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ROLE OF CELLULAR REDOX STATE AND GLUTATHIONE IN ADENYLATE CYCLASE ACTIVITY IN RAT ADIPOCYTES

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Summary

Adenylate cyclase in rat adipocyte membranes was inactivated as a result of treatment with sulfhydryl oxidants or with *p*-chloromercuribenzoate as well as by S-alkylating agents. The inhibition of the basal and isoproterenol- or glucagon-stimulated enzyme activity by the oxidants or the mercurial could be reversed by adding thiols to the isolated membranes. The activity of the enzyme paralleled the cellular glutathione (GSH) content. Lowering of intracellular glutathione by incubating the cells with specific reactants resulted in the inhibition of both basal and hormone-stimulated adenylate cyclase activity in the isolated membranes. Activity could be partly restored by supplying glucose to the incubation medium of intact cells. The fluoride-stimulated adenylate cyclase was also inhibited by the oxidants or the sulfhydryl inhibitors. The results suggest that adenylate cyclase may be partly regulated by oxidation-reduction. Thus, a direct relationship between both basal and hormone-stimulated adenylate cyclase activity and the cellular redox potential, determined by the cellular level of reduced glutathione, may be ascribed to the protection of the catalytic -SH groups of the enzyme from oxidative or peroxidative reactions and maintenance of the redox optimum for the reaction.

Introduction

Adenylate cyclase is one of the major enzyme systems associated with the plasma membrane which mediate hormonal signals to intracellular metabolic

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pathways [1]. Despite notable attempts toward an understanding of the molecular mechanism for the response of this enzyme to stimulation by hormones and drugs [2—4], there is very little information about the possible intracellular factors which may modify or regulate its activity under physiological conditions. The product of this enzymatic reaction, cyclic AMP, is known to be the regulator of various kinetic reactions through specific protein kinases [5]. It is logical to speculate that some product(s) of these metabolic pathways may also control the adenylate cyclase activity. Such cellular regulation of this enzyme activity is still poorly understood because the structure-activity relationships of adenylate cyclase activity have been relatively little explored. However, an important role of some sulfhydryl groups was previously implicated by the studies employing sulfhydryl reagents [6—9]. These data also demonstrate the essential role of some sulfhydryl groups in the catalytic component of the enzyme for both the basal and hormone- or fluoride-stimulated activity which can be severely impaired by any oxidative or peroxidative attack. We report here a relationship between adenylate cyclase activity and variations in the cellular redox potential which is coupled with glucose oxidation within the cells. An important role of cellular glutathione (reduced) in maintaining the active state of adenylate cyclase in these cells is evident from studies on experimentally changing the GSH content of whole cells. It is suggested that adenylate cyclase activity may vary, at any steady state, with the availability or regeneration of cellular glutathione.

Materials and Methods

Adipocytes were isolated by digestion of fat pads from female CD strain rats, 200—225 g body weight (Charles River Co., Boston, MA) with crude collagenase from *Clostridium histolyticum* (Worthington Biochemical Corp.) according to Rodbell [10]. The pooled fat pads (4—5 g) were incubated at 37°C for 1 h in 7—8 ml of Krebs-Ringer phosphate buffer (pH 7.4), containing 3% bovine serum albumin (fraction V, lot No. M47508, Armour Pharmaceutical, Chicago, IL), in the presence of collagenase (1 mg/ml). The cells were filtered through two layers of cheesecloth and washed twice with warm 1% albumin/Krebs-Ringer phosphate buffer. The composition of the buffer (Krebs-Ringer phosphate) was: 126 mM NaCl, 1.4 mM $CaCl_2$, 5.2 KCl, 1.4 mM $MgSO_4$, and 10 mM $NaHPO_4$, at pH 7.4.

For the experiment to alter intracellular glutathione, intact adipocytes were incubated in the Krebs-Ringer phosphate buffer containing 1% albumin and the indicated additions of reagents which preferentially oxidize cellular GSH, such as diamide [11], hydrogen peroxide [12] and *t*-butyl hydroperoxide [13] or which alkylate GSH, such as tosyl-1-lysine chloromethyl ketone [14] or *N*-ethylmaleimide [15] at 37°C for 30 min; this was followed by washing of cells and isolation of membranes. Cells were rapidly washed with warm buffer, resuspended in a lysing medium (5 times diluted buffer) and homogenized in a chilled glass-Teflon homogenizer (4—5 strokes) and centrifuged On Sorvall at 20 000 × *g* for 10 min at 0—2°C. The pellets were used for adenylate cyclase assay.

Adenylate cyclase was assayed in the particular membrane preparations,

resuspended in 75 mM Tris-HCl (pH 7.4), containing 25 mM $MgCl_2$, according to Mukherjee et al. [16] and cyclic [^{32}P]AMP isolated according to Salomon et al. [17]. The final composition of the assay medium was: 30 mM Tris-HCl (pH 7.4)/10 mM $MgCl_2$/1.5 mM theophylline/0.1 mM cyclic AMP/1.5 mM ATP/5 mM phospho*enol*pyruvate/40 μg/ml pyruvate kinase/20 μg/ml myokinase and $1-2 \cdot 10^6$ cpm of [α-^{32}P]ATP. Glutathione in adipocytes was estimated in the cell-free supernatants, according to Hotta [18]. 1.0 ml-aliquots of cells were withdrawn at intervals, followed by homogenization and centrifugation at 27 000 × *g* for 5 min at 0—3° C on a Sorvall centrifuge. Protein was determined by the method of Lowry et al. [19].

Materials. Crystalline porcine glucagon was a gift from Eli Lilly and Company (Ann Arbor, MI). L-Isoproterenol, dithiothreitol, β-mercaptoethanol, *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, tosyl-1-lysine chloromethyl ketone, adenosine 5′-triphosphate, cyclic 3′,5′-adenosine monophosphoric acid (sodium salt), phospho*enol*pyruvate, myokinase and other reagents were purchased from Sigma Chemical Co., St. Louis, MI. Alumina (neutral grade) was from E. Merck Company. Dowex AG 50-X8 was from BioRad. Diamide (diazine dicarboxylic acid-bis-*N*,*N*-dimethylamide) was purchased from Calbiochem. *t*-Butyl hydroperoxide was from Aldrich Chemical Co. Hydrogen peroxide and some of the inorganic chemicals were purchased from Fisher Scientific Company. The radiochemicals, viz. cyclic [^{3}H]AMP and [α-^{32}P]ATP were purchased from New England Nuclear (Boston, MA).

Results

Adenylate cyclase activity in the isolated membranes of adipocytes was inhibited due to the addition of oxidants such as H_2O_2 and sulfhydryl inhibitors such as *p*-chloromercuribenzoate (Fig. 1). The inhibitory effect of the oxidant or the mercurial was reversible in the presence of a molar excess of thiols such as β-mercaptoethanol. We noted that in two out of seven experiments described in Fig. 1, H_2O_2 only at a low concentration (0.1 mM) but not *p*-chloromercuribenzoate, had a reverse effect, viz. a 30% increase rather than a decrease in the enzyme activity. In these cases, addition of a thiol partly depressed the activity. This variation may be explained as due to the possible involvement and availability of an intermediate redox component, which when oxidized, may in turn reduce and activate the catalytic site. However, the inhibitory effect of the S-alkylating agents, e.g. *N*-ethylmaleimide was irreversible. The protective role of glutathione on hormone-sensitive adenylate cyclase against the inactivation by oxidants was also evident by adding GSH (or other thiols) to the assay of the cyclase activity in the isolated membranes which were pretreated with a sulfhydryl oxidant, 2 mM *o*-iodosobenzoate followed by washing. Such treatment of the membranes decreased the basal activity and abolished the response to isoproterenol. As illustrated in Fig. 2, subsequent addition of glutathione to the membranes reversed this inhibitory effect and restored the hormonal sensitivity (Fig. 2). This effect was observed with GSH concentration upto 5 mM. The *S*-methyl derivative of glutathione competed with the effect of GSH; oxidized glutathione (GSSG) had no effect. These data indicate that the enzyme activity is critically dependent on the reduced (sulf-

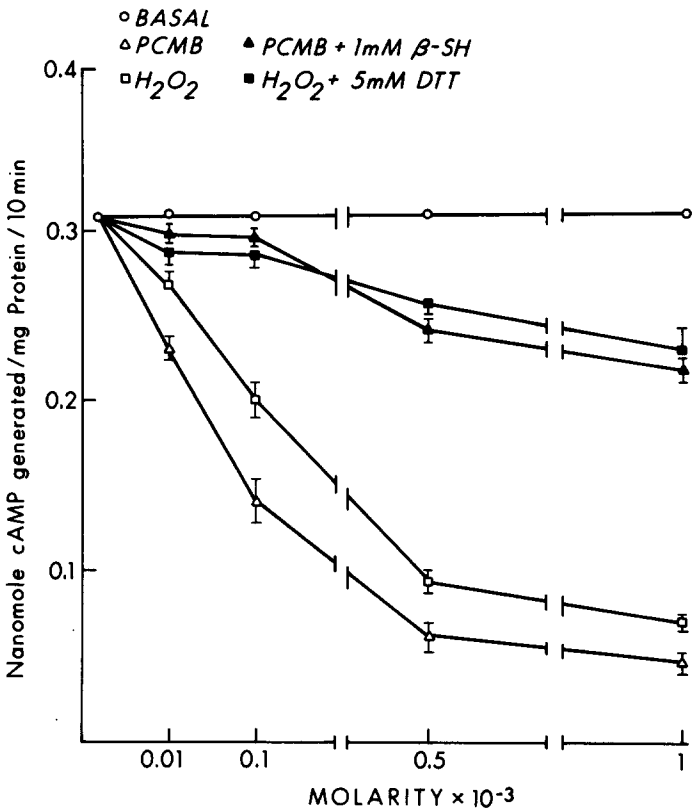

Fig. 1. Inhibition of adenylate cyclase activity in adipocyte membranes by different molar concentrations of an oxidant, hydrogen peroxide or a mercurial, and its reversal with a thiol. The values are averages of four duplicate experiments. Hydrogen peroxide (H_2O_2) or *p*-chloromercuribenzoate (pCMB) was added to the membranes at the concentrations indicated on the abscisa. 5 mM dithiothreitol (DTT) or 1 mM β-mercaptoethanol (β-SH) was added to the assay tubes where indicated at 0° C, 2 min before the oxidant or the mercurial.

hydryl) state of the catalytic component of the enzyme and a physiological regulation possibly involves an oxidation-reduction reaction coupled with other redox components of the cell. It is interesting to note, however, that a full recovery (100%) of the hormonal response could not be achieved by adding the -SH reductants, and an excess of GSH (10 mM) resulted in a partial decline from the rate obtained at 5 mM GSH, rather than linearly increasing the activity. This indicates that the process of hormonal activation may require also some oxidized component of the membrane, as indicated above.

The oxidants and the -SH blocking reagents inhibited the adenylate cyclase activity in the presence of stimulatory hormones such as isoproterenol and glucagon, as well as the activation of NaF (Table I). It was suggested [20,21] that fluoride affects this enzyme directly (i.e beyond the regulatory components, the hormone receptors) by reacting with a non-catalytic site. Alternatively, it has been hypothesized that fluoride may activate the cyclase by removing some inhibitory factors [2]. However, other evidence indicates that

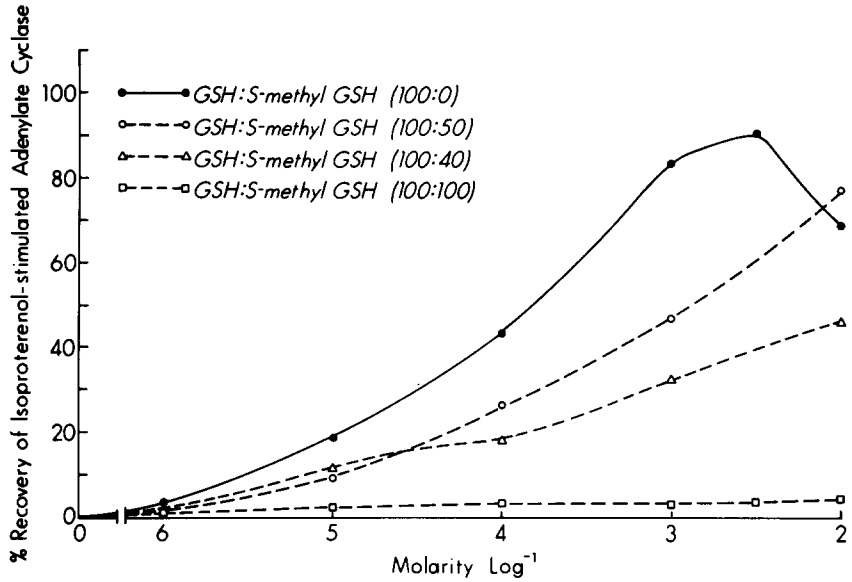

Fig. 2. A competitive inhibitory effect of *S*-methyl glutathione on restoration by reduced glutathione of isoproterenol-sensitive adenylate cyclase activity in adipocyte membranes pre-exposed to a sulfhydryl oxidant. The values are averages of three experiments in duplicate. The basal activity without the treatment with 2 mM *o*-iodosobenzoate was: 0.37 ± 0.015 nmol/10 min per mg, and with 10 μM L-isoproterenol: 1.04 ± 0.02 nmol/10 min per mg; treatment with the oxidant for 5 min at 0°C, followed by washing yielded a basal rate of 0.084 ± 0.006 nmol/10 min per mg. Addition of L-isoproterenol to this preparation yielded 0.124 ± 0.01 nmol/10 min per mg. The basal activity of the oxidant-treated membranes, in the presence of 5 mM GSH was: 0.215 ± 0.012 nmol/10 min per mg. The values are expressed as percentage of isoproterenol-stimulation under this reducing condition in the presence of glutathione vis-a-vis the hormone's effect on the untreated control membranes. The concentration of GSH was varied with different proportions of *S*-methyl GSH in the assay.

the effect of fluoride is synergistic with hormonal stimulation of adenylate cyclase [8,22]. We examined the relative effects of the hormones or fluoride with that of sulfhydryl oxidation, using an oxidant, H_2O_2, a mercurial, *p*-chloromercuribenzoate, and an alkylating agent, *N*-ethylmaleimide (Table I). Pre-exposure of the membrane preparations to the sulfhydryl-reactive agents for 2 min at 0°C resulted in a complete loss of response to the subsequently added isoproterenol, glucagon or fluoride. But prior exposure of the membranes to either of the hormones or the alkylating agents, caused a partial suppression of the stimulated enzyme activity. *N*-Ethylmaleimide, however, inhibited the basal and stimulated enzyme activities more severely and irreversibly (Table I).

These results indicate the possibility that the activation or inactivation of adenylate cyclase involves interconvertible conformational changes in the catalytic component. We have also found that the inhibition of adenylate cyclase activity by oxidants or the -SH blocking agents involved a lowering of the *V* of the enzyme activity, rather than a change in its affinity toward the substrate ATP or the cofactor, Mg^{2+} (Fig. 3). This relationship is generally expected where a conformational alteration by the sulfhydryl groups is involved.

TABLE I

EFFECT OF SULFHYDRYL INHIBITORS ON HORMONE- AND FLUORIDE-STIMULATION OF ADENYLATE CYCLASE ACTIVITY IN ADIPOCYTE MEMBRANES

Values are averages of four paired experiments. L-Isoproterenol (10^{-5} M), glucagon (10^{-5} M) or NaF (10^{-2} M) were added to the isolated membranes before or after the additions of H_2O_2, *p*-chloromercuribenzoate (pCMB) or *N*-ethylmaleimide (NEM), each at 10^{-3} M. The first treatment was done at 0°C, 2 min prior to the second.

First treatment	Second treatment	Cyclic AMP generated (pmol/mg per 10 min)	First treatment	Second treatment	Cyclic AMP generated (pmol/mg per 10 min)
None	—	376 ± 45	H_2O_2	L-Isoproterenol	635 ± 26
	H_2O_2	216 ± 14		Glucagon	268 ± 16
	pCMB	135 ± 22		NaF	872 ± 45
	NEM	48 ± 8	pCMB	L-Isoproterenol	484 ± 25
L-Isoproterenol	—	1265 ± 68		Glucagon	365 ± 28
	H_2O_2	864 ± 28		NaF	724 ± 46
	pCMB	734 ± 16	NEM	L-Isoproterenol	84 ± 14
	NEM	228 ± 18		Glucagon	76 ± 12
Glucagon	—	936 ± 46		NaF	146 ± 22
	H_2O_2	546 ± 26			
	pCMB	472 ± 28			
	NEM	142 ± 18			
NaF	—	2654 ± 125			
	H_2O_2	1765 ± 84			
	pCMB	1440 ± 55			
	NEM	264 ± 30			

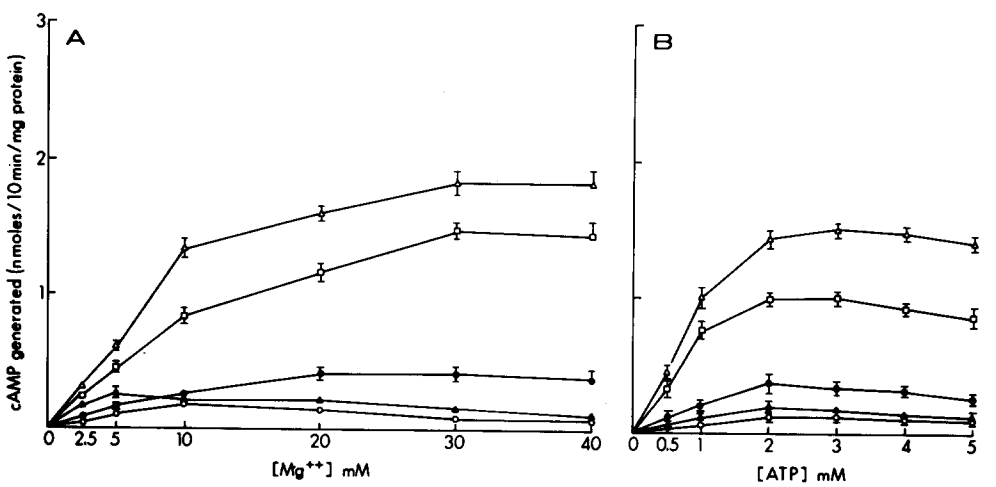

Fig. 3. Changes in the adenylate cyclase activity in adipocyte membranes in the presence of isoproterenol, glucagon, or sulfhydryl oxidants at varying concentrations of Mg^{2+} (A) and adenosine 5'-triphosphate (B). ●, basal; ○, 1 mM *o*-isodosobenzoate; ▲, 1 mM hydrogen peroxide; □, 10 μM glucagon and △, 10 μM L-isoproterenol. The values are the averages ±S.E. of four paired experiments.

A possible in vivo role of reduced glutathione in the adenylate cyclase activity in these cells was examined by pre-incubating whole cells in the presence of reagents which are known to lower cellular GSH (Tables II and III) either by oxidizing it, e.g. diamide [11], H_2O_2 [12], or *t*-butyl hydroperoxide [13] or by S-alkylating GSH, e.g. tosyl-L-lysine chloromethyl ketone [14]. A similar impairment of the cyclase activity was observed by exposing whole cells to *N*-ethylmaleimide employed at a lower concentration (0.1 mM) than the other agents; it should be noted that *N*-ethylmaleimide is reported to react with intracellular GSH as well as to inactivate other enzymes, e.g. glutathione reductase and glutathione peroxidase [15] and at higher concentrations may cause cellular lysis [22,23]. The relationship of adenylate cyclase activity with the cellular GSH is also an important determinant of hormonally stimulated enzyme activity, as illustrated in Table II: the response to hormones, e.g. isoproterenol or glucagon, of the cyclase activity in the membranes isolated from cells treated with the GSH-oxidants was also substantially lost. When D-glucose (1 mM) was added to the pre-incubation medium of cells in the presence of the GSH-oxidiziong or S-alkylating agents, the inhibitory effect of these agents on the cyclase activity, as determined in the isolated membranes, was partly reversed. Presence of D-glucose in the medium prevented the

TABLE II

EFFECT OF TREATMENT OF ADIPOSE CELLS WITH REAGENTS TO LOWER CELLULAR GLUTATHIONE ON THE ADENYLATE CYCLASE ACTIVITY IN ISOLATED MEMBRANES

The cells suspended in Krebs-Ringer phosphate buffer, pH 7.4, containing 1% albumin, were incubated at 37°C for 30 min with or without one of these agents: 1 mM diamide, 4 mM H_2O_2, 1 mM *t*-butyl hydroperoxide, 1 mM tosyl-L-lysine chloromethyl ketone, or 0.1 mM *N*-ethylmaleimide, in the absence or presence of 1 mM D-glucose. The media were removed, the cells washed and membranes isolated for the assay of adenylate cyclase activity. The values are averages ± S.E. of five paired experiments.

Additions to cells in the pre-incubation	Addition to assay	Adenylate cyclase activity (pmol cyclic AMP generated/mg protein per 10 min)	
		Membranes from cells pre-incubated without glucose	Membranes from cells pre-incubated with glucose
None (control)	—	450 ± 64	782 ± 34
	L-Isoproterenol	1958 ± 83	2420 ± 92
	Glucagon	1540 ± 75	1676 ± 88
Hydrogen peroxide	—	254 ± 42	458 ± 32
	L-Isoproterenol	856 ± 52	2130 ± 44
	Glucagon	852 ± 47	1518 ± 65
t-Butyl hydroperoxide	—	215 ± 32	673 ± 22
	L-Isoproterenol	722 ± 36	1625 ± 68
	Glucagon	630 ± 24	1375 ± 34
Diamide	—	116 ± 18	210 ± 32
	L-Isoproterenol	175 ± 30	1928 ± 36
	Glucagon	380 ± 22	1370 ± 35
Tosyl-L-lysine chloromethyl ketone	—	156 ± 20	755 ± 48
	L-Isoproterenol	315 ± 36	2070 ± 82
	Glucagon	356 ± 26	1946 ± 64
N-Ethylmaleimide	—	135 ± 16	246 ± 28
	L-Isoproterenol	418 ± 26	442 ± 35
	Glucagon	312 ± 15	320 ± 16

TABLE III

CHANGES IN THE NON-PROTEIN SULFHYDRYL CONTENT OF ADIPOCYTES EXPOSED TO HORMONES AND GSH-OXIDANTS: EFFECT OF D-GLUCOSE IN THE MEDIUM

The cells were incubated in the absence or presence of 1 mM D-glucose and/or 10^{-4} M L-isoproterenol, 10^{-4} M glucagon, 4 mM H_2O_2, 1 mM *t*-butyl hydroperoxide, 1 mM diamide, 1 mM tosyl-L-lysine chloromethyl ketone or 0.1 mM *N*-ethylmaleimide. The values are averages ± S.E. of four paired experiments.

Additions to medium	Total non-protein sulfhydryl content (nmol/10^7 cells)	
	Glucose absent in medium	Glucose present in medium
None	70.0 ± 5.3	103.6 ± 6.4
Isoproterenol	95.2 ± 4.4	154.0 ± 6.0
Glucagon	105.0 ± 4.2	142.4 ± 5.5
Hydrogen peroxide	53.0 ± 5.5	115.0 ± 6.5
t-Butyl hydroperoxide	26.7 ± 4.5	87.0 ± 4.4
Diamide	38.2 ± 4.0	96.0 ± 3.8
Tosyl-L-lysine chloromethyl ketone	30.6 ± 3.2	82.4 ± 4.0
N-Ethylmaleimide	15.6 ± 1.5	26.6 ± 3.4

inactivation of the enzyme activity, as evident from its nearly normal response to isoproterenol or glucagon (Table III). Cellular GSH is replenished from the oxidized glutathione by the glutathione reductase activity, using NADPH, in addition to its rapid de novo synthesis [24]. The contribution of glucose utilization to the GSH content of cells is documented by the data in Table III.

Discussion

It is likely that a labile and biologically crucial plasma membrane enzyme such as adenylate cyclase, the activity of which appears to depend upon sulfhydryl groups [6—9], may be subjected to an oxidative or a peroxidative attack, as obtained under these experimental conditions, but protected by cellular GSH [25—28]. Our data clearly indicate that the catalytic component of the enzyme is active only in the reduced state of its key sulfhydryl groups and inactive in the oxidized (disulfide) state. Since this site is in the cytoplasmic face of the plasma membrane [2], its physiological regulation may be coupled with intracellular redox potential.

The readily reversible nature of the inhibitory effect of oxidants or the mercurial suggests that a physiological control of the enzyme activity may involve a redox reaction. The present findings are relevant to our recent discovery that insulin or some insulin-mimicking reagents activate in the whole adipocyte plasma membrane an enzyme, pyridine nucleotide oxidase [29,30] the product of which, H_2O_2, or a peroxidative complex of H_2O_2 [15,31,32] may be responsible for the inhibitory effect of insulin on adenylate cyclase. The protective role of cellular GSH in adenylate cyclase activity was examined in two ways: by lowering of intracellular GSH (Tables II and III) and permitting the regeneration of GSH by supplying glucose under conditions wherein the cyclase activity was observed to be inhibited. Both these manipulations, together with measurement of cellular GSH in the presence or absence of

glucose and/or hormones (Table III) confirmed our expectation that GSH level is an important determinant of adenylate cyclase activity. The pathways for glutathione turnover in these cells, other than de novo synthesis [24,33] is coupled with the hexose monophosphate shunt activity as shown below:

1. $NADPH_2 + O_2 \rightarrow NADP^+ + H_2O_2$ [29,30]

2. $H_2O_2 + 2\ GSH \xrightarrow[\text{peroxidase}]{\text{glutathione}} GSSG + H_2O$

3. $GSSG + NADPH + H^+ \xrightarrow[\text{reductase}]{GSSG} 2\ GSH + NADP^+$

4. $NADP^+ + \text{glucose 6-phosphate} \xrightarrow{G6PD} NADPH + H^+ + \text{6-phosphogluconate}$

A number of recent reports documented that adenylate cyclase becomes less responsive to hormonal stimuli under various experimental and pathophysiological conditions [34—37]. Such refractoriness may arise from physicochemical changes in the receptors, in the intermediate effector system, in the enzyme itself or due to alterations in some intracellular metabolic parameter. The present study suggests that changes in the cellular redox state is a major control. The proposal seems the more attractive in view of the previous suggestion that adenylate cyclase is regulated by phosphorylation-dephosphorylation reactions [38] in the pattern of many other enzyme systems which are dependent on cyclic AMP. It is possible that in addition, an oxidation-reduction metabolic control is involved for a dual regulation of this extremely crucial enzyme system.

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