method of Lowry *et al.* [25] with bovine serum albumin as standard.

Chemicals. PIA and 2-allyl-2-isopropylacetamide were gifts from Hoffman–LaRoche Laboratory (Nutley, NJ) and were dissolved in 50% ethanol and saline respectively. 2,4,5,2′,4′,5′-Hexabromobiphenyl was a gift from G. Dannon and S. Aust (Michigan State University, East Lansing, MI), and 3,4,3′,4′-tetrachlorobiphenyl was obtained from Ultrascience (Hope, RI). The biphenyls were dissolved in dimethyl sulfoxide and added to cultures in no more than 2 μl of dimethyl sulfoxide/ml medium. β-Naphthoflavone was obtained from the Aldrich Chemical Co. (Milwaukee, WI) and was dissolved in ethanol (1.2 mg/ml). Reduced GSH, *o*-phthalaldehyde, and trisodium nitrilotriacetate were obtained from Sigma. Ferric nitrilotriacetate was prepared as described [16]. Buthionine sulfoximine was a gift from O. W. Griffith (Cornell Medical School, New York, NY) and was dissolved in saline.

RESULTS

Maintenance of GSH in cultured hepatocytes and effect of insulin. The GSH concentration in pooled 16-day-old embryo livers was 22.9 nmoles/mg protein ± 5.3 (mean ± S.D.) which is equivalent to 3.4 ± 0.8 mM since there is 150 mg protein/g wet liver. Previously [16], we reported that GSH concentrations in cultured hepatocytes were 15.3 ± 5.3 nmoles/mg protein or 2.3 ± 0.8 mM. To determine whether GSH concentrations remained constant in culture, we measured GSH in cells after 18, 42, and 66 hr (Fig. 1). In this experiment, the GSH concentration at 18 hr was 20.7 nmoles/mg protein; there was only a minimal (10–15%) decrease at 42 hr with no further decrease at 66 hr. If exposure of the cells to insulin was continued after 18 hr, GSH expressed as nmoles/mg protein decreased by 32 and 38% at 42 and 66 hr respectively. However, since the amount of protein on each culture plate increased with continuous insulin treatment (0.57 mg protein/plate compared to 0.38 mg in cultures without insulin), the actual number of nmoles of cellular GSH on each plate did not change. Therefore, GSH concentrations appeared stable in culture under either our routine culture conditions (removal of insulin after 18 hr) or with continuous insulin, although in the latter case there was less GSH/mg protein.

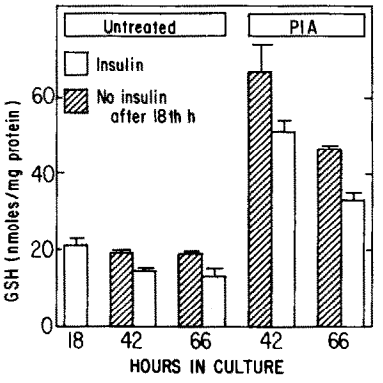

Fig. 1. GSH concentrations in chick embryo hepatocytes: effects of insulin and propylisopropylacetamide (PIA). GSH was determined in cells cultured for the times indicated. Hepatocytes were initially maintained for 18 hr in Williams E medium with dexamethasone (0.3 μg/ml), triiodothyronine (1 μg/ml), and insulin (1 μg/ml). Cells were rinsed twice with and then maintained in medium not containing insulin (hatched columns), or containing insulin (open columns). PIA (140 μM) was added to some plates 18 hr prior to harvesting. Each bar represents the mean ± S.D. of three plates.

Effects of PIA on GSH, cytochrome P-450, and glucuronidation of phenol red. PIA, a phenobarbital-like inducer of cytochrome P-450 in cultured chick hepatocytes [14], markedly increased the cellular GSH content after exposure for 18 hr (Fig. 1). The increase in GSH was greatest with PIA exposure during day 2 of culture (371% of control), but was still marked with PIA exposure during day 3 (252%). The presence of insulin during PIA exposure did not change the extent of increase over control on either day (340 and 255% of control on days 2 and 3 respectively). Therefore, in the experiments reported below, we exposed the cells to chemicals during day 2 in culture in the absence of insulin. The enzymatic method of Tietze [21] corroborated the increase in GSH caused by PIA exposure (66 compared to 20 nmoles/mg protein in untreated cells).

Figure 2 shows the increase in GSH, cytochrome P-450 content, and glucuronidation of phenol red caused by increasing concentrations of PIA. The dose responses for GSH and cytochrome P-450 were identical. The dose response for glucuronidation of phenol red was similar, but the increase plateaued at concentrations of PIA above 35 μM.

Time courses of effects of PIA and BSO on GSH. Figure 3 shows the time course of the increase in

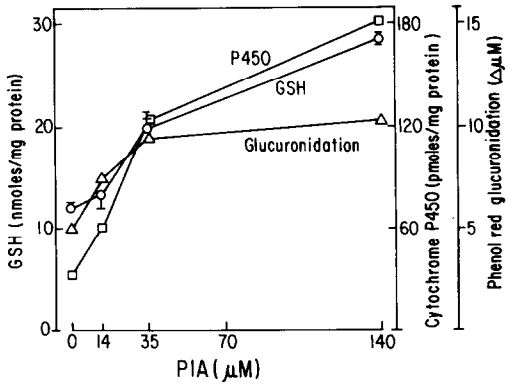

Fig. 2. Dose–response curves for action of PIA on GSH, cytochrome P-450 and phenol red glucuronidation. Hepatocytes were exposed to 140 μM PIA for 18 hr (24th to 42nd hr in culture) in medium without insulin. GSH (○) was determined in cells cultured on 3.5 cm plates (mean ± S.D. of three plates), and cytochrome P-450 (□) and phenol red glucuronidation (△) in cells cultured in 6 cm plates (mean of two plates whose values differed by <10%—no error bar shown).

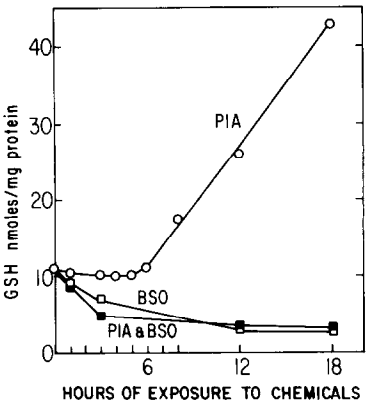

Fig. 3. Time course of GSH increase by PIA and GSH depletion by buthionine sulfoximine (BSO). Cultured hepatocytes on 3.5 cm plates during the 24th to 42nd hr were exposed to 140 μM PIA (○), 0.1 mM BSO (□) or to both chemicals (■) for various times, and the GSH was determined. Each point is the mean GSH content of two or three plates whose values differed by less than 5%.

GSH after exposure to 140 μM PIA. GSH began to increase after an initial lag period of 5 hr, but then the increase was rapid and linear. There was no initial fall in GSH. BSO, a specific inhibitor of γ-glutamylcysteine synthase [17], caused GSH to decrease by 60–70% over 3–5 hr (Fig. 3). The decreased levels persisted without any apparent toxicity to the cells (as observed by phase contrast microscopy). BSO abolished the increase in GSH caused by PIA and still depleted GSH well below control levels. These effects of BSO were also found when GSH was measured by the enzymatic assay.

Effects of different inducers of cytochrome(s) P-450 on GSH concentrations. As shown in Table 1, other phenobarbital-like inducers of cytochrome P-450, such as 2-allyl-2-isopropylacetamide [14] and 2,4,5,2′,4′,5′-hexabromobiphenyl [26], all increased levels of GSH, as well as cytochrome P-450 and glucuronidation of phenol red. In contrast, the 3-methylcholanthrene-like inducers of cytochrome P-450, β-naphthoflavone and 3,4,3′,4′-tetrachlorobiphenyl [27], did not increase either GSH levels or phenol red glucuronidation. Table 1 also shows that, in spite of an 85% depletion of GSH by BSO, the inductions by PIA of cytochrome P-450 and phenol red glucuronidation were unaffected.

Relationship between GSH and induction of ALA synthase by drugs. The results in Fig. 4 show that depletion of GSH by BSO had no effect on induction of ALA synthase. BSO alone had no effect on ALA synthase activity and did not affect either the induction of ALA synthase by PIA or the synergistic induction of ALA synthase caused by the combination of PIA and ferric nitrilotriacetate [16]. Neither β-naphthoflavone nor the combination of β-naphthoflavone with ferric nitrilotriacetate induced ALA synthase in the cultured hepatocytes (Fig. 4).

DISCUSSION

GSH concentrations in untreated cultured chick embryo hepatocytes were well maintained under our routine culture conditions. The concentrations varied from 1.8 to 3.2 mM in different culture preparations. Although the cause of these variations is not

Table 1. Effects of inducers of cytochrome(s) P-450 on GSH levels and phenol red glucuronidation*

Treatment	GSH (nmoles/mg protein)	Cytochrome P-450 (pmoles/mg protein)	Phenol red glucuronidation (Δ μM)
Saline	11.9 ± 0.7	32	4.0
PIA, 140 μM	28.4 ± 0.6	184	10.3
AIA, 140 μM	29.4 ± 0.2	109	10.1
HBB, 10 μM	22.6 ± 0.5	138	7.6
β-NF, 15 μM	10.9 ± 0.5	126†	5.5
TCB, 68 nM	11.2 ± 1.2	96†	5.0
BSO, 0.1 mM	2.3 ± 0.3	46	5.6
PIA, 140 μ M, + BSO, 0.1 mM	4.2 ± 0.2	203	11.2

* Cultured hepatocytes were maintained and assays were performed as described in Fig. 2, they were exposed to chemicals for 18 hr.

† PIA, allylisopropylacetamide (AIA) and 2,4,5,2′,4′,5′-hexabromobiphenyl (HBB) induced cytochrome(s) with a spectral peak at 452 nm, whereas both 3,4,3′,4′-tetrachlorobiphenyl (TCB) and β-naphthoflavone (β-NF) induced cytochromes(s) with a peak at 449 nm.

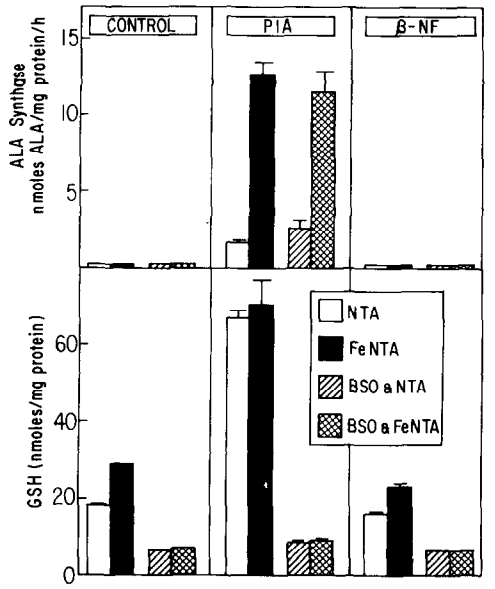

Fig. 4. Relationship between drug-induced increases in δ-aminolevulinate (ALA) synthase and GSH. Hepatocytes were exposed on 3.5 cm plates for 18 hr (during the 24th to 42 hr) to 200 μM nitrilotriacetate (NTA) alone (open bar); 100 μM ferric nitrilotriacetate (Fe^{3+}:NTA = 1:2, solid bar); BSO and NTA (hatched bar); or BSO and FeNTA (cross-hatched) bar, in the presence or absence of 140 μM PIA or 15 μM β-naphthoflavone (β-NF) as indicated. Each value represents the mean value of two plates (whose values differed by less than 5%) or of three plates (± S.D.).

clear, within each culture preparation the GSH concentration was stable for 66 hr (Fig. 1). Since maintenance of GSH in rat hepatocyte cultures has been shown to depend on levels of cysteine or methionine present in the culture medium [28], it is probable that the composition of the Williams E medium that we use (containing 0.3 mM cysteine, 0.08 mM cystine and 0.1 mM methionine) is important for maintenance of GSH in our cells. GSH concentrations reported in cultured rat hepatocytes [28] were higher (27–35 nmoles/mg protein) than the levels we found in the chick embryo hepatocytes (15.3 ± 5.3 nmoles/mg protein). However, GSH in the rat hepatocytes was not measured beyond 24 hr. In several human tumor cells lines [29, 30], GSH concentrations were similar (14–15 nmoles/mg protein) to those observed in the chick hepatocytes.

A major finding of this study is that PIA and other phenobarbital-like inducers of cytochrome P-450 increased GSH concentrations in cultured hepatocytes, whereas the 3-methylcholanthrene-like inducers did not (Table 1). The mechanism for the increase in GSH could be due to increased synthesis, decreased utilization, or decreased efflux of GSH out of the cell. Because BSO, a specific and potent inhibitor of GSH synthesis [17], rapidly depleted GSH even in the presence of PIA (Fig. 3), it appears most likely that the GSH increase caused by PIA is mainly due to increased synthesis. If PIA, instead, blocked GSH utilization or efflux, one would have expected GSH levels to be maintained longer in the presence of both BSO and PIA. The increase in GSH does not appear to be a "rebound" phenomenon secondary to GSH depletion, since even at early time points after adding PIA, GSH concentrations did not decrease (Fig. 3).

In rats, phenobarbital and 3-methylcholanthrene have been reported to transiently increase hepatic GSH content by 30% [9, 10], but neither agent caused the sustained rise in GSH that we observed in the cultured chick hepatocytes. In studies with isolated, perfused rat liver, phenobarbital pretreatment was shown to markedly enhance biliary efflux of GSH, whereas 3-methylcholanthrene pretreatment did not [10]. One possible interpretation, therefore, is that, in the phenobarbital-treated rat liver, more GSH was synthesized than in control or 3-methylcholanthrene-treated rat, but hepatic levels did not increase substantially because the GSH effluxed across the biliary canaliculus. In cultured chick hepatocytes, neither biliary nor sinusoidal efflux seems to be a major route of GSH disposition since only 10–12% as much total glutathione (GSH + GSSG) was recovered in the culture medium as was present within the cells after 18 hr of incubation (unpublished data). However, the recovery of GSH and GSSG in culture medium could be misleadingly low due to formation of mixed disulfides between GSH and medium components. Regardless of this uncertainty, it appears that, in both the intact rat liver and the cultured chick hepatocyte, phenobarbital-like inducers of cytochrome P-450 have an effect on GSH metabolism which the 3-methylcholanthrene-like inducers do not have.

The effect of insulin on GSH levels in the cultured chick hepatocytes was studied because of a recent report suggesting that insulin deficiency in diabetic rats was associated with increased hepatic GSH concentrations [31]. We found that omission of insulin from the culture after 18 hr did not cause a subsequent increase in GSH (Fig. 1). Although retaining insulin in the culture seemed to cause a fall in GSH when the concentration was expressed as nmoles/mg protein, the total content of GSH/plate was unaffected by insulin. Since insulin caused fibroblast proliferation and enlargement of the hepatocytes in the chick culture (unpublished data), the decrease in GSH/mg protein seen with continuous insulin exposure probably reflects the relatively low GSH content of the fibroblasts and/or an increase in hepatocyte protein, but not a change in GSH content of the hepatocytes. Therefore, it does not appear that the continuous presence of insulin is essential in the regulation of hepatocyte GSH under our culture conditions.

BSO rapidly depleted cellular GSH levels in the cultured chick hepatocytes to 15–30% of initial values and the depletion lasted for over 18 hr. The time course of the GSH depletion and the dose of BSO required were similar to what was reported previously in cultured human lymphoid cell lines [29] and tumor cell lines [30]. Although 25 μM diethylmaleate has been used in the past to deplete GSH in the cultured chick hepatocytes [11], BSO seems a better agent to use since the GSH depletion with diethylmaleate is transient [11] and 25 μM diethylmaleate was often toxic (unpublished data).

GSH depletion in the cultured chick hepatocytes

was not associated with any evidence of toxicity since the cells appeared normal morphologically and were still fully responsive for induction of cytochrome P-450, glucuronyltransferase for phenol red, and ALA synthase (Table 1 and Fig. 4).

Although some role for GSH has been implicated in the induction by 2-allyl-2-isopropylacetamide of hepatic ALA synthase in rats and in cultured hepatocytes [11, 12], in the present study depletion of GSH using BSO had no effect on the induction of ALA synthase either by PIA, the propyl analogue of 2-allyl-2-isopropylacetamide, or the combination of PIA and iron (Fig. 4 and Ref. 16). Therefore, our present data do not support an obligatory role of GSH in induction of ALA synthase by drugs.

In summary, we observed an increase in GSH in cultured chick embryo hepatocytes with the phenobarbital-like inducers of cytochrome P-450. However, this increase was completely separable from the other effects of these inducers since depletion of GSH had no effect on the induction of cytochrome P-450, glucuronidation of phenol red, or ALA synthase. We surmise that the mechanism of the GSH increase may be part of a pleiotropic response caused by the phenobarbital-like inducers of cytochrome P-450.

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