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REGULATION OF AROMATASE IN ESTROGEN-PRODUCING CELLS

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Summary—Human adipose stromal cells in monolayer culture aromatize androstenedione to estrone. The rate of aromatization is stimulated 20- to 30-fold by glucocorticoids when fetal calf serum is present in the culture medium and by dibutyryl cyclic AMP in the absence of serum. The action of dibutyryl cyclic AMP to stimulate aromatase activity is potentiated markedly by phorbol esters and inhibited by growth factors, such as EGF. In order to investigate the mechanisms underlying this multifactorial regulation, we have prepared polyclonal and monoclonal antibodies specific for aromatase cytochrome P-450. By use of these antibodies it was demonstrated that the action of these various factors to regulate aromatase activity was caused by alterations in the rate of synthesis of aromatase cytochrome P-450, whereas the synthesis of the reductase component of the aromatase enzyme complex was relatively unaffected. The changes in the rate of synthesis of aromatase cytochrome P-450 were, in turn, reflective of changes in the levels of translatable mRNA specific for this protein. In order to analyze the levels of aromatase cytochrome P-450 mRNA directly, we have isolated a cloned cDNA insert complementary to the mRNA encoding aromatase cytochrome P-450, by screening a λgt 11 human placental cDNA library utilizing the polyclonal anti-aromatase P-450 IgG. Use of this cDNA probe in Northern analysis of RNA extracted from human adipose stromal cells revealed that the changes in translatable mRNA resulting from incubation of the cells with the various regulatory factors were due to changes in the absolute levels of mRNA encoding this protein.

INTRODUCTION

In postmenopausal women, the principal circulating estrogen is estrone, which is formed almost exclusively in extraglandular tissues, primarily adipose tissue [1]. The substrate for aromatization by adipose tissue is plasma androstenedione [1–5] originating primarily by adrenocortical secretion. The transfer constant of conversion of plasma androstenedione to estrone is increased greatly in obese, elderly women [6], as is the incidence of endometrial carcinoma [6–8]. In previous studies [9], in which human adipose tissue was separated into adipocyte and stromal cells by collagenase digestion, followed by differential centrifugation, we found that the adipose stromal cells grew to confluence in monolayer culture, had a fibroblast-like appearance, and maintained the capacity to aromatize androstenedione to estrone. These cells, therefore, provide a convenient model system for study of the regulation of aromatase activity. Utilizing human adipose stromal cells, it was found that glucocorticoids in medium containing fetal calf serum stimulate aromatase activity 20- to 30-fold [10]. A similar stimulation was observed utilizing analogues of cAMP; however, such stimulation was inhibited by serum [11]. This action of serum was mimicked by growth factors such as EGF, PDGF and FGF [12]. On the other hand, the action of dibutyryl cAMP to stimulate aromatase activity was potentiated some 10-fold by phorbol esters [12]. These actions of

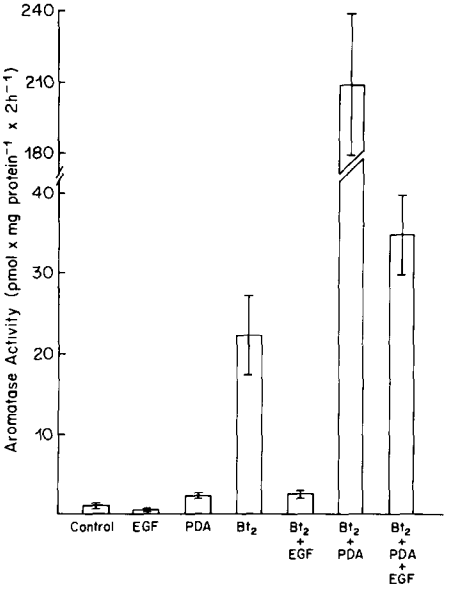

Fig. 1. Effect of various factors on aromatase activity of human adipose stromal cells maintained in monolayer culture. After reaching confluence, the cells were placed in serum-free medium for 24 h and then maintained for 48 h in the absence or presence of EGF (20 ng/ml), PDA (100 nM), Bt_2cAMP (Bt_2; 1 mM), Bt_2cAMP + PDA, Bt_2cAMP + EGF, or Bt_2cAMP + PDA + EGF. At the end of this time, aromatase activity was determined as described [9]. Results are expressed as the mean ± SEM of data obtained using 3 replicate dishes (with permission from the American Society of Biological Chemists, Inc.)

dibutyryl cAMP, EGF and phorbol esters are illustrated in Fig. 1. Thus, it can be seen that aromatase activity is under multifactorial regulation and the object of our current studies is to understand the mechanisms underlying this complex regulation.

REGULATION OF THE SYNTHESIS OF THE AROMATASE ENZYME COMPLEX

Aromatase activity is catalyzed by an enzyme complex present in the endoplasmic reticulum comprised of two proteins, a form of cytochrome P-450 known as aromatase cytochrome P-450 (P-450_{AROM} [13] and a flavoprotein, NADPH cytochrome P-450 reductase, which transfers reducing equivalents from NADPH to the cytochrome P-450. We have recently purified cytochrome P-450_{AROM} and have prepared polyclonal and monoclonal antibodies against this enzyme [14]. Using the polyclonal antibodies, we have investigated effects of various regulatory factors on the synthesis of cytochrome P-450_{AROM} in adipose stromal cells. In these experiments human adipose stromal cells were maintained in culture for 48 h under conditions as illustrated in Fig. 1. At the end of this incubation period, cells were radiolabeled with [^{35}S]methionine and P-450_{AROM} was immunoprecipitated from the cell lysates utilizing the polyclonal anti-P-450_{AROM} IgG. The reductase component also was immunoprecipitated from the cell lysates utilizing a polyclonal IgG specific for pig liver reductase. The immunoprecipitates were separated by SDS–PAGE and visualized by autoradiography. The results of such an experiment are shown in Fig. 2. It can be seen that the incorporation of [^{35}S]methionine into cytochrome P-450_{AROM} was stimulated by dibutyryl cAMP and that this stimulation was inhibited by EGF. The action of dibutyryl cAMP was potentiated markedly by the phorbol ester PDA and this action was again inhibited by EGF. These changes in the synthesis of cytochrome P-450_{AROM} reflect the changes in aromatase activity caused by these stimulatory and inhibitory factors as shown in Fig. 1. By contrast, there was little change in the incorporation of [^{35}S]methionine into the reductase component indicating that the regulation of aromatase activity is primarily a reflection of changes in the synthesis of cytochrome P-450_{AROM}.

In order to investigate the mechanisms whereby the synthsesis of cytochrome P-450_{AROM} is regulated, RNA was extracted from human adipose stromal cells cultured for 48 h under these same conditions. This RNA was then used to program a rabbit reticulocyte lysate *in vitro* translation system in the presence of [^{35}S]methionine. Cytochrome P-450_{AROM} and reductase were immunoprecipitated from the *in vitro* translation mixtures, and the immunoisolates were separated by SDS–PAGE and visualized by autoradiography as described above. An autoradiogram of immunoisolates from such an experiment is shown in Fig. 3. It can be seen that dibutyryl cAMP increased the translatability of

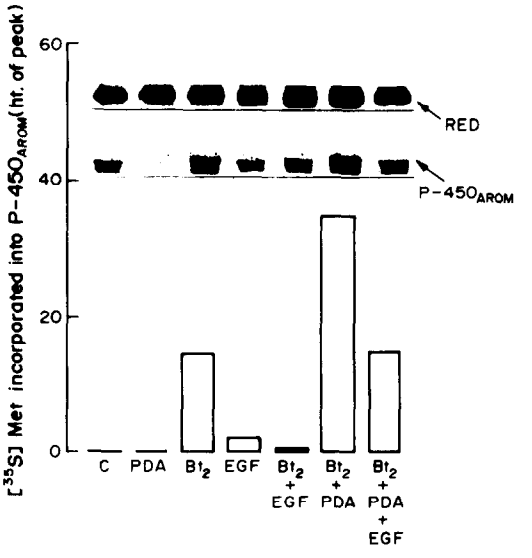

Fig. 2. Effects of various factors on the rates of synthesis of cytochrome P-450_{AROM} and reductase. After incubation in the absence or presence of EGF, PDA and Bt_2cAMP in various combinations, cells were radiolabeled with [^{35}S]methionine, and cytochrome P-450_{AROM} and reductase were immunoprecipitated from the cell lysates. Following SDS-PAGE, the radiolabeled proteins were visualized by autoradiography (top inserts) and analyzed by scanning densitometry. The relative heights of the bands in the immunoisolates of cytochrome P-450_{AROM} are plotted on the bar graphs (with permission from the American Society of Biological Chemists, Inc.).

mRNA encoding cytochrome P-450_{AROM}, and that this action was inhibited by EGF. On the other hand, the effect of dibutyryl cAMP was markedly potentiated by the phorbol ester PDA, and again this action was inhibited by EGF.

These results, concerning changes in the translatability of mRNA encoding cytochrome P-450_{AROM}, agree closely with the changes observed in the synthesis of cytochrome P-450_{AROM} and in aromatase activity in response to the various regulatory factors. By contrast, there was little change in the levels of translatable mRNA encoding the reductase component of the aromatase enzyme complex. Thus it appears that the regulation of aromatase activity is primarily the result of changes in the translatability of the mRNA encoding cytochrome P-450_{AROM}.

ISOLATION AND CHARACTERIZATION OF A cDNA INSERT COMPLEMENTARY TO mRNA ENCODING CYTOCHROME P-450_{AROM}

In order to investigate whether these changes in the levels of translatable mRNA encoding cytochrome P-450_{AROM} were the result of changes in the absolute levels of this mRNA species, it was necessary to isolate a cloned DNA sequence complementary to mRNA encoding this enzyme [15]. Such a cDNA insert was isolated and characterized from a human placental λgt 11 cDNA library prepared by Dr Tanya Z. Schultz of the Department of Cell

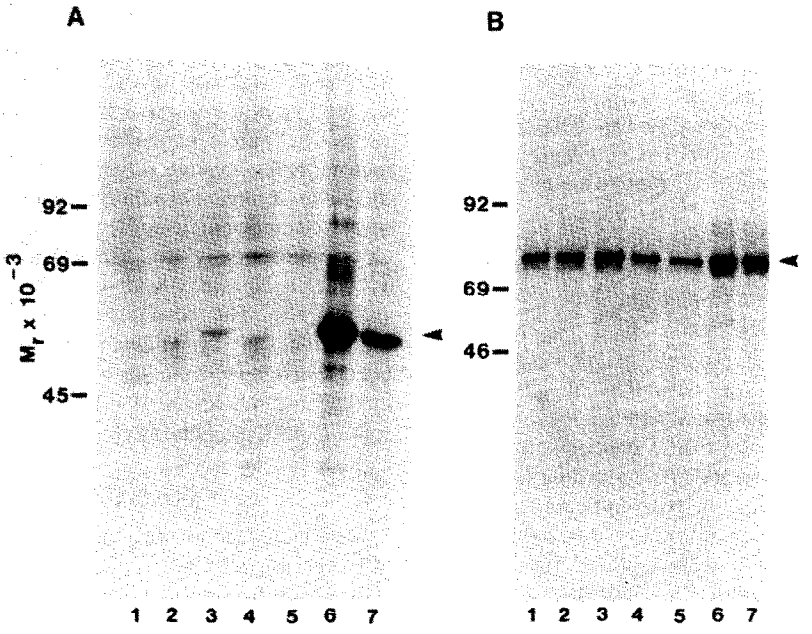

Fig. 3. Effects of various factors on the levels of translatable mRNA encoding cytochrome P-450$_{AROM}$ (Panel A) and NADPH-cytochrome P-450 reductase (Panel B). Confluent cells were incubated for 48 h in the absence (Lane 1) or presence of EGF (20 ng/ml; Lane 2), Bt_2cAMP (1 mM; Lane 3), PDA (100 nM; Lane 4), Bt_2cAMP + EGF (Lane 5), Bt_2cAMP + PDA (Lane 6), Bt_2cAMP + EGF + PDA (Lane 7). RNA was then isolated from the cells, translated, and cytochrome P-450$_{AROM}$ and NADPH-cytochrome P-450 reductase were immunoisolated. Following SDS–PAGE the radiolabeled proteins were visualized by autoradiography. The positions of the 55-kDa cytochrome P-450$_{AROM}$ standard and the 80-kDa reductase standards are indicated by the arrows.

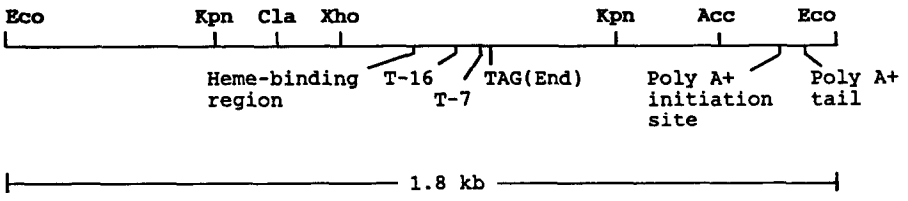

Fig. 4. Restriction map of the 1.8-kb cDNA insert specific for the mRNA encoding cytochrome P-450$_{AROM}$.

Biology, Baylor College of Medicine (Houston, TX). This library was screened using both the polyclonal and monoclonal IgGs against cytochrome P-450$_{AROM}$. A putative cDNA insert of 1.8 kb in length was subcloned into pBR 322 for analysis by restriction digestion. Suitable restriction fragments were subcloned into bacteriophage M-13 for nucleotide sequencing by the dideoxy method of Sanger *et al.*[16]. The restriction map of this 1.8-kb insert is shown in Fig. 4. This insert has about 900 bases of 3'-untranslated region and approximately 60% of the coding sequence of the enzyme. It contains a region near the carboxy terminus which has a high degree of homology with all cytochrome P-450 species that have been studied and which is believed to be the heme binding or HR-2 region of the polypeptide. The homology of this region of cytochrome P-450$_{AROM}$ with that of other cytochromes P-450 is illustrated in Fig. 5. Common to this region in all of the P-450 species is a cysteine which is believed to be the fifth co-ordinating ligand of the heme iron (Fig. 5). Also indicated in Fig. 4 are two peptides which are identical to the peptides T-16 and T-7, which were isolated from tryptic digests of purified cytochrome P-450$_{AROM}$ by Chen *et al.*[17]. It should be noted that peptide T-7 ends with a histidine which, as was pointed out by Chen *et al.*[17], is extremely unusual for a tryptic peptide. Our sequence data provide an explanation in that the histidine is at the carboxy terminal end of the protein. In addition, Chen *et al.*[17] isolated another tryptic peptide which they claimed to be the heme-binding region of the protein. The sequence of this peptide differs from the one which we have identified as the heme-binding region on the basis of the sequence homology with other cytochromes P-450 and by its location relative to the carboxy-terminus of the protein.

NORTHERN ANALYSIS OF RNA

Utilizing this cDNA insert it has been possible to analyze the relative levels of hybridizable mRNA encoding cytochrome P-450$_{AROM}$ in human adipose stromal cells after incubation with various regulatory factors. RNA extracted from cells incubated under the seven different conditions discussed previously, was size fractionated by electrophoresis, transferred electrophoretically to nitrocellulose paper and hybridized to the 1.8-kb cytochrome P-450$_{AROM}$ cDNA[15]. The results of such an analysis are indicative that changes in aromatase activity of these estrogen-producing cells are due to changes in the absolute levels of mRNA encoding cytochrome P-450$_{AROM}$ (data not shown).

CONCLUSIONS

We conclude from these studies that the regulation of aromatase activity in human adipose stromal cells is the result primarily of changes in the levels of the mRNA encoding cytochrome P-450$_{AROM}$ which, in turn, leads to comparable changes in the rate of synthesis and cellular levels of this enzyme. By contrast, there is little change in the levels of the reductase component of the aromatase enzyme complex.

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AMINO ACID SEQUENCE HOMOLOGIES OF THE HR-2 REGION

Human Cytochrome P-450$_{AROM}$	F G F G P R G C A G K Y I A
Human Cytochrome P-450 4	F G M G K R R C I G E V L A
Human Cytochrome P$_1$-450	F G M G K R K C I G E T V A
Bovine Cytochrome P-450$_{17\alpha}$	F G A G P R S C V G E M L A
Bovine Cytochrome P-450$_{scc}$	F G W G V R Q C V G R R I A
Rabbit Cytochrome P-450 4	F G L G K R R C I G E T L A
Mouse Cytochrome P$_3$-450	F G L G K R R C I G E I P A

Fig. 5. Sequence homologies of the heme-binding regions of a number of cytochrome P-450 species.

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