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OXYGEN DEPENDENCE OF OXIDATIVE STRESS

RATE OF NADPH SUPPLY FOR MAINTAINING THE GSH POOL DURING HYPOXIA

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Abstract—NADPH supply for oxidized glutathione (GSSG) reduction was studied in hepatocytes under different steady-state O_2 concentrations with controlled infusions of diamide, a thiol oxidant. When *bis*-chloronitrosourea (BCNU) was used to inhibit GSSG reductase, the rate of GSH depletion approximated the rate of diamide infusion, showing that diamide reacted preferentially with GSH under these experimental conditions. Under aerobic conditions without BCNU treatment, the GSH and NADPH pools were largely unaffected and little diamide accumulation or protein thiol oxidation occurred with diamide infusion rates up to 5.3 nmol/10^6 cells per min. However, at greater infusion rates, GSH and NADPH decreased, diamide and GSSG concentrations increased, and protein thiols were oxidized. This critical infusion rate was easily discernible and provided a convenient means to assess the capacity of cells to reduce GSSG as a function of O_2 concentration. As the O_2 concentration was decreased below 15 μM, the critical infusion rate decreased from the aerobic value of 5.3 to less than 2 nmol/10^6 cells per min in anoxic cells; half-maximal change occurred at 5 μM O_2. Although cells could not maintain normal thiol and NADPH pools at infusion rates above the critical value, analysis of the rates of thiol depletion showed that the maximal NADPH supply rate for GSSG reduction under aerobic conditions was 7–8 nmol/10^6 cells per min and was affected by hypoxia to the same degree as the critical value. Thus, hypoxia and anoxia impair the capability of cells to supply NADPH for the reduction of thiol oxidants. This could be an important factor in the sensitivity of hypoxic and ischemic tissues to oxidative injury.

Toxic hydroperoxides are catabolized in mammalian cells by the GSH peroxidase/oxidized glutathione (GSSG) reductase system [1, 2]. In liver, the specific activity of the peroxidase exceeds that of the reductase so that at maximal rates of peroxide metabolism (20–40 nmol/10^6 cells per min) [3, 4], the process is expected to be limited by the reductase with a specific activity of 16 nmol/10^6 cells per min [5]. However, available evidence indicates that NADPH supply cannot be sustained at this rate and is the major limiting factor for peroxide elimination [6].

NADPH is produced by several $NADP^+$-specific dehydrogenases [7] and from NADH via transhydrogenation in mitochondria [8]. Kauffman *et al.* [7] provided evidence that isocitrate dehydrogenase supplies the majority of NADPH utilized for bioreductions in liver. However, the pentose phosphate pathway represents another important source of NADPH [9, 10]. The regulatory enzyme for this pathway, glucose-6-phosphate dehydrogenase, is inhibited by NADPH at physiological concentrations [9]. This enzyme is activated when the GSH/GSSG and $NADPH/NADP^+$ ratios are decreased and facilitates recovery of the GSH and NADPH pools under oxidizing conditions in a variety of cell types [10]. Accordingly, a decrease in glucose-6-phosphate dehydrogenase activity resulting from treatment with 6-aminonicotinamide [11] or decreased substrate availability, such as occurs in starvation [12] or hypoxia [13, 14], is predicted to impair the ability of cells to withstand oxidative stress. Similarly, decreased NADPH generation due to alterations in the mitochondrial energy state affects oxidant reduction [15].

Hypoxic hepatocytes, which have decreased glucose-6-phosphate concentration and impaired mitochondrial energy production [16], are more susceptible to peroxide-induced toxicity than are aerobic cells [17, 18]. Hypoxic cells show a decreased ability to recover GSH and NADPH pools following exposure to *t*-butylhydroperoxide [18], suggesting that hypoxia impairs GSSG reduction. In this study, we measured the effect of hypoxia on the ability of cells to sustain GSH-dependent reductions. To oxidize GSH in a controlled manner, the oxidant diamide (diazenedicarboxylic acid-*bis*-[*N*,*N*-dimethylamide]) was infused at known rates into suspensions of freshly isolated hepatocytes which were maintained under different steady-state O_2 concentrations. Measurement of GSH, NADPH and diamide pools allowed an analysis of the ability of cells to supply NADPH at rates sufficient to maintain diamide reduction by GSH and, hence, GSSG reduction by NADPH. The results provide estimates of the total NADPH supply rate for GSSG reduction under various conditions and show that hypoxia severely limited the supply of NADPH.

MATERIALS AND METHODS

Materials. Diamide, iodoacetic acid, 1-fluoro-2,4-dinitrobenzene (FDNB), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and

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collagenase (Type IV) were purchased from the Sigma Chemical Co. (St Louis, MO). Trypan blue, *N*-1-naphthylethylenediamine dihydrochloride, and sulfanilamide were from Eastman Kodak (Rochester, NY). 1,3-*bis*(2-Chloroethyl)-1-nitrosourea (BCNU) was obtained from Bristol Laboratories (Syracuse, NY). Silicone oil (density 1.050, high temperature) was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Light mineral oil (paraffin oil, Saybolt viscosity 125/135) was from Fischer Scientific (Fairlawn, NJ). HPLC-grade methanol was obtained from American, Burdick & Jackson (McGaw Park, IL). All other chemicals were of reagent grade and were purchased locally. Deionized water was used throughout, except for HPLC solutions, where distilled, deionized water was used.

Cell preparation and incubation. Hepatocytes were isolated according to the collagenase perfusion method of Moldeus *et al.* [19] from male white rats weighing 150–250 g (Kng(SD)BR, King Animal Lab., Oregon, WI). Hepatocytes prepared by this approach are metabolically similar to intact liver by a number of criteria including the glutathione [20] and pyridine nucleotide content [21]. Cell viability, measured as the ability to exclude 0.2% trypan blue, was routinely 90–99%. Cells were maintained at room temperature in a gyrating water bath for up to 5 hr prior to experimentation without loss of viability.

Incubations (10 mL; 10^6 cells/mL) were performed in siliconized round-bottom flasks in modified Krebs–Henseleit buffer (in mM: NaCl, 140; KCl, 5; $MgSO_4$, 1.5; KH_2PO_4, 1.0; $CaCl_2$, 3.0) supplemented with 10 mM HEPES, pH 7.4. Previous studies showed that these cells from fed animals have ample endogenous substrates to maintain normal respiratory functions without addition of exogenous substrates and that addition of glucose has a relatively minor effect on the availability of glucose-6-phosphate during hypoxia [13]. Moreover, the NADPH supply rates that we obtained were comparable to values obtained by others using other techniques (see below). Therefore, the balanced salts solution was considered suitable for these studies and used for all incubations except as indicated.

Cells were kept in suspension by gentle rotation (30 rpm) using a calibrated Buchi RE121 Rotovapor suspended over a water bath at 37°. Cell suspensions were maintained at steady-state O_2 concentrations ranging from 0 to 214 μM by infusing argon (<0.001% O_2), calibrated O_2-containing gas mixtures (Specialty Gases; Atlanta, GA) or mixed gases as previously described [16]. Experimental manipulations were begun after exposing cells to the appropriate atmospheric conditions for 30 min. Cell viability was greater than 90% for all control incubations (without diamide), but addition of diamide caused some cell death, especially at higher rates of infusion and at longer time points. We therefore used conditions where viability was maintained at 70% or better, and all data are expressed relative to the number of viable cells at the respective time points.

Diamide infusion into cell suspensions. Diamide-containing solutions were infused at a rate of 1.6 mL/hr into incubation flasks by a peristaltic pump equipped with 2 mm i.d. tubing. By varying the concentration of diamide in solution, the rate of diamide infusion was varied from 2.1 to 12.8 nmol/10^6 cells/min. For incubations maintained at ≤ 4 μM O_2, diamide solutions were bubbled with argon for 30 min prior to and during the infusion. For measurement of diamide concentrations, cell aliquots (2 mL) were removed at the indicated times and cells were pelleted in a table-top centrifuge. One milliliter of supernatant fraction was transferred to each of two 1.5-mL disposable cuvettes; one served as the reference, the other as the sample. The diamide concentrations were determined relative to standard solutions from the change in absorbance at 295 nm upon addition of 50 μL of 100 mM GSH. Under the conditions of the experiments with cells, a hydrazide reduction product is expected to accumulate up to about 500 μM (60-min incubation with 9.6 nmol/mL per min infusion). This product can potentially be hydrolyzed and oxidized and contribute to the oxidation–reduction reactions that are being measured with GSH and NADPH. However, the oxidation products of hydrazides are usually very toxic [22] so that it does not appear likely that a substantial contribution of hydrazide metabolism to the measurements occurs. In addition, the rates of NADPH supply by this method are consistent with other methods for measuring NADPH supply, and the rate of GSH oxidation in the presence of BCNU was comparable to that expected from the rate of diamide infusion.

Measurement of GSH, GSSG and the pyridine nucleotides. At the indicated times, cells (0.5×10^6) were separated from the incubation mixture by centrifugation through a silicone–mineral oil mixture (6:1, v/v) into 1 M perchloric acid for the measurement of GSH and GSSG or 0.5 M KOH containing 50% (v/v) ethanol and 35% (w/v) $CsCl_2$ for the measurement of NADH and NADPH. Acid-soluble thiols (consisting primarily of GSH) were measured by the method of Saville [23]; GSH and GSSG were determined by the HPLC method of Reed *et al.* [24] using an Altex Ultrasil NH_2 column (Beckman Instruments, San Ramon, CA). The pyridine nucleotides were determined by HPLC [21] using a C_{18} μBondapak column (Waters Associates, Milford, MA). For measurements of the pyridine nucleotides, cell extracts were maintained on ice and were analyzed within 2 hr of preparation. Values were corrected for loss of cell viability and cell dilution (by the diamide-containing infusate).

Protein thiols were measured in the perchloric-acid precipitated cell pellet obtained from 0.5×10^6 cells following solubilization with sodium dodecyl sulfate and using Ellman's reagent (FDNB) according to the method of Habeeb [25].

RESULTS

Development of the model. Diamide is reduced nonenzymatically by GSH in a two-step sequence:

$$(CH_3)_2NCON{=}NCON(CH_3)_2 + GSH \longrightarrow (CH_3)_2NCON(SG)NHCON(CH_3)_2$$

$$(CH_3)_2NCON(SG)NHCON(CH_3)_2 + GSH \longrightarrow GSSG + (CH_3)_2NCONHNHCON(CH_3)_2$$

where the rate of the overall reaction can be suitably

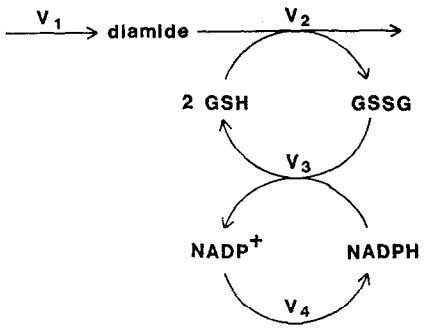

Fig. 1. Diamide-induced GSH redox cycle activity. V_1 = diamide infusion rate; V_2 = diamide reduction rate; V_3 = GSSG reduction rate; and V_4 = NADPH supply rate.

described with the rate constant 300/M/sec [26]. Diamide readily penetrates cells and reacts preferentially with GSH in biological systems [26]. Thus, we used steady-state rates of infusion of diamide (V_1) as a means to provide a constant and known rate of GSSG generation (V_2) in cells (Fig. 1). Under steady-state conditions, the rate of GSSG reduction by GSSG reductase (V_3) will equal the rate of diamide infusion so that V_1 will also equal the total rate of NADPH supply for GSSG reduction from all sources within the cell (V_4). The diamide infusion rate can be increased to obtain conditions in which the cell is unable to maintain a steady-state; in principle, this value represents the maximal rate of NADPH supply for GSSG reduction.

Previous estimates of NADPH supply have been in the range of 5–8 nmol/10^6 cells per min [8, 27–29]. To test whether the diamide reacted specifically and rapidly enough with GSH to provide a useful system for the study of GSSG reduction in this range of NADPH supply rates, we measured the effect of diamide infusion at rates from 3.2 to 11.6 nmol/10^6 cells per min on GSH in cells treated with BCNU to inhibit GSSG reductase (Fig. 2A). The results show that the initial GSH depletion rate was proportional (2 GSH per diamide) to the rate of diamide infusion up to 7.6 nmol/10^6 cells per min, although it was shifted from the maximal theoretical rate of GSH depletion (Fig. 2B). This behavior may be due, in part, to the time required for diamide to reach a steady-state concentration. The concentrations of diamide needed to react with GSH at the rates of diamide infusion were calculated to be in the range of 4.5 to 17.8 μM using the rate constant 300/M/sec. Diamide also reacts with reduced pyridine and flavin nucleotides, ferredoxins and dihydrolipoate, and this could contribute to the displaced curve. However, the rate constants are an order of magnitude lower than that for GSH [26] and the concentrations of these compounds are also typically much lower. In addition, BCNU treatment does not give complete inhibition of GSSG reductase (~85%; [30]), and this is expected to cause some deviation from the predicted rate of GSH depletion.

When diamide was infused at rates greater than 7.6 nmol/10^6 cells per min, the rate of GSH depletion did not increase. This may have been a consequence of residual GSSG reductase activity and activation of NADPH supply, inhomogeneities in the accessibility of the GSH pool to diamide, or the rate of diamide uptake by cells. Studies in non-BCNU-treated cells (reported below) suggest that the latter is not a factor. Thus, at infusion rates less than 7.6 nmol/10^6 cells per min, V_2 approximates V_1 and

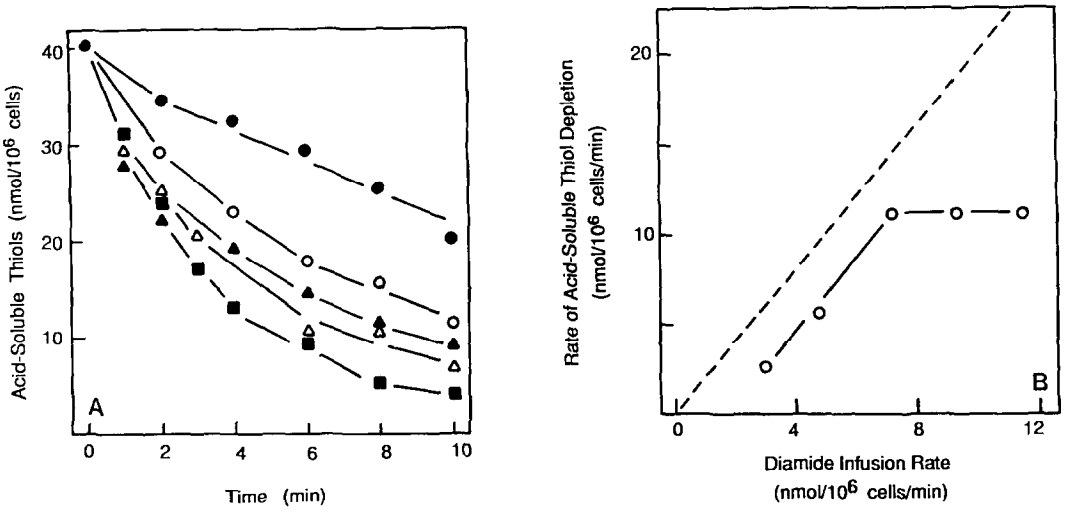

Fig. 2. Diamide-induced depletion of acid-soluble thiols in BCNU-treated hepatocytes. Cells were incubated under air (214 μM O_2) as described in Materials and Methods. Diamide infusion was begun after 15 min of pretreatment with 50 μM BCNU. Cell aliquots (0.5 mL) were removed at the indicated times, and acid-soluble thiol levels were determined by the method of Saville [23]. (A) Time course of thiol depletion upon diamide infusion at rates of 3.2 (●), 5.3 (○), 7.6 (▲), 9.6 (△), and 11.6 (■) nmol/10^6 cells per min. (B) Initial thiol depletion rate versus the diamide infusion rate. Measured rate (○); maximal theoretical rate (– – –). Values are the means of five (3.2 nmol/10^6 cells per min), four (5.3 nmol/10^6 cells per min), and three (7.6 to 11.6 nmol/10^6 cells per min) experiments. The average standard errors (omitted for clarity) were 20% of the mean value.

the diamide infusion rate provides a measure of the total NADPH supply rate. However, at higher infusion rates, the rate of GSSG production may become limiting so that V_1 does not provide a valid estimate of NADPH supply.

Effect of added glucose or citrate on NADPH supply rate. In previous studies with freshly isolated hepatocytes from fed animals, we found that endogenous respiratory substrates are sufficient to maintain normal O_2 consumption rates and that exogenous glucose has little effect on the rates of glucuronidation, which is glucose-limited under hypoxic conditions [13]. To determine whether addition of substrates that can support NADPH supply markedly affects the ability of cells to maintain GSH homeostasis, cells were incubated with either 10 mM glucose or 1 mM citrate and infused with 7.6 nmol diamide/10^6 cells per min. The results showed that glucose had essentially no effect on GSH concentration (20–21% loss of GSH for three preparations under aerobic conditions) compared to controls (20–23% loss) under either aerobic or anaerobic conditions (Table 1). Addition of citrate caused a small preservation of GSH under aerobic conditions but not under anaerobic conditions (Table 1). Thus, additional exogenous substrates do not greatly enhance NADPH supply under these conditions.

Response of the GSSG reductase system to continuous diamide infusion. When diamide was infused into cell suspensions under aerobic conditions (214 μM O_2) at rates from 3.2 to 12.8 nmol/10^6 cells per min, the thiol pool responded as shown in Fig. 3. At 3.2 nmol diamide/10^6 cells per min, no detectable change occurred in cellular thiols. At 5.3 and 6.4 nmol/10^6 cells per min, the thiol content decreased initially but approximated a new steady state within 30 min. At greater infusion rates, a steady state was not achieved within 60 min. Cellular thiol loss was accounted for by a loss of GSH as measured by HPLC (data not shown); GSH loss was accompanied by a parallel increase in extracellular GSSG.

Diamide concentrations were measured in cell suspensions with infusion rates from 4.3 to 9.6 nmol/10^6 cells per min (Fig. 4). Although the diamide concentration did not reach a true steady state in any of the experiments, diamide did not accumulate appreciably at the lower two infusion rates and, thus, was reduced at rates approaching the rate of infusion. Failure to reach a true steady-state concentration may reflect a slow decline in the capacity of the system due to glutathione efflux or the slow rate of cell death. The extent of diamide accumulation was low compared to the rate of infusion; at 9.6 nmol diamide/10^6 cells per min, the amount of diamide which accumulated within 60 min represented only 15% of the amount infused. Thus, even at a rate of diamide infusion which causes almost a 50% reduction in thiols by 30 min, most of the diamide is reduced.

The accumulation of diamide at increased infusion rates probably reflects the inadequacy of NADPH supply in supporting the GSH-dependent reduction and suggests that oxidation of other cellular components, such as protein thiols [31], occurs. To examine this possibility, protein thiol concentrations were measured in cells infused with diamide at rates of 5.3 and 9.6 nmol/10^6 cells per min (Table 2). At the lower rate, no significant oxidation occurred, while at the higher rate protein thiols were decreased about 30% by 60 min. Thus, protein thiols are probably responsible for the reduction of a small percentage

Table 1. Effect of glucose or citrate on GSH loss during diamide infusion

Conditions	GSH (nmol/10^6 cells)
0 min, Aerobic	43.0 ± 1.4
30 min, Aerobic	42.9 ± 1.6
30 min, Anaerobic	41.4, 42.1
30 min, Aerobic + diamide	
Control	33.2 ± 1.5
+ 10 mM Glucose	34.4 ± 1.3
+ 1 mM Citrate	36.1 ± 1.6
30 Min, Anaerobic + diamide	
Control	34.2 ± 3.2
+ 10 mM Glucose	33.8 ± 0.6
+ 1 mM Citrate	34.2 ± 1.7

Cells (10^6/mL) were preincubated with additions as indicated for 30 min under either aerobic or anaerobic conditions. At 0 min, diamide infusion was begun at 7.6 nmol/mL per min. Samples were taken at times indicated, and thiol content was measured by the method of Saville [23]. Values are means ± SE for data from three cell preparations except for anaerobic, 30 min, where only two values were obtained.

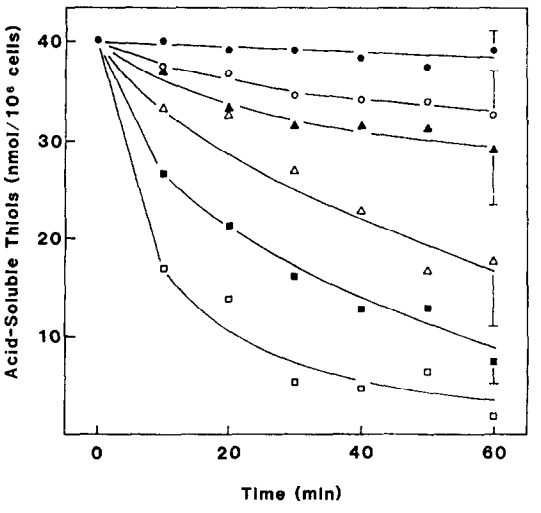

Fig. 3. Diamide-induced thiol depletion in hepatocytes. Cells were incubated under air (214 μM O_2) as described in Materials and Methods. Diamide was infused at rates of 4.3 (●), 5.3 (○), 6.4 (▲), 7.6 (△), 9.6 (■) and 11.6 (□) nmol/10^6 cells per min. Acid-soluble thiols were determined by the method of Saville [23]. Values, corrected for dilution by perfusate, represent the means of two (11.6 nmol/10^6 cells per min), three (7.6, 9.6 nmol/10^6 cells per min), or five (4.3, 5.3, 6.4 nmol/10^6 cells per min) experiments. Standard errors are presented for the 60-min values.

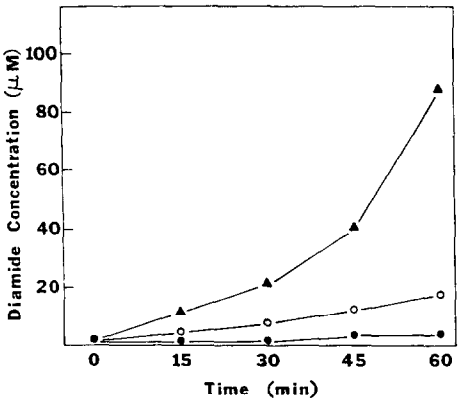

Fig. 4. Concentration of diamide in cell suspensions during diamide infusion. Cell suspensions maintained at a steady-state O_2 concentration of 214 μM O_2, as described, were infused with diamide at rates of 4.3 (●), 5.3 (○) and 9.6 (▲) nmol/10^6 cells per min. The diamide concentration in the supernatant fraction was determined by comparing to standards the GSH-induced loss of absorbance at 295 nm. Values are the average of two experiments.

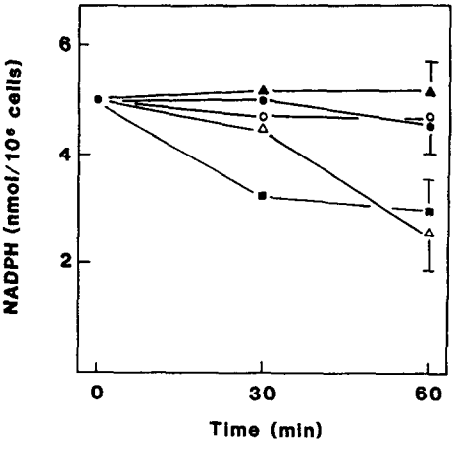

Fig. 5. Diamide-induced NADPH depletion in hepatocytes. Cells were incubated under air (214 μM O_2) as described in Materials and Methods. Diamide was infused into cell suspensions at rates of 2.1 (●), 3.2 (○), 4.3 (▲), 5.3 (△) and 6.4 (■) nmol/10^6 cells per min. Cell aliquots (0.5 mL) were removed 30 and 60 min after beginning diamide infusion, and the NADPH content was determined by HPLC [21]. Values are the means of three experiments and were corrected for dilution by the infusate. Standard errors are presented for the 60-min values.

of diamide at the greater infusion rate. Based on the extent of diamide accumulation and protein thiol oxidation, the capacity of the cell to produce NADPH for GSSG reduction appears to be limited to 70–80% of the rate of infusion at 9.6 nmol/10^6 cells per min. Therefore, the maximal rate of NADPH production for diamide reduction is about 7–8 nmol/10^6 cells per min. However, when the rate of oxidant infusion exceeds the ability of the cell to maintain GSH pools (5.3 nmol/10^6 cells per min), other cellular components are oxidized to a greater extent and the cell is in a state of decline.

Several investigators have shown that factors which decrease the GSH/GSSG ratio also decrease the NADPH/$NADP^+$ ratio [2, 32]. Measurement of NADPH in cells showed that during diamide infusion at rates lower than 5.3 nmol/10^6 cells per min, no detectable decrease occurred within 60 min (Fig. 5). With an infusion rate of 5.3 nmol/10^6 cells per min, NADPH was unaffected by 30 min but had declined about 50% by 60 min. At 6.4 nmol/10^6 cells per min, a decrease in NADPH had occurred within only 30 min and was sustained up to 60 min. Thus, when the rate of diamide infusion was sufficient to deplete thiol pools (5.3 nmol/10^6 cells per min), NADPH pools were also decreased.

These studies show that two oxidant loads are important with regard to defining the capability of cells to supply NADPH for GSSG reduction. The first of these is the critical infusion rate, or the rate of diamide infusion above which the cell cannot maintain homeostasis of thiol status. Under normal aerobic conditions, this value is about 5.3 nmol/10^6 cells per min. Above this rate, the GSH and NADPH concentrations fall, diamide accumulates and protein thiol oxidation occurs. The other oxidant load corresponds to the maximal NADPH supply rate which appears to be in the range of 7–8 nmol/10^6 cells per min. It is not clear whether oxidant loads between the critical and maximal values create steady states that can be maintained indefinitely *in vivo* or whether these conditions exceed the conditions under which

Table 2. Protein thiols in hepatocytes during diamide infusion

Diamide infusion rate (nmol/10^6 cells per min)	Protein thiols (nmol/10^6 cells)		
	0 min	30 min	60 min
5.3	153 ± 33	150 ± 12	179 ± 15
9.6	153 ± 33	90 ± 17*	114 ± 13*

Cell aliquots (0.5 mL) were removed immediately prior to and at 30 and 60 min after beginning diamide infusion, and proteins were precipitated with 1 M perchloric acid. The protein pellet was resolubilized into a 2% solution of sodium dodecyl sulfate. Protein thiols were measured using Ellman's reagent [25]. Values are the means ± SE for three experiments. For the zero values, N = 6; for 30 and 30 min values, N = 3.

* By the paired *t*-test the protein thiol contents between cells infused with 9.6 nmol/10^6 cells per min and the zero time control were significantly different ($P < 0.05$).

cells can survive. However, because protein thiol oxidation and diamide accumulation accompany GSH oxidation as the maximal NADPH supply rate is approached, the critical infusion rate appears to be better for assessing the efficacy of NADPH supply in accommodating oxidant loads under different conditions.

Use of the critical infusion rate to measure the O_2 dependence of NADPH supply. We used the critical infusion rate as a parameter to determine the O_2 dependence of NADPH supply. Soluble thiols were measured in cells maintained at steady-state O_2 concentrations from 0 to 214 μM O_2 upon diamide infusion at rates ranging from 2.1 to 12.8 nmol/10^6 cells per min. The critical infusion rates at the different O_2 concentrations were identified from the concentrations of GSH after 30 min of diamide infusion (see Fig. 3) when plotted as a function of the infusion rate (Fig. 6).

As the O_2 concentration was decreased below 15 μM O_2, the ability of cells to maintain original thiol values was reduced such that, in anoxic cells, the critical infusion rate was estimated to be less than 2 nmol/10^6 cells per min (Fig. 7). Because of the relatively poor response of thiols to infusion of diamide at very low infusion rates (see Fig. 2), the actual rate of NADPH supply in anoxia may be even lower than this estimate. Half-maximal change in the critical value occurred at about 5 μM O_2. Thus, hypoxia and anoxia dramatically decrease the rate at which cells can supply NADPH for the maintenance of GSH under oxidizing conditions.

Because of the complications discussed above, an accurate assessment of the maximal rate of cellular NADPH supply was not possible with this approach. Experiments in aerobic cells suggested that the maximal rate was approximately 75% of the rate of diamide infusion required for the half-maximal GSH depletion (9.6 nmol/10^6 cells per min); at this infusion rate, about 25% of the total diamide infused was shown to react with protein thiols and/or accumulate. A simple analysis of the infusion rates required for half-maximal depletion of GSH at the different O_2 concentrations showed that the O_2 dependence paralleled that of the critical value (Fig. 7). Thus, the maximal rate of cellular NADPH supply has approximately the same O_2 dependence as that found for the critical value.

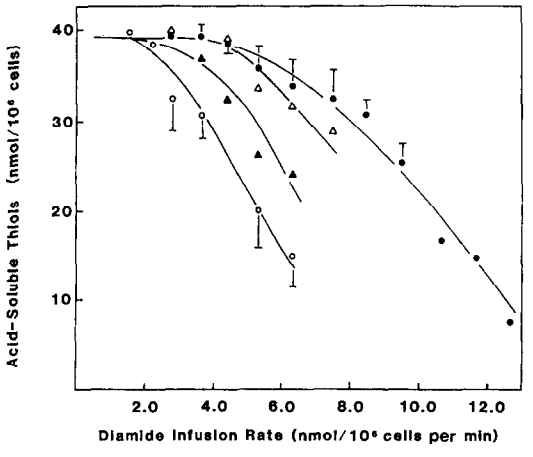

Fig. 6. O_2 concentration dependence of diamide-induced thiol depletion. Diamide was infused at various rates ranging from 2.1 to 12.8 nmol/10^6 cells per min into cell suspensions maintained at steady-state O_2 concentrations of 0 μM (○), 4 μM (▲), 15 μM (△) and 214 μM O_2 (●). Acid-soluble thiols were assayed according to the method of Saville [23]. Values represent the thiol content 30 min after beginning diamide infusion. Standard errors are presented for cells under argon and air for all values where N ≥ 3.

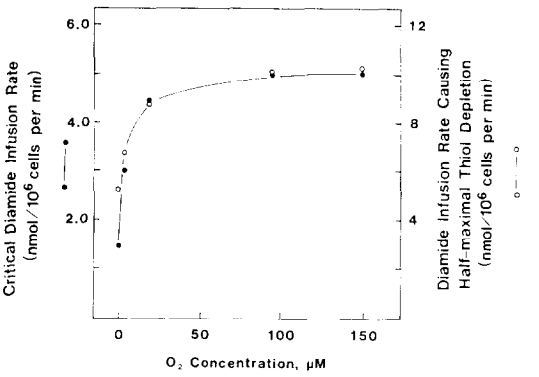

Fig. 7. O_2 dependence for NADPH supply for the maintenance of acid-soluble thiols in hepatocytes. Values represent estimates of the critical infusion rates and those causing half-maximal thiol depletion using the data presented in Fig. 6 and that obtained in cells maintained at 90 μM O_2.

DISCUSSION

Several approaches have been used to measure NADPH supply in cells and tissues. Among these, radiotracer methods for measurement of flux through the NADPH pathway have received the most attention because these are independent of the end use of NADPH and rely directly upon a well-defined pathway of generation, e.g. the pentose phosphate pathway [33, 34]. However, in liver, much of the NADPH supply is of mitochondrial origin [8]; consequently, an accurate estimation of the total NADPH supply requires a method that can measure NADPH supply from mitochondrial and extramitochondrial sources. For this reason, many investigators have utilized the rates of drug metabolism and lipid biosynthesis to estimate NADPH supply rates [2, 27–29].

Because we were interested in measuring NADPH supply to support GSH-dependent reductions, we monitored the response of cells to a thiol-selective oxidant, diamide. Diamide is useful for studies of cellular thiol homeostasis because it is readily permeable to cells and reacts predominantly with GSH under usual conditions in cells [26]. For example, Bump and Reed infused diamide into high-density suspensions of L-5178Y lymphoma cells and measured the effect on the GSH/GSSG ratio [see Ref. 35]. These cells were found to be capable of reducing GSSG at a rate of 2–3 μmol/min while maintaining a constant GSH/GSSG ratio for 3–4 hr. In the current studies, we have extended this

Table 3. Estimates of NADPH supply/consumption rates in liver

Parameter	NADPH supply/consumption (nmol/10^6 cells per min)	Reference
Fatty acid synthesis	8.3	[27]
Aminopyrine demethylation		
Untreated liver	1.6*	[28]
Phenobarbital-treated liver	3.6–6.6*	[8, 27]
Ethylmorphine demethylation	5.0	[29]
GSSG reductase specific activity	16.0*	[5]
Thiol reduction	7–8	Present studies

* Calculated from μmol/g liver using 10 mg as the wet weight of 10^6 cells.

approach to hepatocytes and included measurements of diamide and NADPH to allow analysis of the O_2 dependence of NADPH supply rates.

The values that we obtained for the maximal NADPH supply are comparable to estimates obtained with other methods (see Table 3). However, infusion of diamide at this rate resulted in oxidation of protein thiols, and there was a continued decline in the ability to maintain the GSH pool. Thus, the critical rate at which diamide can be infused without causing these changes in cellular thiols is probably more important with regard to cellular protection.

The current results showed that NADPH supply is impaired dramatically by hypoxia. The half-maximal effect occurred at about 5 μM O_2, a value comparable to the O_2-dependence of a variety of mitochondrial and drug-metabolizing systems. For example, the half-maximal value for oxidation of mitochondrial cytochrome oxidase is 6 μM O_2 [36], for lactate production from pyruvate 12 μM O_2 [37], for drug metabolism by cytochrome P-450 4–10 μM O_2 [38], and for drug glucuronidation 6.6 μM [13]. Because the median O_2 concentration in liver *in vivo* is about 35 μM [39] and includes regions with O_2 concentrations below 10 μM, some cells in the liver may experience hypoxia-related alterations in drug metabolism under normal conditions. These studies show that, in addition to some mitochondrial functions and drug metabolism, cellular defenses against oxidants are sensitive to O_2 concentration under conditions of mild hypoxia which can occur under normal physiological conditions.

Such sensitivity of antioxidant defenses to hypoxia may be of great importance in pathological and toxicological processes. Myocardial and cerebral infarctions due to arteriosclerosis-related impairment of tissue oxygen delivery are among the most frequent lethal disease processes of industrialized nations. In addition, toxicologically important alterations in the metabolism of various compounds are known to occur in hypoxia. Halogenated hydrocarbons such as halothane and carbon tetrachloride are reductively activated under hypoxic conditions [40, 41]. Similarly, in alcoholic liver injury, in which both hypoxia and free radical processes have been implicated in injury [42, 43], the inability of hypoxic cells to protect against oxidative stress could contribute to the extent of injury.

A decreased capacity to produce NADPH also may be important in the extent of injury following post-ischemic reperfusion. Reoxygenation results in the rapid formation of active O_2 species. A marked impairment of NADPH supply could place an additional burden on the post-ischemic cells, impairing their ability to deal with oxidants relative to normoxic cells. Thus, injury from these compounds may be potentiated, rendering otherwise non-lethal amounts lethal. Because of the widespread importance of ischemia in human pathology, this finding may warrant increased efforts to improve antioxidant defenses during reperfusion.

In conclusion, the current study describes an approach to obtain the NADPH supply rate for GSSG reduction. From this analysis, a critical NADPH supply rate was found to occur at 5.3 nmol/10^6 cells per min; this is the maximal rate at which the hepatocytes can reduce oxidants without deterioration of the cellular thiol homeostasis. The maximal NADPH supply rate was approximately 50% higher, but this rate occurred under conditions in which the thiol pool was depleted. Measurement of the critical NADPH supply rate as a function of O_2 concentration showed that NADPH supply was impaired markedly by hypoxia with a half-maximal effect at 5 μM O_2. Impaired NADPH supply may be an important factor in hypoxic and post-ischemic injury associated with a variety of pathophysiological processes.

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