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EVIDENCE THAT THE LIPOLYTIC DEFECT INDUCED BY ESTRADIOL-TREATMENT IN HAMSTER ADIPOCYTES IS RELATED TO AN ESTROGEN RECEPTOR-MEDIATED DEFECT IN THE ADENYLATE CYCLASE CATALYTIC SUBUNIT BUT NOT IN N_s

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This study demonstrates that estradiol-treatment (10 µg per day x 5 days) does not impair the level of N_s, the adenylate cyclase stimulatory regulatory protein, in hamster fat cell membranes. In addition, this report shows that the defective cyclic AMP response induced in intact adipocytes by the estradiol-treatment is either unaltered by the administration of α-bromocryptine or abolished by tamoxifen- or 4-hydroxytamoxifen-treatment. It can thus be concluded that the reduced lipolytic response found in hamster fat cells after estradiol-treatment is related only to an estradiol-receptor-mediated defect in adenylate cyclase catalytic subunit activity which is independent from increased prolactin secretion.

In a recent study (1), we have shown that estradiol (10 µg per day) administered for 5 days to male hamsters results in a 50 % decrease in the in vitro lipolytic and cyclic AMP responses of white adipocytes to catecholamines, ACTH and theophylline. We also reported that these alterations were correlated with parallel decreases in the adenylate cyclase activity which could not be explained either by altered adenylate cyclase activatory receptors such as the β-adrenoceptors or by changes in the activity of the inhibitory pathway controlling adenylate cyclase (2). Moreover, although the status of N_s, the stimulatory regulatory protein of adenylate cyclase (3) was not directly investigated, these experiments suggested that the mechanism through which estradiol-treatment alters lipolysis in hamsters adipocytes is a defect in the adenylate cyclase catalytic subunit rather than in the N_s protein (1).

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The first aim of the present study was therefore to quantify the N_s protein in order to provide convincing evidence that this protein is or not altered by the estradiol-treatment. This was accomplished by comparing the cholera-toxin catalyzed ^{32}P-ADP ribosylation of the functional α_s-subunit of N_s (4) in fat cell membranes from control and estradiol-treated hamsters. Our second purpose was to establish whether the lipolytic changes seen after estradiol-treatment are due to estradiol per se and, if so, to an estrogen-receptor-dependent effect or are the consequence of increased prolactin secretion induced by the estradiol-treatment (5). This was examined by testing the ability of two estrogen-receptor antagonists, tamoxifen (6) and 4-hydroxytamoxifen (7), and the prolactin-secretion inhibitor, α-bromocryptine (8) to modulate the effects of the estradiol-treatment on the cyclic AMP response of hamster fat cells.

MATERIALS AND METHODS

Six-week-old male golden hamsters (100 $\pm$ 10 g) received one daily intramuscular injection of estradiol benzoate (10 µg/animal) in olive oil for 5 days. Control animals received the vehicle only. In some experiments, control and estradiol-treated hamsters received along with their treatment additional daily subcutaneous injections of α-bromocryptine (500 µg/animal), tamoxifen citrate or 4-hydroxytamoxifen (50 µg/animal). Animals were sacrificed on the sixth day.

Epididymal and perirenal adipose tissues were pooled and isolated fat cells prepared as previously described (9). Adipocytes were incubated for 60 min (lipolysis) or 10 min (cyclic AMP) at 37°C in Krebs-Ringer- 25 mM Tris buffer, pH 7.4, containing 5 mM glucose, albumin (3 % for lipolysis and 2 % for cyclic AMP assays) and, when indicated, 0.1 mM theophylline and 50 mU/ml adenosine deaminase. Glycerol release and cyclic AMP production were measured as previously described (10).

ADP-ribosylation of N_s was performed as follows. Fat cell membranes (100 µg protein) prepared as previously described (11) were incubated for 20 min at 30°C in a final volume of 0.1 ml containing 100 mM KH_2PO_4 (pH 7.4), 1mM EGTA, 5 mM $MgCl_2$, 10 mM thymidine, 1 mM ATP, 0.5 mM GTP, 2 mM dithiothreitol, 3 µM [α^{32}P] NAD^+ and 25 µg preactivated cholera toxin (A subunit). Cholera toxin was preactivated in 20 mM dithiothreitol for 10 min at 37°C immediately prior to be used. Following the labeling reaction, membranes were washed, solubilized and subjected to electrophoresis on 10 % polyacrylamide gels in the presence of 0.1 % SDS (12). Gels were stained with Coomassie blue, dried and autoradiographed at - 80°C for 2-3 days using Kodak X-Omat AR film in the presence of intensifying screens. Bands of radioactivity were excised from the dried gels, dissolved in 0.4 ml of 30 % H_2O_2 by heating for 45 min at 70°C, and counted. Proteins were determined by the dye-binding procedure of Bio-Rad Laboratories (13).

α-Bromocryptine was kindly provided by Sandoz Pharmaceuticals and tamoxifen and 4-hydroxytamoxifen by ICI Pharmaceuticals. GTP, ATP, NAD^+ and cholera toxin (A subunit) were from Sigma, adenosine deaminase and thymidine from Boehringer Mannheim, forskolin and bovine albumin from Calbiochem, [^{3}H]-cyclic AMP and cyclic AMP binding protein from Amersham, collagenase (CLS, spec. act. 148 U/mg) from Cooper and [^{32}P] NAD^+ (spec. act. 37.5 Ci/mmol) from N.E.N.

RESULTS AND DISCUSSION

Confirming our recent observation (1), isoproterenol (1 µM)- stimulated lipolysis was depressed by 40 % after estradiol-treatment. The same was also observed

TABLE I

Lipolytic response of white fat cells from control and estradiol-treated hamsters

Addition	Glycerol production (umol/g lipids/60 min)	
	Control	Estradiol-treated
None	1.2 ± 0.2	1.0 ± 0.2†
(-)-Isoproterenol (1 μM)	18.5 ± 1.5	10.9 ± 0.8*
Forskolin (0.1 mM)	21.3 ± 1.6	13.0 ± 1.2*
Theophylline (0.1 mM)	18.5 ± 0.7	10.8 ± 1.6*
Dibutyrylcyclic AMP (5 mM)	17.9 ± 1.3	16.9 ± 1.5†

Fat cells were incubated as described under Materials and Methods in the presence of adenosine deaminase (50 mU/ml). Each value represents the mean ± S.E.M. of 3 to 5 experiments. † = non-significant ; * = $P < 0.01$.

when cells were incubated with the receptor-independent adenylate cyclase activator (14) forskolin (10 μM) or the phosphodiesterase inhibitor theophylline (0.1 mM) (Table I). In contrast, lipolysis stimulated by the cyclic AMP analog dibutyryl cyclic AMP (5 mM) was unaltered by estradiol-treatment (Table I), a finding which adds further support to our previous suggestion that impaired adenylate cyclase function is the basis of the defective lipolytic response seen in hamster adipocytes after estradiol- treatment.

As shown in Table II, essentially the same results were found when cyclic AMP productions were examined. In fact, in the presence of adenosine deaminase (50 mU/ml) and theophylline (0.1 mM) to prevent both adenylate cyclase inhibition by endogenous adenosine and phosphodiesterase interference, basal and isoproterenol- (1 μM) or forskolin- (10 μM) stimulated cyclic AMP productions were all depressed in estradiol-treated hamsters (45 to 68 % below the control values). As also shown in this Table, administration of bromocryptine alone reduced basal, isoproterenol and forskolin-stimulated cyclic AMP productions as well. (The mechanism of this effect of bromocryptine remains unclear, although in vivo treatment with prolactin influences cyclic AMP in hamsters fat cells-unpublished observation). When bromocryptine- treated hamsters were simultaneously injected with estradiol, however, a further decrease in cyclic AMP productions was observed, and it is important to note that the magnitude of this effect was similar to that induced by estradiol in control hamsters. As the bromocryptine-treatment used in these experiments

TABLE II

Influence of α-bromocryptine (α-Br) and tamoxifen (Tam) treatments on basal, (-)-isoproterenol- and forskolin-stimulated cyclic AMP production in white adipocytes from control (C) and estradiol- (E_2)-treated hamsters

Addition	Cyclic AMP production (nmoles/g lipids/10 min)					
	C	E_2	α-Br	α-Br + E_2	Tam	E_2 + Tam
None	1.37 ± 0.21	0.44 ± 0.05* (-68)	0.81 ± 0.11*	0.24 ± 0.02** (-70)	0.72 ± 0.06*	0.80 ± 0.09† (+11)
(-)-Isoproterenol (1 uM)	46.11 ± 2.87	25.30 ± 1.75* (-45)	29.70 ± 1.81*	17.22 ± 1.53** (-42)	29.00 ± 2.07*	26.19 ± 1.85† (-10)
Forskolin (10 uM)	32.02 ± 2.86	15.34 ± 1.05* (-52)	19.86 ± 1.80*	9.56 ± 0.59** (-52)	11.14 ± 1.25*	10.40 ± 0.91† (-7)

Fat cells were incubated as described under Materials and Methods in the presence of theophylline (0.1 mM) and adenosine deaminase (50 mU/ml) alone or in combination with the indicated added compounds. Each value is the mean ± S.E.M. of 5 experiments performed in duplicate. Numbers shown in parentheses represent the effects of the estradiol-treatment expressed as percentage of the values found in the control, α-Br or Tam groups, respectively. * = $P < 0.01$ compared to control value ; ** = $P < 0.01$ compared to the α-Br values ; † = non-significant compared to the Tam values.

has been shown to be adequate to abolish prolactin secretion in various species (15-16), the above results indicate that the impaired adenylate cyclase function induced by estrogen in hamster adipocytes is not related to increased prolactin secretion.

In hamsters treated only with tamoxifen (Table II) or 4-hydroxytamoxifen (Fig. 1), basal and stimulated cyclic AMP productions were also reduced, but the potency of 4-hydroxytamoxifen in decreasing forskolin-stimulation was weaker than the potency of tamoxifen. Although unexpected, we cannot exclude that these effects are due to a partial estrogen-agonistic action of these compounds in hamster fat cells as was previously reported in rat uterus for tamoxifen (17, 18) and in rat adipose tissue for nafoxidin (19), another estrogen-antagonist. At all events, data in Table II and Fig. 1 clearly demonstrate that when tamoxifen- or 4-hydroxytamoxifen- treated hamsters received estradiol, no further decrease in fat cell cyclic AMP productions could be observed. These experiments, which fail to show any additivity between the inhibitory actions of estradiol and these estrogen-antagonists, strongly suggest that the mechanism underlying the defective adenylate cyclase response found in estradiol-treated hamster is estrogen-

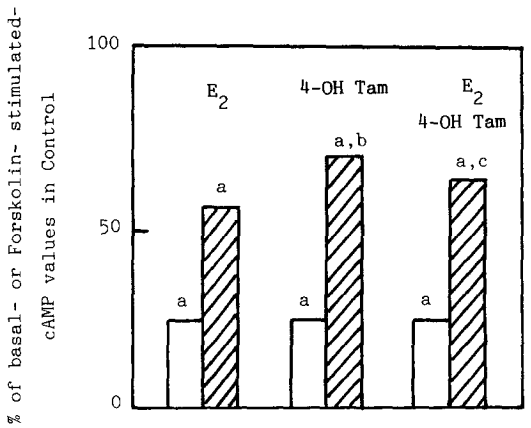

Fig. 1. Influence of 4-hydroxytamoxifen (4-OH Tam) treatment on basal and forskolin-stimulated cyclic AMP production in white adipocytes from control and estradiol- (E_2) treated hamsters. Fat cells were incubated as described under Table II. Each value is the mean of 3 experiments performed in duplicate. Open and hatched bars represent basal and forskolin-stimulated cyclic AMP productions, respectively, and are expressed as percentage of the basal (0.95 $\pm$ 0.18) or forskolin-stimulated values (31.36 $\pm$ 3.82 nmoles cyclic AMP/g lipids/10 min) found in the controls. (a) = $P < 0.01$ compared to control (b) = $P < 0.01$ compared to E_2 ; (c) = non-significant compared to E_2 and 4-OH Tam.

receptor-dependent. Whether the concerned receptors belong to the adipocytes (20) or to other tissues cannot yet be answered.

Finally, recent reports having shown that estrogens (21) and other steroid hormones (22) can alter the amount of the adenylate cyclase regulatory protein N_s in some tissues, this study was completed by comparing the amount of the α_s-subunit of N_s in adipocyte membranes from control and estradiol-treated hamsters. This α_s-subunit, which is responsible for the activation of the adenylate cyclase catalytic site (3), is ADP ribosylated by cholera toxin (4) and can thus be directly quantified by measuring the amount of ^{32}P NAD^+ incorporated into its 43-48 Kdaltons peptides (23, 24). Fig. 2 shows the SDS polyacrylamide gel electrophoresis autoradiograms of cholera toxin-catalyzed radiolabeling of fat cell membrane peptides in control and estradiol- treated animals. As can be seen, the electrophoretic mobility of α_s was unaltered in the estrogen-group. In addition, calculation of the relative amount of ^{32}P label incorporated into the 43-48 Kdaltons peptides under optimal conditions (25 µg/ml cholera toxin, 50 µM NAD^+) failed to reveal any significant difference between control and estradiol-treated animals (820 $\pm$ 85 vs 890 $\pm$ 78 fmol ^{32}P/mg protein, respectively, n = 3). These results, together with our previous observation (1) that the estradiol- induced defect in adenylate cyclase activity still persists after complementation of the fat cell mem-

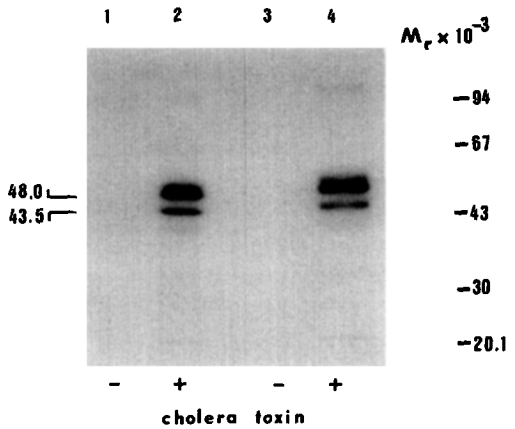

Fig. 2. SDS-polyacrylamide gel analysis of cholera toxin [α ^{32}P] ADP-ribosylated peptides of adipocyte membranes from control and estradiol-treated hamsters. Fat cell membranes from control (lanes 1 and 2) and estradiol-treated hamsters (lanes 3 and 4) were incubated with [α ^{32}P] NAD^+ plus cold NAD^+ (50 μM) in the absence or presence of cholera toxin (25 μg/ml). Conditions of incubation, electrophoresis and autoradiography are described under Materials and Methods. These data are from one experiment representative of two others. Mr values of the labeled peptides were established by comparison with protein standards.

branes with erythrocyte N_s, clearly demonstrate that neither the amount, nor the function of N_s is altered by the estradiol-treatment.

Therefore, this study allows to firmly conclude that the defective lipolytic response found in male hamster adipocytes after estradiol-treatment is due to a decreased activity of the adenylate cyclase catalytic subunit and that this decrease is mediated by an estrogen-receptor dependent mechanism. At our knowledge, this is the first example of a steroid hormone- induced modulation of the adenylate cyclase catalytic activity in a mammalian cell. Because the plasma estrogen levels reached at the end of the treatment used here (1) are similar to those found in female hamsters at the end of pregnancy (24), it is tempting to speculate that the estradiol-effects reported here may play a significant role in the important lipolytic changes that occur in adipose tissue during late pregnancy and on the onset of lactation (25, 26).

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