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Lack of Evidence for Estrogen and Progesterone Receptors in Human Adipose Tissue

M. Brőnnegård,[1]* M. Ottosson,[2] J. Bőős,[1] C. Marcus[1] and P. Bjőrntorp[2]

[1]*Department of Pediatrics, Endocrine Research Unit, Karolinska Institute, Huddinge University Hospital, 141 86 Huddinge and* [2]*Wallenberg Laboratory, Department of Heart and Lung Disease, Sahlgrenska Hospital, University of Gothenburg, Sweden*

We have previously presented data indicating the absence of estrogen and progesterone receptors from human adipose tissue by the use of specific antibodies (Abbott) as well as specific ligands. In addition, specific estrogen and progesterone cRNA probes did not hybridize to any mRNA species in either abdominal or gluteal/femoral adipose tissue as demonstrated by solution hybridization and Northern blot. In order to demonstrate even extremely small quantities of gene products we have now used the Polymerase chain reaction-technique to study estrogen- and progesterone receptor gene expression. Sequences corresponding to each specific cDNA were demonstrated indicating small amounts of estrogen- and progesterone receptor mRNA not detected by RNA/RNA or RNA/TNA (total nucleic acids) hybridization assays. The estrogen receptor-regulated gene pS2, however, was not induced by estrogens in human adipose tissue in contrast to a significant increase in pS2 mRNA levels after estrogen exposure to the estrogen receptor(+) cell line MCF7. From these results we conclude that estrogen- and progesterone receptors are absent from human adipose tissue and that the extremely low level of transcription of the corresponding genes is not sufficient to allow translation of the message into functional proteins.

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INTRODUCTION

One of the most controversial issues in endocrinology today is the number of steroid hormone receptors necessary to obtain a biological response. In spite of the classical three step model in the mode of action of steroid hormones (ligand > receptor > biological response) [1, 2] there is substantial evidence that several other factors such as heat shock proteins contribute to determine target tissue responsiveness[3]. Therefore, this model should be referred to with caution when trying to explain specific biological effects in terms of ligand concentration and receptor structure and/or number. Furthermore, the actions of steroids appear to involve the cell membrance as well as the genome, and (although it has not been demonstrated) it is even conceivable that actions of steroids at the cell surface might be able to trigger changes in gene expression indirectly [4, 5]. During the last 10 years evidence has accumulated that synergistic interactions occur between non-genomic and genomic actions of steroids meaning that a specific tissue or cell type may actually lack intracellular receptor proteins or alternatively express extremely low levels of receptors, but be highly "membrane-responsive" [6]. Thus, cell- and tissue specificity of response to a whole-body signal is determined by local pre-receptor, receptor and genomic differences.

Adipose tissue is one of the most abundant pools of estrogen [7]. However, in spite of the remarkably high concentrations of estrogen and progesterone in comparison to other human tissues, the actual effect of these steroids in human adipose tissue has been debated [7]. Previous studies have indicated a high level of glucocorticoid receptors (GR) as well as androgen receptors in adipose tissue [8, 9]. On the contrary, the expression of estrogen (ER)- and progesterone (PR) receptors both at protein and mRNA level has been difficult to demonstrate [8]. In this study, we have applied the polymerase chain reduction (PCR)-technique in order to evaluate the expression of corresponding mRNAs for the ER and

*Correspondance to M. Brőnnegård.
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PR in human adipose tissue. We have also determined the expression levels of the glucocorticoid- and progesterone-regulated metallothionein (MTIIA) gene and the estrogen-regulated gene pS2 which, if induced, would indicate a receptor-mediated process [10].

MATERIALS AND METHODS

Subjects and cell cultures

Subcutaneous, abdominal adipose tissue was obtained from three pre-menopausal women, 20, 41 and 47 years of age, undergoing abdominal surgery for non-malignant diseases. Body mass index was 23.8, 28.5 and 25.8 kg/m^2, respectively. None were taking oral contraceptives and none were diabetic. The patients had been fasting overnight and surgery was performed under general anesthesia. About 20 g of adipose tissue was excised during the initial phase of the operation. The study was approved by the Ethics Committee of the University of Gothenburg.

Cells from the human breast cancer cell line MCF7 were grown in RPMI medium 1640 (GIBCO) supplemented with 10% fetal calf serum. All experiments were performed after four cell passages with MCF7 cells growing in monolayer culture. Cells were maintained in serum-free medium for 8 h and then treated with cortisol, progesterone and β-estradiol at the concentrations indicated in the legends to the figures, for 6 h. Before RNA or TNA (total nucleic acids) preparation, the cells were washed twice with phosphate-buffered saline. pH 7.4.

Immediately after excision, the adipose tissue was placed in sterile Parker medium 199 (SBL, Stockholm, Sweden) supplemented with 10 mM HEPES [*N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid; Sigma, St Louis, U.S.A.], at room temperature and pH 7.4. Pieces of adipose tissue (5–20 g) were prepared under sterile conditions and used for incubations in plastic tubes (600 mg tissue/20 ml medium). Connective tissue and blood vessels were removed. The adipose tissue was pre-incubated in control medium (Parker medium 199 supplemented with 12.5 mM $NaHCO_3$, 10 mM HEPES, 1% human serum albumin; Immuno AG, Vienna, Austria] and 0.1 mg cephalothin (Keflin, Lilly France S.A., France) and 1000 μU insulin per ml (Actrapid, Novo Nordisk A/S, Denmark), pH adjusted to 7.4) for 3–5 days. The medium was changed daily. After pre-incubation, at the time for steroid hormone exposure ($t = 0$), about 500 mg of adipose tissue was frozen in liquid nitrogen as a control. The rest of the tissue was divided into three groups and was given fresh medium. One third was incubated in medium with cortisol (control medium supplemented with cortisol, 10^{-6} M), one third in medium with estradiol (control medium supplemented with β-estradiol, 10^{-6} M) and one third was continuously incubated in the control medium. After $t = 1$, 2, 4, 6, 9, 12 and 24 h of incubation 500 mg of tissue from each medium was frozen in liquid nitrogen. In one incubation experiment, the 9 h samples were excluded. In selected experiments, adipose tissue was also incubated with progesterone (control medium supplemented with progesterone, 10^{-6} M) and 500 mg of tissue from the medium were frozen in liquid nitrogen at indicated time intervals. The samples were stored at -70°C until preparation of total nucleic acids. At each time point, 50–60 mg of adipose tissue in duplicate from each medium was used for determination of heparin-releasable lipoprotein lipase (LPL) activity [11]. This was made to check the conditions under which the tissue was incubated. Under optimal conditions cortisol induces LPL activity within 24 h (after pre-incubation) in human adipose tissue indicated by a 2.6-, 3.3- and 1.3-fold induction, respectively, as compared with the initial LPL activity in the three patients investigated (data not shown).

Preparation of RNA and TNA

Total RNA from adipose tissue (200 mg) and cultured MCF7 cells (approx. 10^7 cells) was prepared by the single step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction according to the protocol of Chomczynski [12]. For preparation of TNA, adipose tissue or MCF7 cells were homogenized in a sodium dodecyl sulfate (SDS) buffer (1% SDS; 10 mmol/l of Tris–HCl, pH 7.5, and 5 mmol/l of EDTA), digested with 100 mg of proteinase K for 45 min at 45°C, and subsequently extracted with phenol–chloroform after the addition of isoamylic alcohol (24:1, v/v) [13]. RNA and TNA concentrations were determined spectrophotometrically. The integrity of RNA samples was verified by gel electrophoresis and ethidium bromide staining.

Hybridization analysis of RNA

Oligonucleotide probes (50 base pairs) were synthesized and cloned into the *Pst*I/*Hind*III sites in pGEM TM-I (Promega Biotechnology, Madison, WI). The sequence of these inserts in pGEM TM-I was confirmed by DNA sequencing using the dideoxy chain-termination method [14]. These plasmids were used for *in vitro* synthesis of cRNA using SP6 RNA polymerase and the opposite mRNA strand using T7 RNA polymerase as described below. The sequence of the MTIIA oligonucleotides used has been reported previously [15]. For the pS2 gene the sequence of the oligonucleotides used corresponded to nucleotides 55–106 [10]. RNA hybridization was analyzed using a solution hybridization protocol [16]. TNA samples were hybridized to approx. 20,000 cpm/sample of [^{35}S]UTP labeled RNA probes and hybrids were allowed to form in 40 μl of a buffer consisting of 0.6 mol/l of NaCl, 30 mmol/l of dithiothreitol (DTT),

Table 1. Primers used for the reverse transcription and PCR reactions

Sequence	cDNA site
1. 5′-GCC GGC CTC GCG CAC CGH GTA GCC G-3′	643–667 (as)
2. 5′-GAG GGC GCC GCC TAC GAG TTC AAC G-3′	398–442 (s)
3. 5′-GCA GCC GCT CGC GCC CGG CGC CTT G-3′	1441–1464 (as)
4. 5′-TTG GGG CCA CCG CCC CCG CTG CCG C-3′	1255–1278 (s)
5. 5′-CTC GGG GAA TTC AAT ACT CAT GGT C-3′	2360–2385 (as)
6. 5′-GCA GGA TAT GAT AGC TCT GTT CCA GAC-3′	1768–1794 (s)

Sequences used for PCR reactions are from published cDNA sequences of respective steroid receptor. 1–2, estrogen receptor [28]; 3–4, progesterone receptor [29]; 5–6, glucocorticoid receptor [30]. Antisense (as) and sense (s) sequences are indicated.

and 25% formamide at 68°C. After an overnight incubation, samples were digested with RNase by adding 1 ml of a solution containing 40 μg RNase A and 2 μg RNase Tl (Boehringer-Mannheim, Mannheim, Germany). Digestion was performed for 45 min at 45°C after which RNase-resistant RNA was precipitated by the addition of 0.1 ml of 6 mol/l of trichloroacetic acid. Precipitates were collected for scintillation counting by filtration on glass fiber filters (Whatman GF/Clifton, NJ). The amount of pS2 and MTIIA mRNA of a sample was determined in duplicate and calculated from a linear standard curve constructed from incubations with known amounts of *in vitro* synthesized mRNA complementary to the ^{35}S-labeled probe as described previously [15, 16]. Results are expressed as amol mRNA/μg TNA. Means and SD were calculated for the various experimental groups. Means were compared by the Mann–Whitney U test, when appropriate and differences between groups considered significant for P values of 0.05 or less.

Synthesis of cDNA and PCR

cDNA was synthesized from total cellular RNA using oligo (dT) primers and M-MLV reverse transcriptase (BRL). A mix of 1 × M-MLV RT buffer (50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.01 M DTT, 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 0.2 μg oligo (dT) and 200 U M-MLV RT in 20 μl were incubated at 37°C for 60 min. The PCR reaction was carried out in a reaction mix containing 1 × amplification buffer [1.5 mM $MgCl_2$, 0.2 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 μm of each primer, 1 μg cDNA and 2.5 U taq polymerase (Promega)], in a total volume of 100 μl. The following PCR program was used for amplification of DNA; 1 cycle of denaturation at 95°C for 2 min; annealing at 55°C for 1 min; extension at 72°C for 2 min, followed by 29 cycles of denaturation at 95°C for 1 min; annealing at 55°C for 2 min; extension at 72°C for 2 min, and a final cycle of extension at 72°C for 7 min. For each PCR a control reaction with no addition of cDNA was carried out, excluding any contamination of foreign DNA in the samples. The sequence of each specific primer is shown in Table 1.

RESULTS

Expression of pS2 mRNA

In MCF7 cells, β-estradiol caused a dose-related and significant increase of pS2 mRNA. Figure 1 shows a typical dose–response curve with a maximal inductive effect on pS2 at a concentration of 10^{-6} M. No effect on pS2 mRNA expression levels was observed after incubation of MCF7 cells with progesterone or cortisol (Fig. 1). The time course of β-estradiol action on pS2 mRNA levels in MCF7 cells showed a maximal effect 6 h after onset of treatment (data not shown) and based on these data MCF7 cells were treated with each specific steroid for 6 h before TNA preparation. In human adipose tissue, however, no induction of pS2 mRNA by β-estradiol was observed (Fig. 2). It should be noted that the basal level of expression of pS2 mRNA in both MCF7 and adipose tissue is very low and did not indicate any differences between the two cell types. The fact that pS2 mRNA was not induced in adipose tissue indicate that ER is not present whereas expression of a functional ER is a requirement for the induction of ER-regulated genes.

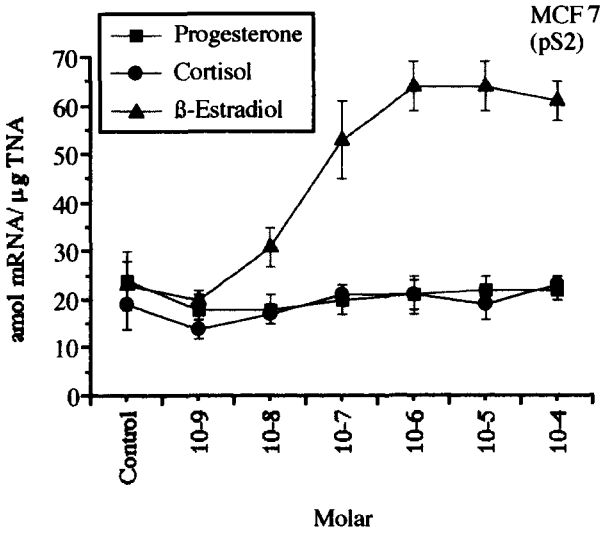

Fig. 1. Dose–response curve for pS2 mRNA in MCF7 cells. MCF7 cells were incubated with increasing concentrations of progesterone, cortisol or β-estradiol for 6 h, TNA was prepared and solution hybridization carried out with a cRNA probe for pS2 as described in Materials and Methods.

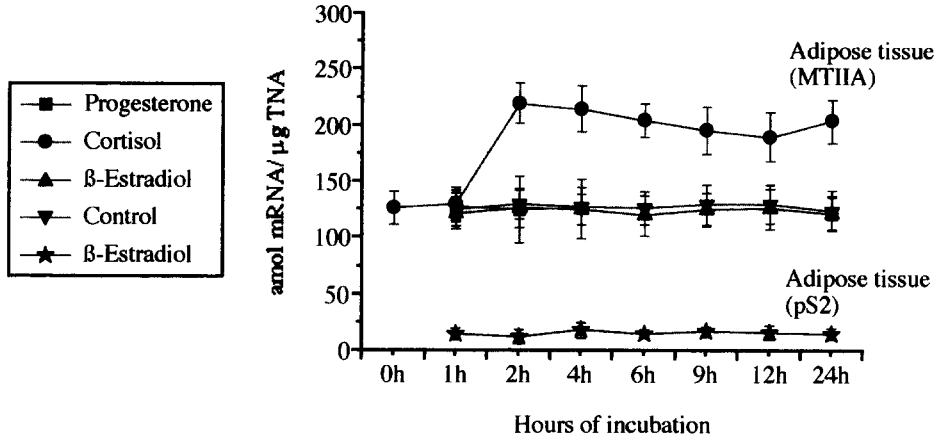

Fig. 2. Time course curves of progesterone, cortisol and β-estradiol action on MTIIA mRNA and pS2 mRNA levels in human adipose tissue. Human adipose tissue was incubated with 10^{-6} M of respective steroid, TNA prepared at indicated time intervals and solution hybridization carried out with cRNA probes for MTIIA and pS2, respectively, as described in Materials and Methods.

Expression of MTIIA mRNA

MTIIA mRNA is known to be positively regulated by both glucocorticoids, heavy metals and progesterone [17]. In MCF7 cells, cortisol as well as progesterone caused a dose-related increase of MTIIA mRNA. Figure 3 shows a typical dose–response curve where both cortisol and progesterone caused a maximal inductive effect on MTIIA at a concentration of 10^{-5}–10^{-6} M. The time course of cortisol and progesterone action on MTIIA mRNA levels showed a maximal effect 6–7 h after onset of treatment (data not shown) and based on these data, cells were treated with each specific steroid for 6 h before TNA preparation. No induction of MTIIA was observed after treatment of MCF7 cells with β-estradiol (Fig. 3).

Treatment of MCF7 cells with cortisol (10^{-5}–10^{-6} M) caused a 3-fold increase in MTIIA mRNA levels whereas only a 1.8-fold increase was observed in adipose tissue ($P < 0.01$) (Figs 2 and 3). No differences in basal expression of MTIIA mRNA were observed between the two cell types. The reason for this cell-specific difference in MTIIA induction is not understood and has to be further evaluated.

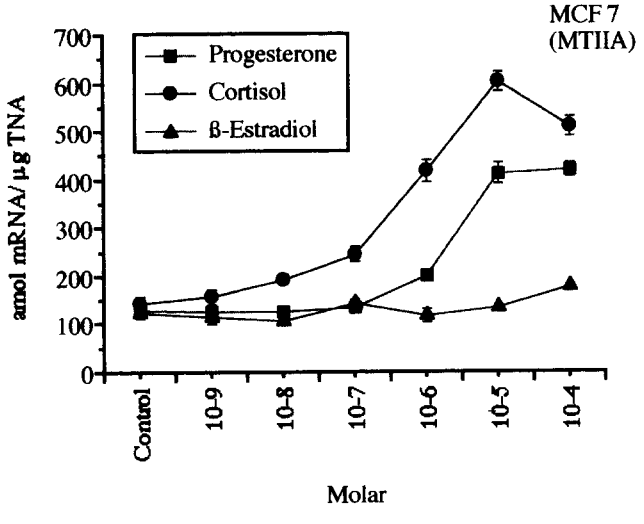

Fig. 3. Dose–response curves for MTIIA mRNA in MCF7 cells. MCF7 cells were incubated with increasing concentratrions of progesterone, cortisol and β-estradiol for 6 h, TNA prepared and solution hybridization carried out with a cRNA probe for MTIIA as described in Materials and Methods.

As shown in Fig. 2, cortisol at a concentration of 10^{-6} M clearly induced the expression of MTIIA mRNA, whereas no effect was obtained with either β-estradiol or progesterone at the same concentration. It is known that due to differences in receptor affinity for different steroids progesterone has to be added in significantly higher concentrations as compared to glucocorticoids in order to obtain an effect on gene transcription via GR [17]. When adipose tissue was incubated with progesterone, however, at a concentration of 10^{-3}–10^{-4} M (data not shown), the mRNA levels for MTIIA increased 1.2–1.3-fold. This concentration of progesterone is significantly higher than that needed for the induction of MTIIA mRNA in MCF7 cells and probably reflects the fact that high concentrations of progesterone bind, with poor affinity, to the GR to stimulate transcription.

Amplification of ER, PR, and Gr cDNA in MCF7 and adipose tissue

As indicated in Fig. 4, PCR products of ER and PR in MCF7 cells, were easily detected after 30 cycles of amplification. All PCR products were of expected sizes corresponding to sequences indicated by selected primers in Table 1. In adipose tissue amplification of an equal amount of cDNA (mRNA) resulted in small, barely detected amounts of PCR products for both ER and PR (Fig. 4). No PCR products were seen in the control samples (=no cDNA added) (Fig. 4). GR cDNA, however, was easily amplified in both adipose tissue and MCF7 cells (Fig. 5). All PCR reactions were

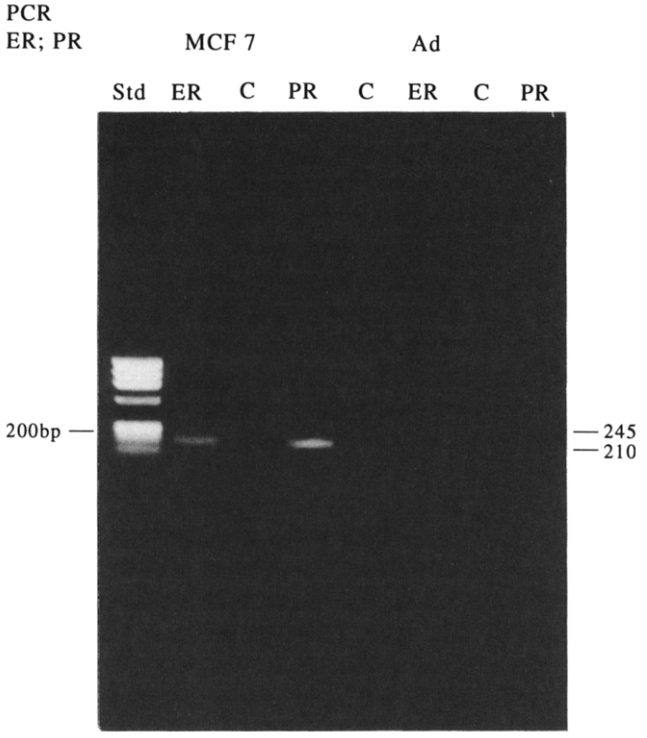

Fig. 4. PCR products of estrogen (ER) and progesterone (PR) receptors from adipose tissue (Ad) and MCF7 cells. The ethidium bromide-stained 1% agarose gel shows that PCR amplified ER and PR cDNA using primers presented in Table 1, yielded the expected 245 and 210 bp fragments, respectively. Control PCR reactions with no template added (C) and a DNA molecular weight marker, phiX174 (Std), are indicated.

optimized according to the highest annealing temperature generating a PCR product.

DISCUSSION

In these studies we examined the relationship between the cellular ER, PR and GR mRNA content and the steroid response. The study was prompted by evidence that estrogens and progesterone have documented effects in human adipose tissue in spite of undetectable receptor levels [8, 18] and that there are substantial data supporting important roles for these steroids in the regulation of human adipose tissue metabolism, morphology and distribution [19].

In the ER- and PR-positive cell line MCF7 in contrast to human adipose tissue, the ER-regulated gene pS2 displayed a classical dose–response curve when the estradiol responses were compared with increasing concentrations of β-estradiol. To our knowledge, there is no gene specifically regulated by progesterone. To indirectly address the question of PR expression in human adipose tissue we therefore investigated the induction of the MTIIA gene, which is known to be positively regulated by both progesterone and glucocorticoids via PR and GR, respectively [17]. In adipose tissue, progesterone at concentrations where a significant MTIIA-induction in MCF7 cells were observed, did not increase MTIIA mRNA expression levels, thus indirectly indicating the lack of PR gene expression. However, at supraphysiological concentrations of progesterone a minor increase in MTIIA mRNA was observed. This effect on gene transcription by progesterone is postulated to be mediated via binding of progesterone to GR and subsequently to glucocorticoid and/or progesterone response elements [20]. Consequently, the specificity of hormone response in a

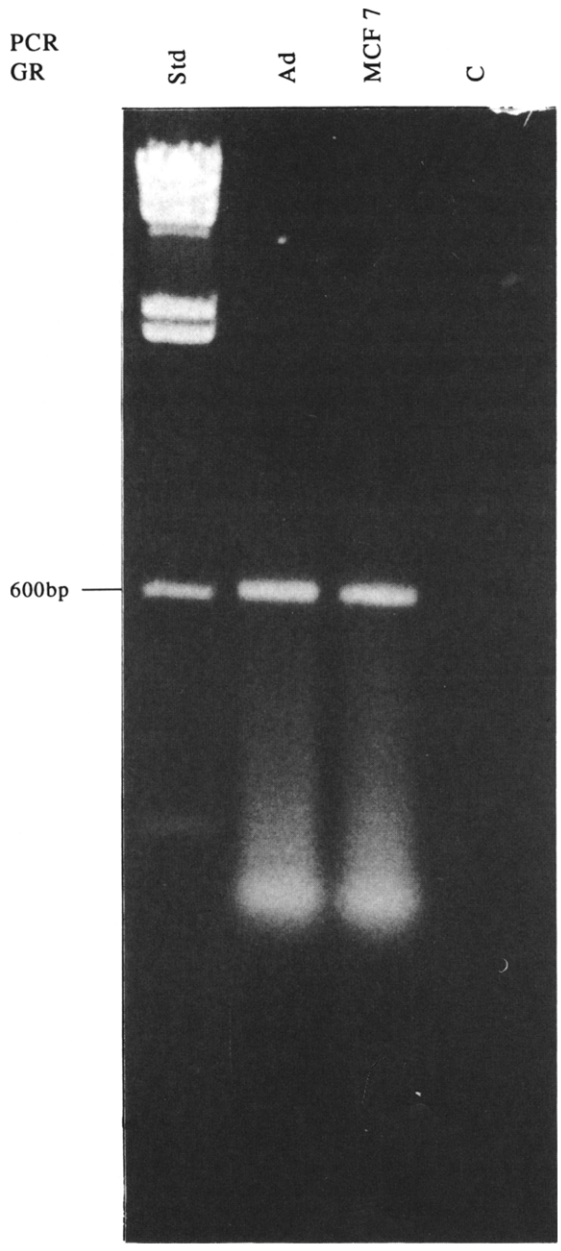

Fig. 5. PCR products of the GR from human adipose tissue (Ad) and MCF7 cells. The ethidium bromide-stained 1% agarose gel show that PCR amplified GR cDNA using primers 5 and 6 (Table 1), yielded the expected 600 bp fragment. Control PCR reactions with no template added (C) and a DNA molecular weight marker, phi174 (Std), are indicated.

given tissue is dependent, in part, upon the expression of a particular receptor in that tissue.

Previous studies have shown that the degree of glucocorticoid response of a defined target gene is linearly related to the number of ligand-bound GRs [21]. It has also been shown that receptor action at low receptor titers is limited by binding of receptor to its response element and that estrogen response element is probably markedly undersaturated by receptor at physiological conditions [22]. Similar studies have not been carried out for PR. Therefore, extremely low ER expression levels would not generate any cellular response in terms of increased transcription of ER-regulated genes. The question is then why ER and PR are not expressed in human adipose tissue.

Our results indicate a critical level of mRNA for translation to take place and raise the question of the biological significance of very low and by generally used techniques undetectable mRNA levels. Price and O'Brien [23] recently demonstrated ER mRNA levels in human adipocytes as well as in adipose stromal cells by PCR. Adipose stromal cells as well as adipocytes contain significant concentrations of estrogens and it is possible that the ER-gene is down-regulated. If this is the case, it contrasts well established actions of estrogens in breast tissue where female sex steroids up-regulate both ER and PR [24]. The second question is then how and if there are any effects of estrogens and/or progesterone in human adipose tissue.

It is possible that steroid (e.g. progesterone) metabolites may act by way of "orphan" receptors, which are members of the steroid receptor superfamily [25]. Such a possibility has been suspected for a long time but not until recently been considered again to explain high affinity binding of 5-dihydroprogesterone in some tissues [26]. It is also established that female sex steroids per se evoke biological responses by way of non-genomic processes [4, 5]. These effects are often mediated through the release of Ca^{2+} from intracellular stores and probably triggered by inositol 1,4,5-triphosphate generated by a steroid receptor-induced hydrolysis of membrane phosphatidyl-inositol 4,5-bisphosphate[27].

In summary, we have presented additional evidence indicating the absence of ER and PR from human adipose tissue based on the following findings; (i) failure to induce the ER-regulated gene pS2 by β-estradiol and (ii) failure to induce the Gr and PR-regulated gene MTIIA by progesterone at physiological concentrations. Further studies of the effects on human adipose tissue metabolism by steroid hormones are needed in order to clarify and explain these issues.

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