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Rapid and Specific Efflux of Glutathione before Hepatocyte Injury Induced by Hypoxia

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Hypoxia caused the efflux of glutathione (GSH) from hepatocytes before membrane lysis occurred. Dithiothreitol (DTT), a thiol reductant, greatly increased the hypoxia induced GSH efflux as well as the subsquent membrane lysis. The NADH generating nutrients sorbitol and β-hydroxybutyrate as well as ethanol also enhanced hepatocyte GSH efflux and cell injury, whereas on the other hand NADH oxidising metabolic intermediates, e.g., acetoacetate or the artificial electron acceptor methylene blue, partly prevented GSH efflux and membrane lysis. Hypoxia induced GSH efflux and cytotoxicity were also prevented by oxypurinol, a xanthine oxidase inhibitor, as well as by the polyphenolic antioxidant quercetin, suggesting that reactive oxygen species contributed to the GSH efflux and cell lysis. The above results suggest that reductive stress caused by hypoxia activates the redox sensitive sinusoidal GSH transporter that is likely responsible for the GSH efflux before membrane lysis ensues.

Hypoxic injury which results from ischemia is of major clinical importance especially in organ transplantation and cardiac reperfusion injury. The biochemical changes that precede cell injury by hypoxia include a depletion in ATP levels (1), an increase in cytosolic calcium and sodium levels (2, 3), phospholipase A2 activation, and membrane phospholipid degradation (4). Recently, we have shown that hepatocyte injury caused by hypoxia or mitochondrial respiratory inhibitors occurs following a period of marked reductive stress as reflected by an increase in the hepatocyte lactate/pyruvate ratio and β-hydroxybutyrate/acetoacetate ratio. Furthermore, increasing the reductive stress with NADH generators results in increased cytotoxicity whereas offsetting the reductive stress with NADH oxidisers averts cytotoxicity. Cytotoxicity was also prevented by desferoxamine, a ferric ion chelator and ROS scavengers. This reductive stress resulted in the intracellular release of iron and formation of reactive oxygen species (ROS) (5, 6). GSH depleted hepatocytes were also much more susceptible to hypoxia induced cell injury (5). The results presented in this communication indicate that hypoxia induced reductive stress and ROS results in extensive GSH efflux before cytotoxicity ensued.

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MATERIALS AND METHODS

Acetoacetate, dithiothreitol, 1-fluoro-2,4-dinitrobenzene, GSH, β-hydroxybutyrate, iodoacetic acid, methylene blue, sorbitol and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO). 1-Bromoheptane, meta phosphoric acid were purchased from Aldrich Chemical Co. (Milwaukee, WS). Collagenase (from Clostridium histoliticum) was purchased from Worthington Biochemicals Corporation (Freehold, NJ). Hepes, and bovine serum albumin were obtained from Boehringer-Mannheim (Montreal, Canada). All other chemicals used were of the highest purity that was commercially available.

Hepatocytes were isolated from adult male Sprague-Dawley rats (220-250g), by collagenase perfusion of the liver as described by Moldeus et al (7). Cell viability was measured by Trypan blue exclusion method, and the hepatocytes used in this study were at least 85-90% viable. Cells were preincubated at a concentration of 1×10^6 cells/ml in rotating round-bottom flasks at 37°C in Krebs-Henseleit buffer, pH 7.4, supplemented with 12.5 mM Hepes under an atmosphere of 95% O_2, 5% CO_2. The cells were then incubated under an atmosphere of 5% CO_2/95% N_2 to develop a hypoxic environment. Oxygen electrode measurement showed that the oxygen concentration in the cellular medium was $<0.01\%$ (1.0 - 1.5 μM) 40 min after the hepatocytes were added to the flasks under the N_2 atmosphere. Stock solutions of chemicals and nutrients were made either in incubation buffer or in DMSO and added to the hepatocyte suspensions at the indicated time points. The DMSO vehicle (0.2%) alone neither depleted GSH nor caused cytotoxicity.

Cells were removed from the incubation mixture by rapid centrifugation (microcentrifuge) at various time intervals. Supernatents (incubation media) were transferred to another set of tubes and the cell pellet was washed once with the incubation buffer and then resuspended in 0.8 ml of incubation buffer. The total amount of GSH and GSSG in the cells as well as in the medium was measured in deproteinized samples (5% metaphosphoric acid) after derivatization with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene, by high-pressure liquid chromatography, using a μBondapak NH_2 column (Water Associates, Milford, MA) (8). GSH and GSSG were used as external standards. A Waters 6000A solvent Delivery system, equipped with a Modal 660 solvent programmer, a Wisp 710A automatic injector, and a Data Module were used for analysis.

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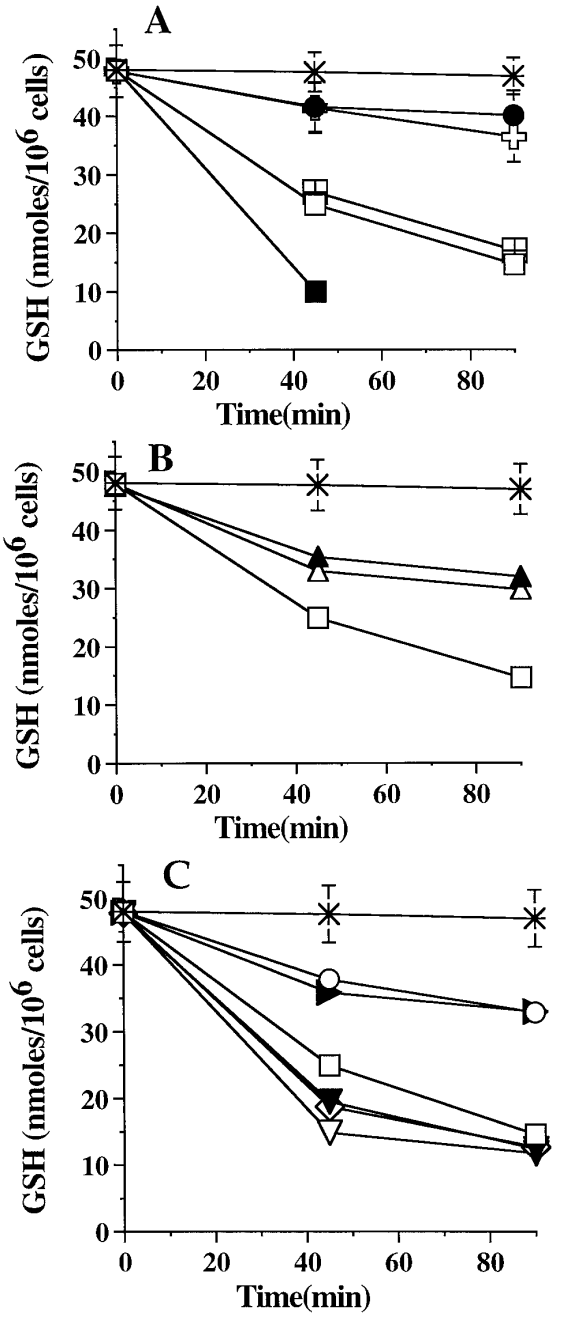

FIG. 1. Modulation of hepatocyte GSH levles under hypoxia. Hepatocytes (10^6cells/ml) were incubated under an atmosphere of 95% N_2/ 5%CO_2 with the following: Aerobic (*), Hyoixia (□); (A) + L-Cystine (0.5 mM) (●), + DTT (3.0 mM) (■), + Sulfobromophthalein (0.1 mM) (⊞), Aerobic + DTT (3.0 mM) (✣); (B) + Quercetin (0.1 mM) (▲), Oxypurinol (0.05 mM) (△) and (C) + Methylene blue (0.085 mM) (○), + Acetoacetate (10 mM) (▶), + Sorbitol (10 mM) (▼), + Ethanol (40 mM) (◇), + β-Hydroxybutyrate (10 mM) (▽). GSH levels were determined by HPLC analysis as described under Materials and Methods. Values are expressed as means ± S. E. of at least three separate experiments.

RESULTS AND DISCUSSION

As shown in Figure 1 and 2, hypoxia caused hepatocyte GSH efflux well before cytototoxicity ensued (Table 1). The decrease in intracellular GSH level caused by the hypoxic environment (Figure 1) was accompanied by a similar amount of GSH appearing in the medium (Figure 2) and could therefore be attributed to GSH efflux. Previously we showed that hypoxia markedly increased the lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratio in isolated hepatocytes which was sustained until membrane lysis ensued. Furthermore, addition of NADH generating nutrients- sorbitol and β-hydroxybutyrate and ethanol further increased the $NADH/NAD^+$ ratio

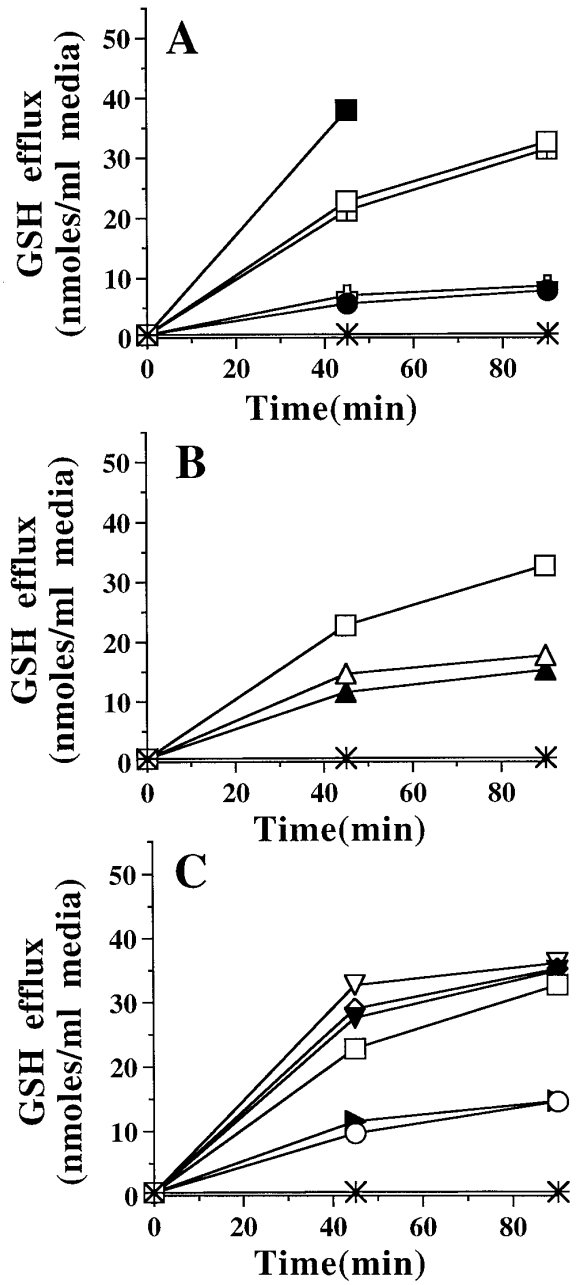

FIG. 2. Modulation of hepatocyte GSH efflux under hypoxia. Details as in Figure 1.

TABLE 1

Modulation of Hypoxia Induced Cytotoxicity in Isolated Rat Hepatocytes

Addition	% Trypan blue uptake at time (min)		
	60	120	180
Aerobic hepatocyte	18 ± 2	18 ± 3	20 ± 3
Hypoxic hepatocytes	34 ± 4	51 ± 5	86 ± 8*
+ DTT (3 mM)	45 ± 4	69 ± 6	100†
+ L-Cystine (0.5 mM)	27 ± 3	30 ± 4	53 ± 5†
+ L-Methionine (5 mM)	30 ± 4	32 ± 4	41 ± 4†
+ Sulfobromophthalein (0.1 mM)	37 ± 3	55 ± 5	84 ± 6
+ Quercetin (0.1 mM)	20 ± 3	23 ± 4†	34 ± 4†
+ Oxypurinol (0.05 mM)	24 ± 3	28 ± 4	42 ± 4†
+ Sorbitol (10 mM)	45 ± 5	74 ± 7	100†
+ β-Hydroxybutyrate (10 mM)	43 ± 4	71 ± 6	100†
+ Ethanol (40 mM)	61 ± 6	80 ± 7†	100†
+ Acetoacetate (10 mM)	27 ± 3	35 ± 4	44 ± 5†
+ Methylene blue (0.085 mM	31 ± 3	37 ± 4	49 ± 5†

Note. Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer (pH 7.4) at 37°C. Hypoxic environment was maintained as described in "Materials and Methods." Cell viability was determined as the percentage of cells taking up Trypan blue. Values are expressed as means of three separate experiments (± S.D.).

* Significant difference in comparison with aerobic control ($p<0.001$).

† Significant difference in comparison with hypoxic control ($p<0.001$).

which markedly accelerated subsequent cell injury whereas NADH oxidizing metabolic intermediates acetoacetate or the artificial electron acceptors methylene blue restored the NADH/NAD^+ ratio and prevented hypoxic injury (5). Hepatocyte GSH also prevents hypoxic injury as GSH depleted hepatocytes were much more susceptible (5). Reductive stress also seems to contribute to GSH efflux from hepatocytes as NADH generating nutrients accelerated GSH efflux whereas NADH oxidizing nutrients or artificial electron acceptors inhibited GSH efflux (Figure 1, 2). Others have reported that 40% of hepatocyte GSH depletion by the mitochondrial inhibitors- cyanide and antimycin A was attributed to GSH efflux and was hypothesized to result from ATP depletion (9). The nutrients or chemical agents at concentrations used in this study did not affect hepatocyte viability nor caused significant GSH efflux under aerobic conditions (results not shown).

Kaplowitz et al (10, 11) have shown that normal rat hepatocytes have a sinusoidal GSH transporter located in the basolateral membrane which governs GSH efflux into blood, a canalicular GSH transporter for GSH efflux into the bile and an inner mitochondrial membrane GSH transporter for transporting cytosolic GSH into the mitochondria. The canalicular transporter was inhibited by sulfobromophthalein (12) whereas the sinusoidal transporter was activated by dithiothreitol and inhibited by L-cystine or methionine (10, 11) indicating that the thiol-disulfide status of certain sulfhydryl group(s) of the transporter modulated hepatic sinusoidal GSH transport. Our finding that hypoxia causes a marked increase in GSH effflux and dithiothreitol (DTT) markedly increased hypoxia induced cytotoxicity and GSH efflux whereas cystine or methionine were cytotoprotective and prevented GSH efflux (Table 1, Figure 1, 2), suggests that the sinusoidal GSH transporter is likely responsible for the GSH efflux. Under aerobic conditions DTT at the concentration used in this study did not affect hepatocyte viability (results not shown) and increased normal GSH efflux to a much smaller amount than under hypoxia (Figure 1, 2). Sulfobromophthalein however did not affect hypoxia induced hepatocyte GSH efflux. The rate or extent of ATP depletion on the other hands in hypoxic hepatocytes was not affected by DTT or cystine (results not shown). The modulation of hypoxia induced cytotoxicity by DTT or cystine is not therefore mediated by modulation of cellular ATP depletion but is related to GSH efflux presumably following activation of the hepatic GSH transporter by reductive stress. ROS scavengers as well as xanthine oxidase inhibitors partly prevented the GSH efflux and cell injury (Table 1, Figure 1, 2) which suggests that ROS also contributes to the GSH efflux.

In summary, the results from present communications suggest that hypoxia induced GSH efflux and subsequent cell lysis is caused by reductive stress and ROS formation which modifies and activates the thiols of the GSH transporter.

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