ABNORMAL TOTAL NADP$^+$ AND GLUTATHIONE AND IMPAIRED GLUCOSE METABOLISM OF LARGE RAT ADIPOCYTES

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1. Introduction

Adipocytes isolated from older fatter rats are known to have decreased basal rates of fatty acid synthesis [1,2] and impaired insulin responsiveness of both the pentose phosphate cycle (PPC) and fatty acid synthesis [3,4] which are not explained by defects in insulin binding or glucose transport [5,6,2]. It has been suggested that the primary block is in lipid synthesis, since both acetyl-CoA carboxylase and fatty acid synthetase activities are diminished several-fold in such cells compared to controls [7,8], since glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities are normal [7], and since the PPC but not fatty acid synthesis of the large cells responds normally to a shift in [NADPH] : [NADP$^+$] ratios induced by the electron acceptor vitamin K_5 [7]. These studies were undertaken to investigate further the role of abnormal NADPH content or [NADPH] : [NADP$^+$] ratios in the impaired glucose metabolism of large rat cells. The results indicate that the large cells have decreased total contents of (NADPH + NADP$^+$) and (GSG + GSSG) and that abnormalities in the cellular ratios of these substances may contribute to the diminished glucose metabolism observed.

2. Materials and methods

Type I crude *Clostridium histolyticum* collagenase (lot 49H009) was obtained from Worthington Biochem., Freehold, NJ. Fraction V bovine serum albumin (lot S11008) was purchased from Reheis Chem., Kankakee, IN, and dialyzed against 2 changes of distilled water and 3 changes of Krebs-Ringer bicarbonate buffer (containing 50% the recommended [Ca^{2+}]) at pH 7.4. Sigma Chem., St Louis, MO, supplied GSH, GSSG, NADP$^+$, NADPH, 5,5'-dithiobis (2-nitrobenzoic acid), nicotinamide, glutathione reductase (type III), glucose 6-phosphate dehydrogenase (type IX), and phenazine methosulfate. Thiazolyl blue was purchased from Fisher Scientific. D-[1-^{14}C]-Glucose and D-[3-^{3}H]glucose were obtained from Amersham.

2.1. *Preparation and incubation of cells*

Isolated epididymal fat pad adipocytes were prepared by the collagenase-digestion method in [9] from male Wistar rats (Charles River Labs) at 180-220 g or 350–400 g body wt. The rats were fed similar laboratory chow ad libitum. Small rat cells were digested 60 min with shaking at 60 cycles/min, large rat cells 30–45 min at 30 cycles/min because of increased fragility. Cells at the appropriate concentration were incubated for 1 h with shaking 60 cycles/min at 37°C in 20 mg/ml albumin buffer. Incubations for assay of (NADPH + NADP$^+$), NADP$^+$, and GSH contained 0.6–1.5 × 10^6 cells, while those for measurement of glucose metabolism contained 0.6–1.5 × 10^5 cells. Final incubations were 2 ml including additives.

2.2. *Assay of coenzymes and glutathione*

Freshly prepared or incubated adipocytes were packed by centrifuging 5 min at 1800 × *g* in plastic pipet tips, the ends of which had been heat-sealed. The infranatant buffer was removed and discarded by cutting the end of the pipet tip and cells were washed into 1 ml appropriate buffer for homogenization. Cells were homogenized by 40 strokes of a hand-held Teflon–glass homogenizer.

Total glutathione (GSH + GSSG) was determined in cells homogenized in 0.1 M phosphate/0.005 M

EDTA buffer (pH 7.5). The homogenates were centrifuged 5 min at 18000 × *g*, and the aqueous infranatant removed. The cloudy infranatant was treated with cold trichloroacetic acid (final conc. 6%), and the precipitated protein removed after centrifugation at 1800 × *g* for 30 min at 0°C. Trichloroacetic acid was removed with 5-diethyl ether extractions. Aliquots (0.1–0.2 ml) were assayed with the enzymatic cycling technique [10] in a Beckman DB-DG spectrophotometer. Weighed GSH standards were prepared daily.

Total (NADPH + $NADP^+$) was measured by homogenizing cells in 0.1 M glycyl–glycine buffer (pH 7.4) which contained 0.1 M nicotinamide; $NADP^+$ was measured following homogenization in 0.02 N HCl/0.1 M Na_2SO_4 and incubation at 60°C for 45 min to destroy NADPH [11]. All samples were centrifuged at 100 000 × *g* for 1 h at 0°C and the aqueous infranatant removed and assayed for $NADP^+$ or (NADPH + $NADP^+$) using the enzymatic cycling method in [12]. Coenzyme standards were prepared daily. Using cycling periods up to 10 min coenzyme amounts as low as 3 ng/cuvette could be reliably detected. Recoveries of coenzymes added to adipocyte homogenates treated to destroy endogenous coenzymes were >90%.

2.3. *Measurement of glucose metabolism*

Oxidation of D-[1-^{14}C]glucose and incorporation of D-[3-^{3}H]glucose into lipid were measured as in [13].

3. Results

Intracellular concentrations of $NADP^+$, (NADPH + $NADP^+$), and (GSH + GSSG) from large and small rats are shown in table 1 expressed on a per cell basis. $NADP^+$ concentrations were not always detectible in fresh adipocyte homogenates. Very likely conversion of the larger amounts of NADPH occurred during homogenization and subsequent steps and this allowed measurement of $NADP^+$. Total (NADPH + $NADP^+$) content of the small rat adipocytes was significantly greater than that in the large rat adipocytes. Similarly, total glutathione content was also greater in the small rat cells than in the large rat cells (table 1). Cells from large rats after 1 h incubation had lower basal rates of glucose C-1 oxidation (table 1) and lipid synthesis (not shown) as well as lower relative insulin responses than cells from small rats (tables 1,2).

In an attempt to shift intracellular [NADPH] : [$NADP^+$] and [GSH] : [GSSG] ratios, cells from large and small rats were incubated with the respective oxidized forms as well as with insulin (table 2). The large rat cells again showed decreased relative insulin effects in glucose oxidation and lipid synthesis compared to the small cells. $NADP^+$, but not GSSG, stimulated both activities in the large rat cells to a similar degree as in the small cells, particularly when GSSG was also present. However, neither $NADP^+$, GSSG or both could restore the deficient relative insulin responses in the large cells, nor did these agents enhance the insulin responses of the small rat cells.

4. Discussion

Total (NADPH + $NADP^+$) and (GSH + GSSG) were found to be decreased on a per cell basis in adipocytes from older fatter rats compared to cells from younger

Table 1
Total (NADPH + $NADP^+$) and (GSH + GSSG) and insulin responsiveness in adipocytes from large and small rats[a]

Measurement		Large Rats	*p*	Small rats
$NADP^+$ (ng/10^6 cells)		2.6 ± 1.4	NS	3.4 ± 1.7
(NADPH + $NADP^+$) (ng/10^6 cells)		25 ± 7.2	<.05	48.8 ± 7.6
(GSH + GSSG) (μg/10^6 cells)		1.92 ± 0.09	<.001	2.45 ± 0.13
D-[1-^{14}C]Glucose oxidation	Control	3.2 ± 0.4	<.01	7.7 ± 0.4
(nmol/10^5 cells . h)	Insulin	4.5 ± 0.5	<.01	21.4 ± 2.2

[a] Values shown are $\bar{x}$ ± SE for 5 separate expt. Insulin was present at 5×10^{-9} M. Control insulin-treated glucose oxidation for large rat cells: $p < 0.02$. Statistical comparisons were made using the Student's paired '*t*'-distribution

Table 2
Responsiveness (% of control) of large and small adipocytes to changes in redox state[a]

Addition	D-[1-^{14}C]Glucose oxidation			D-[3-^{3}H]Glucose incorp. into lipid		
	Large Rats	p	Small rats	Large rats	p	Small rats
Insulin	210 ± 29	<0.05	359 ± 34	153 ± 25	<0.05	241 ± 18
$NADP^+$	147 ± 8	n.s.	172 ± 21	172 ± 18	n.s.	179 ± 28
GSSG	98 ± 5	n.s.	109 ± 7	99 ± 8	n.s.	98 ± 9
$NADP^+$ + GSSG	171 ± 13	n.s.	175 ± 21	163 ± 22	n.s.	175 ± 31
Insulin + $NADP^+$	238 ± 27	<0.05	393 ± 21	196 ± 16	n.s.	246 ± 26

[a] Values shown are $\bar{x}$ ± SE for 9 separate expt. Statistical analysis was as in table 1. $NADP^+$ vs $NADP^+$ + GSSG for large rat glucose oxidation: $<p$ 0.05. Concentrations employed: $NADP^+$, 0.5 mM; GSSG, 1 mM and insulin, 5×10^{-9} M

leaner rats. This appears a valid method for expressing the data since the difference in size between the two cell types is related to the size of the triglyceride globule and not to cytoplasmic protein content [3]. It is possible that these differences were caused by greater loss of nucleotide or glutathione during pad digestion and cell preparation, since large adipocytes are more fragile. Since care was taken to avoid cell breakage in the large cell digestions, it is more likely that the differences observed were related to diminished PPC activity and fatty acid synthesis in the cells from the large rats. Similar decreases in NADPH were found [14] in fat pads from alloxan-diabetic rats, which also exhibit insulin resistance [15].

Because of low concentrations of $NADP^+$ it is not possible to derive an accurate estimate of [NADPH] : [$NADP^+$] ratios from these results, although as expected, much of the total (NADPH + $NADP^+$) was in the reduced form [14].

Even in the presence of medium glucose concentrations (0.28 mM) at which glucose transport is probably rate-limiting for metabolism [6], the larger rat cells had diminished insulin responses in both glucose carbon-1 oxidation (a measure of PPC activity) and lipid synthesis (table 1,2). Measurement of incorporation of ^{3}H from carbon-3 of glucose into lipid has the additional advantage of reflecting the contribution of NADP ^{3}H produced in the PPC to fatty acid synthesis, since it has been shown particularly under stimulated conditions that the use of pentose phosphate cycle NADPH in fatty acid synthesis is very efficient [16]. Large rat adipocyte glucose carbon-1 oxidation but not fatty acid synthesis responds normally to the electron acceptor vitamin K_5 [7], which causes a decrease in the [NADPH] : [$NADP^+$] ratio. Direct incubation with $NADP^+$, particularly in the presence of GSSG (table 2), resulted in similar relative increases in glucose carbon-1 oxidation and transfer of ^{3}H from glucose to lipid in the large and small cells. Assuming diffusion of some $NADP^+$ into cells, these results suggest that both the PPC and fatty acid synthesis of the large cells are capable of responding to a decreased [NADPH] : [$NADP^+$] ratio. However, since $NADP^+$ did not significantly improve the impaired insulin responsiveness in the large cells (table 2), altered [NADPH] : [$NADP^+$] ratios may not be the only factors involved in that defect.

It is concluded that diminished basal rates of PPC activity and lipid synthesis in large adipocytes are associated with decreased total (NADPH + $NADP^+$) contents, and that some of the decrease in basal rates may be due to abnormal [NADPH] : [$NADP^+$] ratios from diminished NADPH consumption in the fatty acid synthetic pathway.

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