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RAPID COMMUNICATION

EFFECTS OF ALTERED CALCIUM HOMEOSTASIS ON THE EXPRESSION OF GLUTATHIONE S-TRANSFERASE ISOZYMES IN PRIMARY CULTURED RAT HEPATOCYTES

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Abstract - The effects of altered Ca^{2+} homeostasis on glutathione *S*-transferase (GST) isozyme expression in cultured primary rat hepatocytes were examined. Isolated hepatocytes were cultured on Vitrogen substratum in serum-free modified Chee's essential medium and treated with Ca^{2+} ionophore A23187 at 120 hr post-plating. GST activity increased slightly, albeit significantly, in a concentration-dependent manner in A23187-treated hepatocytes relative to untreated controls. Western blot analysis using GST class α and μ specific antibodies showed an ~1.6- and 1.5-fold increase in the class α, Ya and Yc subunits, respectively, whereas no significant increase (~1.2-fold) in class μ GST expression was observed following A23187 treatment. Northern blot analysis revealed an ~5-fold increase in GST class α and an ~7-fold increase in class μ GST mRNA levels in ionophore-treated hepatocytes compared to untreated cells. Results of the Western and Northern blot analyses of the ionophore-treated hepatocytes were compared with those obtained for *tert*-butyl hydroperoxide-treated cells. Immunoblot analysis showed a significant increase in the expression of GST class α, Ya and Yc subunits, ~1.8- and 1.7-fold, respectively, for *tert*-butyl hydroperoxide-treated hepatocytes as compared to controls, with little or no increase in class μ GSTs. Northern blot analysis showed ~3- and 2-fold increases, respectively, in class α and μ GST mRNA levels, following the *tert*-butyl hydroperoxide treatment. The results of the present investigation show that alterations in Ca^{2+} homeostasis produced by either Ca^{2+} ionophore A23187 or *tert*-butyl hydroperoxide treatment of hepatocytes enhanced the expression of GST isozymes in primary cultured rat hepatocytes.

The glutathione *S*-transferases (GSTs)† (EC 2.5.1.18) are a family of multifunctional enzymes which play an important role in catalyzing the conjugation of a variety of electrophilic metabolites, including carcinogens and cytotoxic drugs, to glutathione [1]. The GSTs exist as homo- or heterodimers with subunit molecular weights ranging from 22 to 27.5 kDa. To date, ten cytosolic GST subunits have been identified and these have been classified into three gene families on the basis of their primary structures, namely α (subunits 1, 2, 8 and 10), μ (subunits 3, 4, 6, 9 and 11) and π (subunit 7) [2]. Similar to other xenobiotic metabolizing enzymes, the hepatic GSTs are known to be differentially expressed among various species [3] and are inducible in rat liver by a large number of chemicals including phenobarbital (PB), polycyclic aromatic compounds, dietary factors and compounds producing oxidative stress [4-6].

Reduced glutathione and thiols are very important in regulating inner membrane permeability by maintaining intramitochondrial and cellular -SH groups in a reduced state. Certain proteins are highly sensitive to changes in cellular thiols, such as the Ca^{2+}-dependent ATPases which serve as membrane bound Ca^{2+} pumps to maintain low levels of cytoplasmic Ca^{2+} [7]. An alteration in Ca^{2+} homeostasis is believed to trigger cell injury and, if not reversed, will eventually lead to cell death. Cellular-thiol and Ca^{2+} status appear to be closely related and an imbalance in one likely affects the other. In view of this close relationship between Ca^{2+} homeostasis and reduced cellular thiol levels, the present study was designed to examine the expression of GST isozymes following treatment of primary cultured rat hepatocytes with the ionophore A23187 or with *tert*-butyl hydroperoxide (tBHP). The results of the present study demonstrate that tBHP addition or alterations in hepatocyte intracellular Ca^{2+} homeostasis produced by A23187 enhanced the expression of class α and μ GSTs as evidenced by Western and Northern blot analyses.

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†Abbreviations: GST, glutathione *S*-transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CDNB, 1-chloro-2,4-dinitrobenzene; PBS, phosphate-buffered saline; BSA, bovine serum albumin; BCA, bicinchoninic acid; DMSO, dimethyl sulfoxide; tBHP, *tert*-butyl hydroperoxide; PB, phenobarbital; and 3-MC, 3-methylcholanthrene.

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MATERIALS AND METHODS

Chemicals. Vitrogen was obtained from the Collagen Corp. (Palo Alto, CA) and collagenase (type I) from the Worthington Biochemical Corp. (Freehold, NJ); calcium ionophore A23187 (Bromo), tBHP, bovine serum albumin (BSA), bicinchoninic acid (BCA) and tricine were purchased from the Sigma Chemical Co. (St. Louis, MO). Chee's essential medium was purchased from Gibco-BRL (Grand Island, NY) and 1-chloro-2,4-dinitrobenzene (CDNB) from the Aldrich Chemical Co. (Milwaukee, WI). Oligonucleotides, sequence-specific to GST α or μ, were obtained from Research Genetics (Huntsville, AL). [γ-^{32}P]dATP (>5000 Ci/nmol) was purchased from Amersham International (Arlington Heights, IL). Prestained SDS-PAGE standards were purchased from BioRad Laboratories (Richmond, CA). All other chemicals were of the highest grade commercially available.

Isolation of hepatocytes and treatment in culture. Rat hepatocytes were isolated from adult male Sprague-Dawley rats (weighing 180-200 g) purchased from Harlan Laboratories Inc. (Indianapolis, IN), using slight modifications of the double collagenase perfusion procedure originally described by Seglen [8]. Approximately 3.5 to 6.5 x 10^8 cells as counted by hemocytometer, were routinely recovered from an adult rat liver with 95% viability as assessed by trypan blue exclusion. The isolated hepatocytes were suspended in modified Chee's essential medium and layered on Vitrogen-coated 100 mm tissue culture dishes (1 x 10^6 cells/mL) [9]. Cells were allowed to attach to the dishes for 4 hr at 37° in an atmosphere of 95% air, 5% CO_2 with a relative humidity of 98%. Unattached cells were aspirated after 4 hr and fresh Chee's modified medium was added to the cultured hepatocytes. Medium was subsequently renewed every 48 hr. Cells cultured for 120 hr were treated with Ca^{2+} ionophore A23187, dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.5 μM for 10 min, followed by replacement with Chee's modified essential medium, and were allowed to remain in culture for an additional 24 hr (144 hr). Cells were treated with tBHP dissolved in DMSO (10 μM) at 120 hr and allowed to remain in culture for an additional 24 hr. Control cell cultures were treated with an equal amount of DMSO for the corresponding period of time to eliminate the vehicle effects. The cells were then harvested from each group and cytosolic/microsomal fractions were prepared by centrifuging postmitochondrial supernatant at 105,000 *g* for 90 min at 4°. Protein content of the cytosolic fraction was determined using a BCA reagent kit as per the manufacturer's (Sigma Chemical Co.) instructions with BSA as the standard.

Metabolic assays and Western blot analysis. Cytosolic GST activity was assayed by measuring the rate of GSH conjugation to CDNB at 340 nm on a COBAS FARA Centrifugal Analyzer essentially according to the procedure of Habig *et al.* [10]. Cytosolic proteins were resolved by SDS-PAGE using 12% polyacrylamide gels and stained using Coomassie Brilliant Blue [11]. Immunoblot analysis was performed according to published procedures using class specific antibodies, generated in our laboratory [4] against GST class α and μ isozymes. To examine the relative changes in protein bands, the immunoblots were scanned using a Molecular Dynamics model 300 Series scanning laser densitometer (Sunnyvale, CA).

Northern blot analysis. Poly(A)$^+$ mRNA was isolated from total RNA using an oligo(dT)-cellulose column, according to the method of Jacobson [12]. Samples were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde and transferred to nitrocellulose Durablot (Sigma) membranes. Hybridizations were performed at 54° for 18-24 hr in prehybridization solution containing 2 x 10^6 cpm/mL of oligonucleotide probe. The sequences of the oligonucleotides employed in these experiments were chosen from the primary sequence data for GST α, Ya subunit (5'-AATGGCTCTGGTCTGCACCAGCTTCAT-3') [13] and GST μ, Yb subunit (5-'GACGTCCCAGTATCCCAGTGTCAT AGG-3') [14]. Filters were washed and RNA bands were visualized by exposing the nitrocellulose membranes at -80° to Kodak X-Omat AR film. Examination of mRNA loaded onto the blot was accomplished by hybridization of stripped membranes with ^{32}P-end labeled poly(dT)$_{16}$ and loading was assessed using scanning laser densitometry (data not shown).

Statistical analysis. Catalytic activity data were analyzed by Student's t-test or by one- or two-way ANOVA as appropriate. Results were considered to be significant at $P<0.05$. Xenobiotic-treated cultured hepatocytes were compared to their respective controls. The values are means $\pm$ SEM for three independent experiments performed in triplicate.

RESULTS

Effect of calcium ionophore A23187 on hepatocyte viability and GST metabolic activity. Since calcium plays a crucial role in cell injury leading to cell death, the time- and concentration-dependence of Ca^{2+} ionophore A23187 treatment on cultured hepatocyte viability and GST expression were examined. At 120 hr post-plating of cells, the

hepatocytes were exposed to 0.01, 0.1, 0.5, 1.0 or 10 μM concentrations of A23187 for 2, 4, 6, 8, 10, 15 or 20 min in order to determine optimal conditions for viability and enhanced GST expression. Following these times, the ionophore-containing medium was aspirated from the cells and the hepatocytes were incubated for an additional 24 hr in modified Chee's essential medium. Cells were examined under the phase contrast microscope and viability was checked by trypan blue exclusion and lactate dehydrogenase release. With the exception of the 10 μM ionophore concentration, none of the ionophore concentrations were found to be cytotoxic to the hepatocytes under the experimental conditions employed (data not shown). Metabolic activity of the GSTs, when measured using CDNB as a substrate, was increased significantly (~15 - 39%) in the 0.5 and 1.0 μM ionophore-treated hepatocytes as compared to untreated hepatocytes (Fig. 1). A 10-min incubation with 0.5 μM ionophore was chosen for monitoring induction of GSTs in response to altered Ca^{2+} homeostasis.

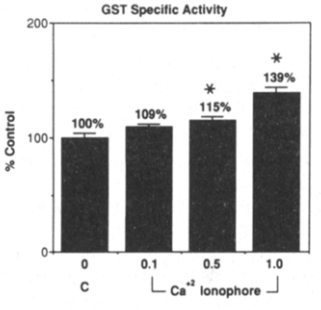

Fig. 1. Effect of A23187 on GST metabolic activity in primary cultured rat hepatocytes. Hepatocytes at 120 hr were treated with 0.1, 0.5 or 1.0 μM Ca^{2+} ionophore A23187 for 10 min after which the ionophore-containing medium was replaced and the cells were allowed to remain in culture for an additional 24 hr. The cells were then harvested, the cytosolic fractions isolated, and the metabolic activity towards CDNB was monitored. The control value for activity was 37 nmol of conjugate/min/mg protein (100%). The values are the means ± SEM for three independent experiments in triplicate. *Values are significantly different ($P<0.05$) from the untreated control hepatocytes.

SDS-PAGE and immunoblot analyses. SDS-PAGE analysis of cytosolic proteins from A23187- and tBHP-treated cells is shown in Fig. 2A. An increase in the intensity of class α (Ya/Yc) protein bands was observed in ionophore-(lane 4) and tBHP- (lane 5) treated hepatocytes relative to untreated hepatocytes (lane 3). Immunoblot analysis of the cytosolic proteins (Fig. 2B) with class-specific antibodies generated in our laboratory revealed ~1.6- and 1.5-fold increases in the class α Ya and Yc bands, respectively, following ionophore treatment (lane 4), while ~1.8- and 1.7-fold increases in the Ya and Yc bands were observed following tBHP treatment (lane 5) relative to control hepatocytes (lane 3) based on scanning laser densitometry. Analysis of the class μ (Yb) GST (Fig. 2C) expression revealed that GST μ protein levels failed to increase significantly (e.g. ~1.2-fold) following either ionophore (lane 4) or tBHP (~1.1-fold) (lane 5) treatment relative to control levels (lane 3).

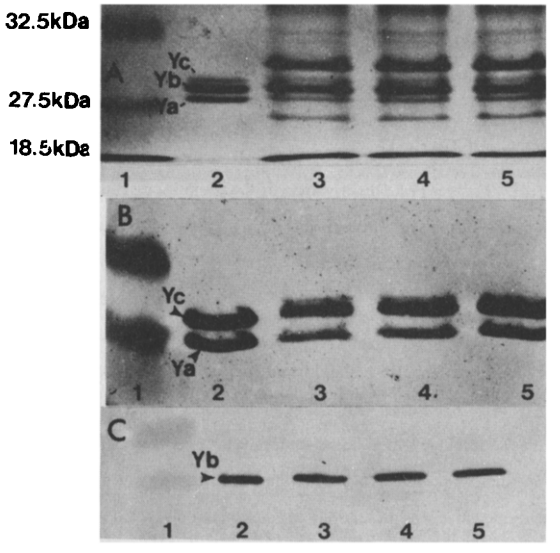

Fig. 2. SDS-PAGE and immunoblot analyses of cytosolic proteins (10 μg/lane) from primary cultured rat hepatocytes treated with Ca^{2+} ionophore A23187 or tBHP. At 120 hr, cultured hepatocytes were treated with Ca^{2+} ionophore A23187 (0.5 μM) or tBHP (10 μM). Gels were stained with Coomassie Brilliant Blue for SDS-PAGE analysis (**panel A**) or transferred electrophoretically to nitrocellulose membranes for immunoblot analysis. Immunoblots were probed with class specific antibodies for GST α (**panel B**) or GST μ (**panel C**). **Lane 1**, prestained molecular weight markers: lysozyme (18.5 kDa), soybean trypsin inhibitor (27.5 kDa), carbonic anhydrase (32.5 kDa); **lane 2**, affinity purified GST proteins; **lane 3**, cytosol from 144 hr untreated cultured rat hepatocytes; **lane 4**, cytosol from cultured rat hepatocytes treated with Ca^{2+} ionophore A23187 (Bromo); **lane 5**, cytosol from cultured rat hepatocytes treated with tBHP.

Effect of ionophore on GST mRNA levels in cultured rat hepatocytes. Northern blot analysis was performed on poly(A)$^+$ RNA isolated from cultured rat hepatocytes using oligonucleotide probes complementary to GST class α and

GST class μ RNA sequences. Northern blot analysis of the class α GSTs (Fig. 3A) revealed that hybridization signals for GST α mRNA levels were increased ~5.0-fold in ionophore-treated cells (lane 2) while an ~3.0-fold increase was observed for class α GST mRNA levels in tBHP-treated hepatocytes (lane 3) compared to untreated hepatocytes (lane 1). An ~7-fold increase was noted in class μ GST mRNA levels (Fig. 3B) in ionophore-treated hepatocytes (lane 2), while tBHP-treated hepatocytes (lane 3) showed an ~2-fold increase in hybridization signals, compared to untreated cells. Densitometric analysis of the Northern blot revealed that hybridization signals for the α and μ class GSTs were increased ~5- and 7-fold, respectively, in ionophore-treated cultured rat hepatocytes, whereas GST α and μ mRNA levels were elevated ~3- and 2-fold, respectively, in tBHP-treated cells as compared to untreated cells. The Northern blot data suggest that A23187-treated hepatocytes were slightly more responsive in the enhanced expression of α and μ class GST mRNAs as compared to tBHP-treated hepatocytes.

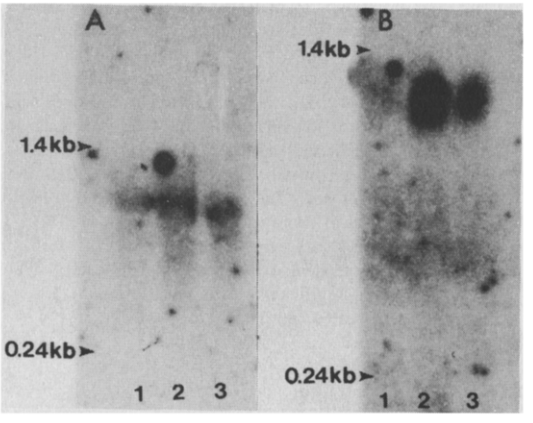

Fig. 3. Northern blot analysis of the poly(A)$^+$ RNA for GST α **(panel A)** and GST μ **(panel B)** isolated from cultured rat hepatocytes treated with A23187 or tBHP. Details of the treatments are described in the Materials and Methods. Quantitation of mRNA loading was done by stripping the probes from nitrocellulose membranes and rehybridizing with a ^{32}P- labeled poly(dT)$_{16}$ probe (data not shown). **Lane 1,** poly(A)$^+$ RNA from untreated cultured rat hepatocytes at 144 hr; **lane 2,** poly(A)$^+$ RNA from A23187- (0.5 μM) treated cultured rat hepatocytes at 144 hr; **lane 3,** poly(A)$^+$ RNA from tBHP- (10 μM) treated cultured rat hepatocytes.

DISCUSSION

The results of the present study demonstrate that Ca^{2+} ionophore A23187 and tBHP enhanced the expression of GST isozymes, suggesting that this process occurs in response to alterations in cellular Ca^{2+} homeostasis. The observation that both GST class α and μ mRNAs were elevated by ionophore or tBHP treatment while only the class α GST (Ya/Yc) protein levels were increased significantly is in accord with previous observations from induction studies employing PB or 3-methylcholanthrene (3-MC) *in vivo* [3, 4, 15]. In general, the rates of transcription of rat hepatic GST genes following treatment with PB or 3-MC are less than those associated with other enzymes, such as CYP2B1/2B2 or CYP1A1/1A2. The rate of transcription of the GST Ya/Yc and Yb genes was reported to be elevated ~5-fold at 8 and 6 hr, respectively, following PB administration, whereas 3-MC administration increases the transcriptional rate of the GST Ya/Yc gene 8-fold at 16 hr and the transcriptional rate of the Yb gene ~5-fold at 6 hr following treatment [16]. Although these inducers increase GST α and μ class mRNAs ~5- to 10-fold [16, 17], only an ~1.5- to 2-fold increase in protein levels occurs. In addition, differential effects on Ya and Yc expression were also noted with Ya showing the greatest relative increase in levels following 3-MC treatment. Transcriptional activation of the glutathione *S*-transferase α gene by phenolic antioxidants and oxidants such as H_2O_2, *tert*-butyl hydroquinone and 3,5 di-*tert*-butylcatechol, acting through the antioxidant responsive element (ARE), has been reported by Rushmore *et al.* [6]. The ARE core-sequence has been shown recently to contain a high affinity recognition motif (AP-1-like sequence) for a transacting factor(s) [18]. AP-1-like binding sites have been reported to be present in the murine GST Ya subunit gene [19]. Since altered Ca^{2+} homeostasis can also result in enhanced expression of the proto-oncogenes c-fos and c-jun, perhaps this accounts for, at least in part, the activation of the GST genes in hepatocytes following treatment with calcium ionophore. The present data suggest that altered Ca^{2+} homeostasis may be a primary initial step in a cascade of cellular events which result in activation of GST genes and enhanced GST expression. Further work is in progress to study the mechanism(s) involved in regulating the expression of glutathione *S*-transferase genes in terms of altered calcium homeostasis, the calcium messenger system and early-intermediate transcription factor expression.

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REFERENCES

1. Jakoby WB and Habig WH, Glutathione transferases. In: *Enzymatic Basis for Detoxication* (Ed. Jakoby WB) Vol. II, pp. 63-93. Academic Press, New York, 1980.

2. Mannervik B, The isozymes of glutathione transferase. *Adv Biochem Rel Areas Mol Biol* **57**:357-417, 1985.

3. Primiano T, Kim SG and Novak RF, Differences between rats and rabbits in hepatic cytosolic glutathione *S*-transferase expression in response to nitrogen heterocycle and other inducers. *Toxicol Appl Pharmacol* **113**:64-73, 1992.

4. Primiano T and Novak RF, Enhanced expression, purification, and characterization of a novel class α glutathione *S*-transferase isozyme appearing in rabbit hepatic cytosol following treatment with 4-picoline. *Toxicol Appl Pharmacol* **112**:291-299, 1992.

5. Kretzschmar M and Klinger W, The hepatic glutathione system - influences of xenobiotics. *Exp Pathol* **38**:145-164, 1990.

6. Rushmore TH, Morton MR and Pickett CB, The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J Biol Chem* **266**:11632-11639, 1991.

7. Olafsdottir K, Pascoe GA and Reed DJ, Mitochondrial glutathione status during Ca^{2+} ionophore-induced injury to isolated hepatocytes. *Arch Biochem Biophys* **263**:226-235, 1988.

8. Seglen PO, Autophagy and protein degradation in rat hepatocytes. In: *Isolation, Characterization and Use of Hepatocytes* (Eds. Harris RA and Cornell NW) pp. 153-162. Elsevier, New York, 1982.

9. Dwivedi RS, Primiano T and Novak RF, Enhanced expression of class α and π glutathione *S*-transferase (GSTs) genes by phenobarbital (PB) and 3-methylcholanthrene (3-MC) in primary rat hepatocyte culture. *Biophys J* **61**:A456, 1992.

10. Habig WH, Pabst MJ and Jacoby WB, Glutathione *S*-transferases: The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**:7130-7139, 1974.

11. Laemmli UK, Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* **227**:680-685, 1970.

12. Jacobson A, Purification and fractionation of poly(A)$^+$ RNA. In: *Methods in Enzymology* (Eds. Berger SL and Kimmel AR), pp. 254-261. Academic Press, Boca Raton, Fl, 1987.

13. Telakowski-Hopkins CA, Rodkey JA, Bennett CD, Lu AYH and Pickett CB, Rat liver glutathione *S*-transferases: Construction of a cDNA clone complementary to a Yc mRNA and prediction of the complete amino acid sequence of a Yc subunit. *J Biol Chem* **260**:5820-5825, 1985.

14. Lai H-CJ and Tu C-PD, Rat glutathione *S*-transferases supergene family. Characterization of an anionic Yb subunit cDNA clone. *J Biol Chem* **261**:13793-13799, 1986.

15. Igarashi T, Irokawa N, Ono S, Ohmori S, Ueno K, Kitagawa H and Satoh T, Difference in the effects of phenobarbital and 3-methylcholanthrene treatment on subunit composition of hepatic glutathione S-transferase in male and female rats. *Xenobiotica* **17**:127-137, 1987.

16. Ding VD-H and Pickett CB, Transcriptional regulation of rat liver glutathione *S*-transferase genes by phenobarbital and 3-methylcholanthrene. *Arch Biochem Biophys* **240**:553-559, 1985.

17. Pickett CB, Donohue AM, Lu AYH and Hales BF, Rat liver glutathione *S*-transferase B: The functional mRNAs specific for the Ya Yc subunits are induced differentially by phenobarbital. *Arch Biochem Biophys* **215**:539-549, 1982.

18. Nguyen T and Pickett CB, Regulation of rat glutathione *S*-transferase Ya subunit gene expression. *J Biol Chem* **267**:13535-13539, 1992.

19. Friling RS, Bergelson S and Daniel V, Two adjacent AP-1 like binding sites from the electrophile-responsive element of the murine glutathione *S*-transferase Ya subunit gene. *Proc Natl Acad Sci USA* **89**:668-672, 1992.