Determinants of Hydroperoxide Detoxification in Diabetic Rat Intestine: Effect of Insulin and Fasting on the Glutathione Redox Cycle

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The capacity for hydroperoxide detoxification in diabetic (DM) intestine was studied in streptozocin-induced DM rats by quantification of the intestinal glutathione (GSH) redox cycle, a key cellular pathway for peroxide elimination. A role for luminal glucose in regulation of redox cycle activity was examined in insulin-treated or 24-hour–fasted DM animals. Intestinal activities of the redox enzymes, GSH peroxidase, GSSG reductase, and glucose-6-phosphate dehydrogenase (G6PD), were significantly decreased by 17 hours' insulin treatment, whereas only G6PD was decreased by fasting. Mucosal GSH levels were also markedly decreased under these conditions. These results are consistent with an overall suppression of intestinal GSH redox cycle function by short-term administration of insulin. Insulin treatment for 7 consecutive days increased hepatic G6PD activity by fourfold but was without effect on intestinal G6PD, suggesting tissue specificity in insulin regulation of G6PD. The rate of metabolism of *tert*-butyl hydroperoxide (*t*BH) in isolated enterocytes was low in the absence of substrates (0.51 ± 0.07 nmol/10^6 cells/min) but was increased fivefold by exogenous glucose (2.70 ± 0.11 nmol/10^6 cells/min), indicating that glucose availability is an important contributor to intestinal detoxification of toxic hydroperoxides. Collectively, the current results show that GSH redox cycle enzymes in DM intestine are under coordinate insulin control, and that this control appears to be downregulated by short-term insulin treatment.

PREVIOUS STUDIES have shown that an elevated level of serum oxidized lipoprotein is associated with human and experimental diabetes mellitus (DM).[1-3] This enhanced circulating lipid peroxide level is implicated in DM complications.[3] Although the mechanism for increased plasma accumulation of peroxides is unclear, recent studies suggest that an increase in absorption of dietary oxidized lipids may be primarily responsible for the elevated plasma levels of lipid peroxides observed in the streptozocin-induced DM rat.[1] A contributing factor to an enhanced peroxide uptake and subsequent peroxide transport into the lymph could be a decreased intracellular hydroperoxide metabolism[4-6] in DM intestine.

We have recently shown that the glutathione (GSH) redox cycle[7,8] (Fig 1) is an important cellular pathway for detoxification of lipid hydroperoxides by rat small intestine.[4-6] The rapid and efficient reduction of hydroperoxides is accomplished by the coordinate function of GSH peroxidase, GSSG reductase, and the supply of GSH, a major cellular reductant (Fig 1). Thus, regulation of intestinal redox enzyme activities and of GSH supply could ultimately control the output of hydroperoxides into the systemic circulation. In addition, a key step in the supply of GSH is the continuous regeneration of GSH from GSSG at the expense of NADPH (Fig 1). A major contributor to the total cell NADPH pool is the pentose phosphate pathway,[9] and glucose utilization by this pathway is regulated by glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting step.[10] Hence, elimination of hydroperoxides by DM small intestine could depend on the function of G6PD and the availability of glucose. Because tissue utilization of glucose is altered by insulin insufficiency in DM, this change in tissue glucose status could have a significant impact on the overall regulation of hydroperoxide detoxification in the DM intestine.

The objective of the current study is therefore to quantify the determinants of hydroperoxide detoxification in the DM intestine. To address this objective, we used the streptozocin-induced diabetic rat as the animal model and defined intestinal detoxification capacity in terms of tissue enzyme activities and reductant levels of each component of the GSH redox cycle. In addition, we have directly quantified the rate of hydroperoxide metabolism without or with glucose supplements in isolated enterocytes. The results provide important insights into the function of the GSH redox cycle in the DM intestine, and underscore the importance of maintaining glucose status to support redox cycle activity in intestinal hydroperoxide detoxification in DM animals.

MATERIALS AND METHODS

Materials

Streptozocin, *tert*-butyl hydroperoxide (*t*BH), and the glucose assay kit were purchased from Sigma Chemical (St Louis, MO). Ultralente insulin and lente insulin were from Novo Nordisk Pharmaceuticals (Bagsvaerd, Denmark). The protein dye reagent was obtained from Bio Rad (Hercules, CA). All other chemicals were of reagent grade and were purchased from local sources.

Induction of DM in Rats

Male Sprague-Dawley rats (170 to 200 g) were maintained ad libitum on standard rat chow for 5 days before streptozocin treatments. Streptozocin was dissolved in 0.01 mol/L citrate buffer, pH 4.5, and each rat was injected intraperitoneally (70 mg/kg) within 5 minutes after drug preparation. On day 7 after streptozocin administration, attainment of DM was confirmed by measurements of plasma glucose level. Animals with plasma glucose levels of 12 mmol/L or greater were considered DM, and typically 70% to 80% of the rats developed DM under these conditions. Rats that

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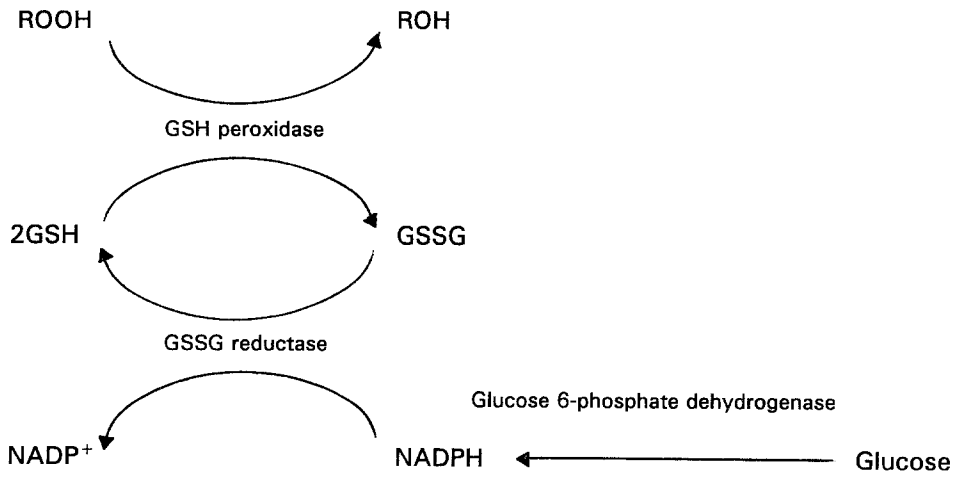

Fig 1. Scheme depicting GSH redox cycle in hydroperoxide detoxification. ROOH, hydroperoxide; ROH, hydroxide.

developed DM were maintained on rat chow for another week before experimentation.

Experimental Protocols

DM rats were divided into four groups as follows: group 1, DM ad libitum–fed controls; group 2, DM rats injected with 5 U lente insulin and killed at 17 hours after insulin administration (17-hour insulin treatment); group 3, DM rats injected with 5 U ultralente insulin once per day for 7 consecutive days (7-day insulin treatment); and group 4, DM rats fasted for 24 hours before killing. In all instances where indicated, insulin was administered subcutaneously.

Control and experimental rats were anesthetized with halothane and killed by exsanguination. The entire small intestine and the liver were carefully removed and placed on ice. Collection of intestinal mucosa was conducted on ice, and centrifugation steps were performed at 4°C. The intestine was divided into two equal segments, namely the proximal and distal intestine, and each segment was rinsed thoroughly with ice-cold 0.9% saline. The intestinal mucosa was harvested by gentle scraping using a glass slide. A 10% (wt/vol) tissue homogenate was prepared and centrifuged at 700 × *g* in a table-top, refrigerated Beckman GPR centrifuge (Beckman Instruments, Fullerton, CA) to remove connective tissues and cell debris. The liver was minced in ice-cold saline (0.9% NaCl), and liver homogenate was prepared according to the procedure reported by Rudack et al.[11] Aliquots of liver and intestinal homogenates were used for determination of enzyme activities, GSH concentrations, and protein contents.

Isolation of Intestinal Cells From DM Intestine

Enterocytes were prepared from the proximal intestine of streptozocin-treated rats according to the method reported by Masola and Evered.[12] Briefly, DM rats were killed under halothane anesthesia, and the proximal half of the small intestine was excised. The intestinal lumen was washed twice with cold 0.9% saline to remove particulate matter. The lumen was filled with Krebs-Henseleit buffer, pH 7.4 (buffer A), containing 10 mmol/L dithiothreitol (DTT) and incubated at 37°C for 10 minutes to remove excess mucus. The lumen was then refilled with buffer A containing 5 mmol/L EGTA and 10 mmol/L DTT and further incubated at 37°C for 15 minutes. Thereafter, the intestinal segment was massaged gently to release the enterocytes. The cells were filtered through gauze and washed twice with buffer A containing 10 mmol/L DTT and 0.25% bovine serum albumin. The enterocytes were resuspended in buffer A to a final cell concentration of 3.5 × 10^6/mL. Cell viability was assessed with 0.1% trypan blue, and viability was routinely between 85% and 95%. Enterocytes were used within 1 hour of isolation.

Cell Incubation With tBH Without or With Glucose

Enterocytes (3.5 × 10^6 cells/mL) were incubated in buffer A in 5-mL rotating round-bottomed flasks at 37°C as previously described.[13,14] The cells were preincubated for 5 minutes before addition of *t*BH to a final concentration of 300 μmol/L. During the preincubation time, glucose was added to the cell suspension to a final concentration of 10 mmol/L. At 0, 5, 15, and 30 minutes, 0.5-mL samples were removed, and cells were separated from incubation media by centrifugation in a microfuge (700 × *g* at 4°C) for 2 minutes. At all time points, there was no significant loss of cell viability in control and glucose-supplemented cells. The media were saved on ice, and *t*BH levels in the media were measured within 60 minutes after the end of the experiments. Samples kept on ice were stable as verified by standard *t*BH solutions processed under identical conditions.

Biochemical Assays

Tissue enzyme activities were determined spectrophotometrically using previously established assays for G6PD,[15] GSH peroxidase,[16] GSSG reductase,[17] and malic enzyme.[18] In each instance, the change in NADPH was monitored at 340 nm and quantified using 6.22 as the millimolar extinction coefficient for NADPH.[19] Enzyme activities were expressed as nanomoles per minute per milligram protein. Mucosal GSH concentrations were determined by the method reported by Owens and Belcher.[20] *t*BH was determined spectrophotometrically according to the method reported by Heath and Tappel.[21] Protein level was measured according to the Bradford method.[22] Blood glucose was assayed by the glucose oxidase method using a glucose assay kit.

Statistical Analysis

Values are the mean ± SE. Data were evaluated by one-way ANOVA, and multiple comparisons were made by the method of least-significant difference.

RESULTS

In preliminary studies, we found that treatment of rats with a single dose of streptozocin 70 mg/kg resulted in a twofold increase in food intake (from 24.9 ± 0.8 to 45.3 ± 1.1 g/d) and an associated fourfold increase in blood glucose level (from 7.5 ± 0.1 to 31.5 ± 0.6 mmol/L) within

1 week, consistent with development of DM in the rats. These results are in agreement with previously published values on induction of DM in rats.[23-25]

Effect of Fasting on Intestinal Redox Cycle Enzymes

Our previous studies show that in nondiabetic control rats, the supply of glucose to support function of the GSH redox cycle is an important determinant of the overall efficiency of detoxification of lipid hydroperoxides by the small intestine.[14] These results suggest that the altered glucose homeostasis in DM could impact the intestinal capacity for handling hydroperoxides. To test this suggestion, glucose status in DM intestine was altered by a 24-hour fast. Thereafter, the enzymes of the GSH redox cycle (G6PD, GSH peroxidase, and GSSG reductase) and reductant (GSH) levels in the small intestine were determined. The results in Fig 2 show that fasting significantly decreased intestinal G6PD activity, but exerted little effect on GSSG reductase and GSH peroxidase. This result indicates that intestinal G6PD activity is a function of nutritional status of the animal, and suggests that availability of luminal nutrients such as glucose may affect overall intestinal handling of toxic peroxides.

Effect of Glucose on tBH Metabolism in Isolated Enterocytes

To verify that hydroperoxide detoxification in DM intestine is in fact influenced by glucose availability, we measured the rate of hydroperoxide metabolism in isolated enterocytes using *t*BH as a model peroxide substrate. The results show that *t*BH elimination in intestinal cells under basal conditions in the absence of substrates was small (Fig 3). The initial rate of elimination was 0.51 ± 0.07 nmol/10^6 cells/min, and the addition of 10 mmol/L glucose caused a fivefold increase in *t*BH elimination (2.70 ± 0.11 nmol/10^6 cells/min). This dependence of hydroperoxide elimination on exogenous glucose confirms that luminal glucose is an important determinant of mucosal catabolism of toxic hydroperoxides in DM small intestine.

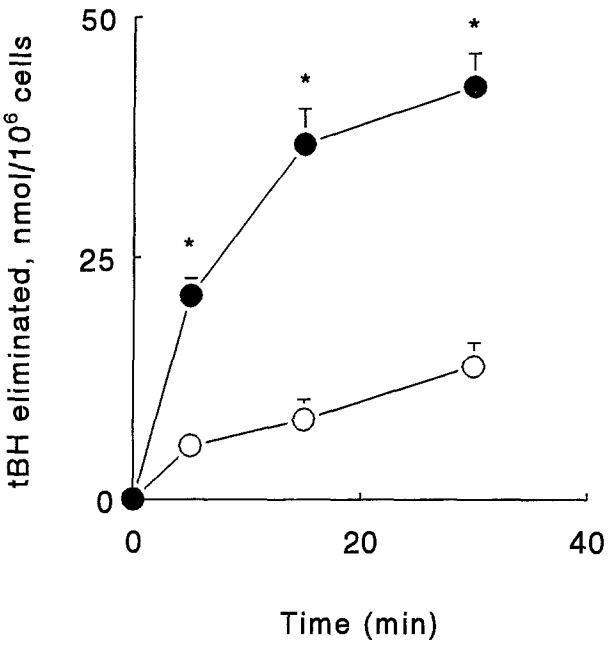

Fig 3. Glucose-dependent *t*BH elimination in isolated enterocytes from DM rats. (○) Baseline; (●) plus glucose. Results are the mean ± SE for 6 cell preparations. *Statistically different from control, $P < .001$.

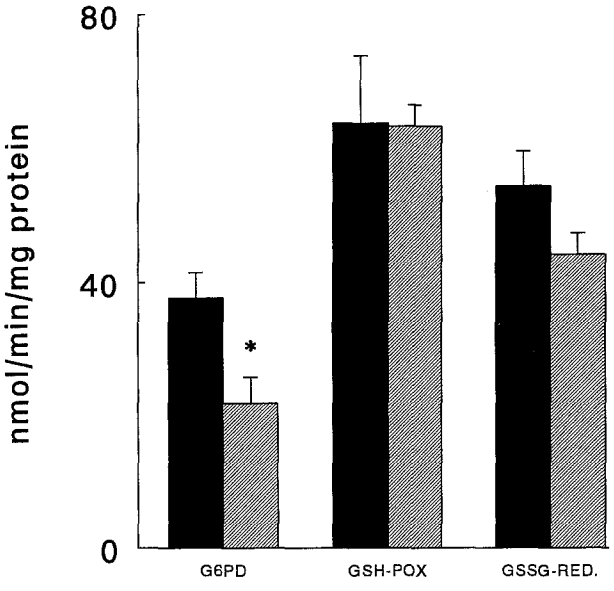

Fig 2. Effect of a 24-hour fast on intestinal redox cycle enzyme activities. Results are the mean ± SE for ad libitum–fed DM rats (■, n = 10) and 24-hour–fasted DM rats (▨, n = 4). *Statistically different from ad libitum–fed DM animals, $P < .05$.

Effect of Insulin on Intestinal G6PD

Because brush-border Na^+-dependent glucose uptake in the DM intestine is downregulated by insulin,[26] we examined the effect of insulin administration on GSH redox cycle enzymes. Administration of a single dose of insulin to animals before death 17 hours later has little effect on food consumption (data not shown). However, this maneuver significantly decreased intestinal G6PD activity (Fig 4). The decrease was seen in both the proximal and distal intestine. Interestingly, long-term insulin treatment, once daily for 7 consecutive days, had minimal effects on G6PD specific activity (Fig 4), suggesting that the insulin effect is short-term, and is consistent with a short half-life (2 to 3 days) of intestinal epithelium.[27] By comparison, short-term insulin treatment also appears to decrease hepatic G6PD activity, whereas administration of insulin for 7 days significantly increased activity of the liver enzyme by fourfold (Fig 5). The latter finding was in agreement with previous studies.[28-31] Taken together, the results suggest that the function of G6PD along the entire small intestine is responsive to short-term insulin treatment, and that downregulation of the intestinal enzyme probably occurs secondary to an insulin-induced decrease in mucosal glucose uptake.[26]

The responsiveness of G6PD activity to luminal nutrient availability suggests that the glucose-mediated supply of NADPH may determine intestinal hydroperoxide reduction (Fig 1). To investigate whether insulin and fasting

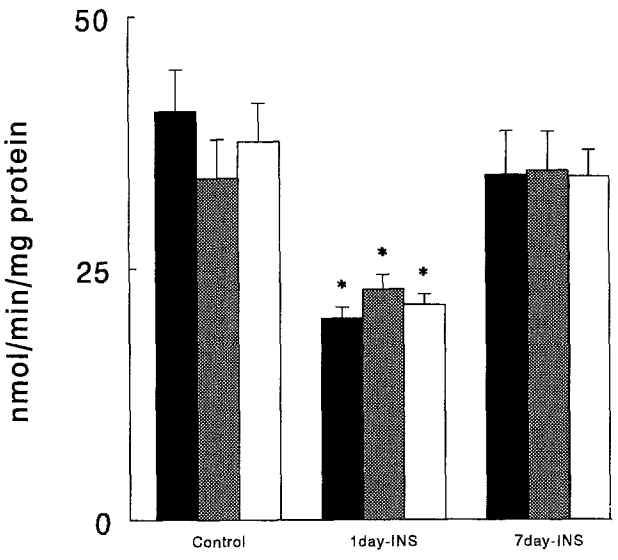

Fig 4. Insulin effects on mucosal G6PD activity in DM rats. 1day-INS, insulin administered for 17 hours before death. 7day-INS, insulin given for 7 consecutive days before death. Results are the mean ± SE for ad libitum–fed DM control (n = 10), 1day-INS (n = 10), and 7day-INS (n = 4). *Statistically different from DM control ($P < .01$). (■) Proximal; (▨) distal; (□) total.

collectively affect other NADPH-generating systems, we quantified the activity of malic enzyme, an important contributor to the cellular NADPH pool. The results show that mucosal malic enzyme activity was decreased by fasting and by short-term insulin treatment in a manner similar to that of G6PD (Table 1), thus supporting a generalized control of cellular NADPH supply in the DM intestine.

Effect of Insulin on GSSG Reductase and GSH Peroxidase

Mucosal activities of GSH peroxidase and GSSG reductase were quantified to determine whether insulin also affects GSH-dependent redox enzymes. The results show

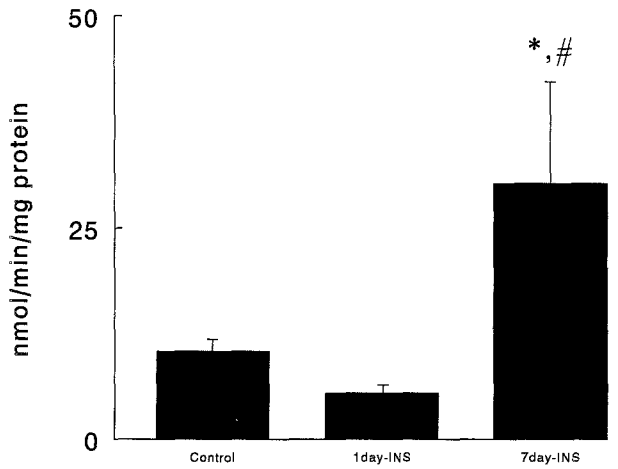

Fig 5. Insulin effects on hepatic G6PD activity in DM rats. Values are the mean ± SE for ad libitum–fed DM control (n = 10), 1day-INS (n = 10), and 7day-INS (n = 4). *Statistically different from DM control ($P < .05$). #Statistically different from 1day-INS ($P < .05$).

Table 1. Effect of Insulin Treatment and Fasting on Malic Enzyme Activity in Proximal and Distal Small Intestine of DM Rats

Group	No. of Rats	Malic Enzyme (nmol/min/mg protein) Proximal Intestine	Distal Intestine
Ad libitum–fed	9	9.67 ± 1.35	11.25 ± 1.58
24-hour–fasted	4	5.45 ± 2.10	5.35 ± 1.60*
1-day insulin	9	4.84 ± 0.39*	6.64 ± 0.70*

NOTE. Results are the mean ± SE for the number of animals indicated for each condition.

*Statistically different from ad libitum–fed DM animals ($P < .05$).

that administration of insulin for 17 hours caused significant decreases in GSH peroxidase and GSSG reductase activities (Fig 6A and B, respectively). As with G6PD, long-term treatment of DM rats with insulin for 7 days produced values similar to those for the DM control (Fig 6A and B), consistent with regulation of redox enzymes by short-term insulin administration. Collectively, these results show that insulin mediates the simultaneous regulation of function of the three enzymes of the GSH redox cycle, and that the effect occurred within 17 hours after hormone administration.

Effect of Insulin on Mucosal GSH Levels

The coordinate control of GSH redox enzyme activities suggests that availability of the reductant, GSH, may similarly be affected by insulin. To test this, we quantified mucosal GSH concentration. The results show that tissue GSH contents in the proximal and distal intestine were markedly decreased after short-term insulin administration but not after long-term insulin treatment (Table 2), similar to the changes in activities of mucosal GSH redox enzymes. However, measurements of γ-glutamyl cysteine synthetase, the rate-limiting step in GSH synthesis, show that activity of the enzyme was unaltered by insulin (data not shown). Thus, the results suggest that the decrease in tissue GSH concentrations by insulin was probably not associated with changes in levels of the biosynthetic enzyme per se, but may be related to decreased precursor cysteine supply or to decreased mucosal GSH uptake from the intestinal lumen or plasma.

DISCUSSION

Previous studies have shown that the function of the GSH redox cycle is a key determinant of intestinal metabolism of lipid hydroperoxides in nondiabetic animals.[4-6,14] In the current study, we have shown that this system may similarly play an important role in hydroperoxide detoxification in the DM small intestine. The results implicate a coordinate regulation of GSH redox cycle function by short-term insulin administration. The parallel decreases in activities of the core enzymes and mucosal GSH levels following 17 hours' insulin treatment are consistent with this interpretation. The sum total of decreased intracellular hydroperoxide detoxification in the DM intestine due to lower redox cycle activity would mean enhanced peroxide output into lymph. The increase in lymphatic peroxide transport could contribute to the elevations of plasma

oxidized lipids that are often associated with the DM state in humans and experimental animals.[1-3]

Interestingly, G6PD activity is also affected by dietary factors. The decrease in G6PD activity by fasting suggests that luminal glucose may contribute to intestinal G6PD regulation. Because G6PD controls glucose flux through the pentose phosphate pathway[9,10] and because the supply of NADPH is crucial for maintaining constant cell GSH (Fig 1), the function of G6PD could limit NADPH availability during high rates of hydroperoxide metabolism.[9,10,14] This means that the overall efficiency in preserving high mucosal GSH for redox cycle function in DM intestine could largely be governed by the availability of glucose and its subsequent flux through the pentose phosphate shunt. Consistent with this suggestion, we found a fivefold stimulation of *t*BH elimination by glucose in isolated enterocytes. These considerations are important, since DM small intestine epithelium is subjected to large variations in luminal and plasma glucose concentrations consequent to varied dietary carbohydrate intakes.

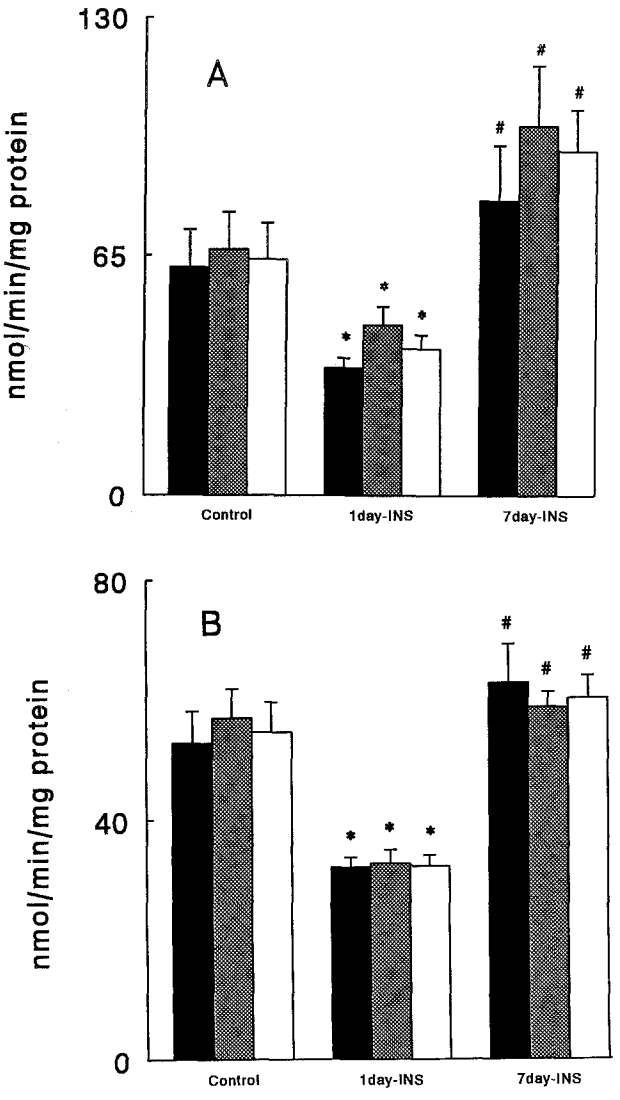

Fig 6. Insulin effects on intestinal GSH peroxidase (A) and GSSG reductase (B) activities in DM rats. Results are the mean ± SE for ad libitum–fed DM control (n = 10), 1day-INS (n = 10), and 7day-INS (n = 4). *Statistically different from DM control ($P < .01$). # Statistically different from 1day-INS ($P < .05$). (■) Proximal; (▨) distal; (□) total.

Table 2. Insulin Effects on Mucosal GSH Concentrations in Proximal and Distal Intestine of DM Rats

Group	No. of Rats	Mucosal GSH Concentration (nmol/mg protein)	
		Proximal Intestine	Distal Intestine
Ad libitum–fed	9	36.4 ± 5.3	32.8 ± 4.8
Insulin treatment			
1 day	9	26.0 ± 3.6*	19.7 ± 2.6*
7 days	4	43.3 ± 4.5	40.8 ± 3.4

NOTE. Results are the mean ± SE for the number of animals indicated for each condition.

*Statistically different from ad libitum–fed DM control ($P < .05$).

Our finding that insulin modulates hepatic G6PD activity is consistent with hormonal control of the liver enzyme in the DM state, and is in agreement with previous studies in nondiabetic animals.[28-32] Interestingly, in contrast to the liver enzyme, the temporal decrease in intestinal G6PD activity occurred within 17 hours after insulin administration, and by 7 days, insulin treatment was without effect on activity of the intestinal enzyme. This differential time-dependent effect of insulin on intestinal and hepatic G6PD suggests tissue specificity in hormonal regulation of the enzyme in these two organs.

Mechanistically, the effect of insulin on GSH redox cycle function may be twofold. Based on the results of insulin treatment and fasting on intestinal G6PD, the effect of insulin may be secondary to its role in mediating enterocyte glucose uptake from the intestinal lumen. This suggestion is supported by studies reported by Madsen et al,[26] who showed that insulin downregulates intestinal transport of glucose. On the other hand, Vinnik et al[33] found that insulin has no effect on intestinal glucose transport. Alternatively, the insulin effect may be independent of intestinal glucose transport and utilization, ie, insulin may act directly to decrease mucosal enzyme activities, as evidenced by the dissociation of the effects of insulin and fasting on GSH peroxidase and GSSG reductase. Regardless of the mechanism, our results demonstrate that the overall regulation of function of the GSH redox cycle in peroxide detoxification in the DM intestine could be highly sensitive to the insulin and tissue glucose status of the DM animal.

Our data that exogenous glucose enhances hydroperoxide metabolism further suggest that, in general, the availability of glucose for NADPH production in the DM state could markedly impact a variety of other NADPH-dependent metabolic pathways, such as drug oxidation by microsomal P-450[34] and biosynthesis of fatty acids.[35-37] The current results show similar decreases in G6PD and malic enzyme activities in response to insulin and fasting, indicating that

cellular NADPH-generating systems in the DM intestine may be coordinately regulated. Studies are currently under way to examine the mechanism of regulation of NADPH supply enzymes using an established enterocyte-like human carcinoma cell line (CaCo 2 cells). Preliminary results show a dose-dependent relationship of G6PD activity with glucose concentrations in the culture medium.

To summarize, we found that the function of the GSH redox cycle is critical for hydroperoxide handling in the DM intestine. The key components of the redox cycle appear to be responsive to insulin administration, and the regulation of NADPH supply to support function of the cycle relies on glucose availability. The results of these studies are directly pertinent to understanding the disposition of luminal peroxidized lipids in the DM state, the capacity of DM intestine in hydroperoxide metabolism, and the importance of maintaining glucose status to support intestinal detoxification of toxic hydroperoxides in DM animals.

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