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Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE_2 via cyclic AMP, leading to activation of promoter II of the *CYP19* (aromatase) gene

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ABSTRACT In the present report, we show that prostaglandin (PG) E_2 is the most potent factor which stimulates aromatase expression via cyclic AMP and promoter II. PGE_2 acts via EP_1 and EP_2 receptor subtypes to stimulate both the PKC and PKA pathways. The combined stimulation of both of these pathways results in maximal expression of promoter II-specific *CYP19* transcripts. Since PGE_2 is a major secretory product both of breast tumor epithelial cells and fibroblasts, as well as of macrophages infiltrating the tumor site, then this could be the mechanism whereby estrogen biosynthesis is stimulated in breast sites adjacent to a tumor, leading in turn to increased growth and development of the tumor itself.

INTRODUCTION

Tissue-specific regulation of aromatase P450 (the enzyme responsible for estrogen biosynthesis) in humans is regulated in part by the use of tissue-specific promoters via alternative splicing mechanisms (1,2). As a consequence, aromatase P450 transcripts have tissue-specific 5′-termini spliced into a common site upstream of the start of translation (2-4). Expression in adipose tissue appears to utilize three separate promoters, namely a distal promoter I.4, the proximal promoter II located just upstream of the translational start site, and a third promoter, I.3, located upstream of promoter II with which it shares common regulatory elements (2). In normal adipose tissue, transcripts specific for promoter I.4 predominate. We and others have observed that aromatase expression is increased in adipose tissue at sites adjacent to a breast tumor, compared to expression in other sites of the breast (5-8), and compared to breast adipose tissue of cancer-free individuals. This suggests that breast tumors produce a factor(s) which stimulates aromatase expression in the surrounding mesenchymal tissue. Surprisingly, this increased expression is associated with promoter switching, such that promoter II-specific transcripts predominate at sites proximal to a tumor, as well as within the tumor itself (8). Promoter II-specific expression of aromatase in adipose stromal cells is stimulated by cyclic AMP and this expression is potentiated by phorbol esters (2,9), suggesting that both PKA and PKC pathways are involved in optimal expression, although phorbol esters by themselves have no stimulatory activity. The regulatory elements involved in this signaling pathway have not as yet been delineated in adipose cells, although an SF-1 site may be involved (10).

In the present report, we show that PGE_2 is a potent activator of aromatase expression in adipose stromal cells, via promoter II. Furthermore, we propose that PGE_2 is the factor produced by breast tumors which stimulates local estrogen biosynthesis in the surrounding adipose tissue.

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EXPERIMENTAL PROCEDURES

Materials: Prostaglandins E_2, D_2, $F_{2\alpha}$, I_2, and J_2 were from Cayman Chemical (Ann Arbor, Michigan). 17-Phenyl trinor PGE_2 11-deoxy-16, 16-dm PGE_2 sulprostone and 1-O-hexadecyl-2-O-methyl-*sn*-glycerol were also from Cayman Chemical. Forskolin was purchased from Calbiochem. Dibutyryl cAMP (Bt2cAMP) and phorbol myristate acetate were from Sigma. CAMP enzymimmunoassay (EIA) system was from Amersham Life Science (Buckinghamshire, England).

Cell Culture: Subcutaneous adipose tissue was obtained from women at the time of reduction abdominoplasty or reduction mammoplasty. Consent forms and protocols were approved by the Institutional Review Board, University of Texas Southwestern Medical Center at Dallas. Adipose stromal cells were prepared as described. Adipose stromal cells in primary culture were maintained in Waymouths enriched medium containing 10% Nu serum (10% v/v) (Collaborative Research Inc.) and allowed to grow to confluency (5-6 days) before stimulation.

Aromatase Assay: Aromatase activity was determined by incubating the cells for 2 h with [1β-^{3}H] androstenedione, and assaying the release of [^{3}H] water as described.

RNA isolation and RT-PCR: Human total RNA was isolated from the frozen tissues by the guanidinium thiocyanate-cesium chloride method (11). RT-PCR was performed according to a recently standardized competitive RT-PCR method which we developed for this purpose (12). RNA was initially treated with DNase I to remove any contaminating DNA. Total RNA was then reverse transcribed using random hexamers. Complementary DNA was used in subsequent PCR amplifications for 25 cycles. Specific 5′-end primers were used to amplify the various 5′-termini. The 3′-end primer was the same in all reactions (12). A trace amount of [^{32}P] dCTP was added to each PCR mix. The reaction products were analyzed on 4% non-denaturing polyacrylamide gels. Gels were either autoradiographed with x-ray film or scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and quantitatively analyzed using ImageQuant software.

Analytical Methods: Protein concentrations were determined with the bicinchoninic acid protein assay agent (Pierce). Quantitation of cAMP secreted into media was performed as recommended.

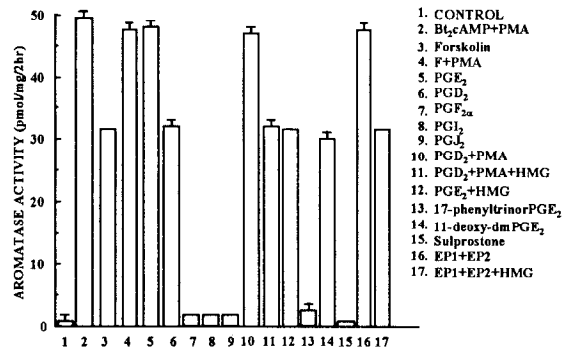

Fig. 1 Aromatase activity of adipose stromal cells in the presence of various prostanoid derivatives. Adipose stromal cells were prepared from subcutaneous adipose tissue of women at the time of reduction abdominoplasty or reduction mammoplasty, as previously described (13). Confluent monolayers of these cells were incubated for 24h in serum-free medium in the absence (control) or presence of the substances indicated. At the end of this time, aromatase activity was assayed by the release of tritium from [β-^{3}H] androstenedione into [^{3}H]water as described (13). The following concentrations were employed: Bt_2 cAMP, 1 mM; PMA, 1 nM; forskolin, 25μM; PGs, 10^{-6}M; HMG, 10^{-8}M.

RESULTS

In a search for factors that might stimulate aromatase expression of adipose stromal cells via an increase in cyclic AMP, we previously found that ACTH, β-adrenergic agonists and gonadotropins are without effect, or cause at best extremely modest stimulation (14). By contrast, as shown in Figure 1, PGE_2 stimulates aromatase activity of human adipose stromal cells to the same extent as Bt_2 cAMP or forskolin plus phorbol esters, resulting in a ~ 50-fold increase as compared to control cells. This suggests that PGE_2 is capable of activating both PKA- and PKC- mediated signaling pathways. This was further evidenced by the observation that when PGE_2 was added together with HMG, an inhibitor of PKC, the stimulation was reduced to that observed in the presence of forskolin alone. At least 4 receptor subtypes have been characterized which bind PGE_2 (15). Of these, the EP_2 subtype activates Gαs, resulting in stimulation of adenylate cyclase, whereas the EP_3 subtype inhibits adenylate cyclase. By contrast the EP_1 subtype activates phosphoinositide hydrolysis, presumably resulting in increased intracellular levels of free Ca^{++} and diacylglycerol, which in turn can lead to activation of PKC. We used ligands specific for each of these receptor subtypes to delineate their role in the stimulation of aromatase (16-18). As expected, use of 11-deoxydm PGE_2, an agonist specific for the EP_2 subtype, resulted in aromatase activity equivalent to that seen in the presence of forskolin alone; on the other hand, addition of 17-phenyltrinor PGE_2, an agonist specific for the EP_1 receptor subtype, resulted in aromatase activity equal to that of control cells and of cells treated with phorbol esters alone. When the EP_1 and EP_2 ligands were added together, activity identical to that seen in the presence of PGE_2 was attained. When HMG was added together with these ligands, activity equivalent to that observed with the EP_2 ligand alone, or with PGE_2 plus HMG, was observed. By contrast, use of an EP_3 ligand, sulprostone, resulted in activity no different from that of control cells. In order to determine whether the addition of PGE_2 to human adipose stromal cells resulted in an increase in cyclic AMP levels, a comparison was made between the time-course of the action of PGE_2 on cyclic AMP formation on the one hand, and aromatase activity on the other (Figs. 2 and 3). As can be seen, PGE_2 elicited a time-dependent increase in both cyclic AMP formation and aromatase activity, although the time-course of cAMP formation was much more rapid.

Of other ligands tested, PGD_2 also was effective in stimulating aromatase activity (Fig. 1). However, the maximum fold- stimulation in the presence of PGD_2 was equivalent to that seen in the presence of forskolin alone. However, when phorbol ester was added together with the PGD_2, then activity equivalent to that seen in the presence of PGE_2 was attained. Thus it appears that PGD_2 does not activate the PKC pathway. Other putative agonists such as PGI_2, $PGF_{2\alpha}$, PGJ_2 or PAF were ineffective in stimulating aromatase activity (Fig.1).

These results support the concept that PGE_2 interacts with two receptor subtypes in adipose stromal cells, one of which, presumably EP_2, is coupled to stimulation of cyclic AMP formation, and a second, presumably EP_1, which is coupled to PKC activation. As a consequence, the stimulation elicited by PGE_2 is equivalent to that seen in the presence of both forskolin plus phorbol esters.

Consistent with previous studies on aromatase regulation (19), the time-course of activation of aromatase by PGE_2 is indicative of a mechanism involving elevated transcript levels and increased protein synthesis. Consequently, the effect of PGE_2 on promoter-specific expression was investigated. In order to determine which promoter-specific transcripts of aromatase P450 were expressed in adipose stromal cells in response to PGE_2, we used competitive RT-PCR to amplify each of the promoter-specific transcripts from RNA extracted from adipose stromal cells maintained under the culture conditions indicated in Fig. 4. This method has been verified previously (12). As can be seen, adipose stromal cells maintained under control conditions contain primarily promoter II- and exon I.3-specific transcripts in low abundance, together with some exon I.4-specific transcripts. Stimulation of expression in the presence of dexamethasone plus IL-11 resulted in an increase in aromatase expression due primarily to an

increase in exon I.4-specific transcripts, as previously shown (2,12). On the other hand, increased expression in the presence of either PGE_2, forskolin or dibutyryl cAMP plus phorbol esters resulted in an increase predominantly of promoter II-specific transcripts, with a lesser increase in I.3-specific transcripts. It should be noted that no attempt was made to quantify expression in this study. Previously we have shown by northern analysis that treatment with dibutyryl cAMP plus phorbol esters on the one hand, or dexamethasone plus serum on the other, results in a marked increase in transcript expression, using either coding region or exon-specific sequences as probes (2). The present results indicate that PGE_2 can mimic the effects of cyclic AMP to stimulate aromatase expression in human adipose stromal cells, and that this expression is a consequence primarily of an increased formation of promoter II-specific transcripts.

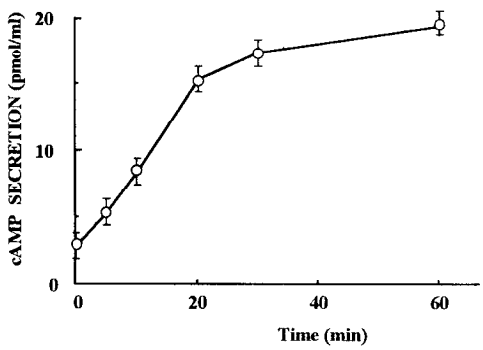

Fig. 2 cAMP formation by adipose stromal cells in the presence of PGE_2. PGE_2 (10^{-6}M) was added to adipose stromal cells in confluent monolayer culture in serum-free medium. Aliquots of media were removed at various times thereafter, as indicated, for assay of cAMP which was conducted using a cAMP enzymimmunossay (EIA) system from Amersham Life Science (Bucks., UK).

DISCUSSION

Since it is now well documented that there is increased expression of aromatase transcripts in adipose tissue adjacent to a breast tumor as compared to distal sites (5-8), it would appear that this increased expression is due to a factor or factors secreted by the cancer cells or else by macrophages infiltrating into the tumor site. Furthermore, since promoter II is responsible for this increased expression, the action of this factor must be mediated by cAMP. Such a limitation rules out many potential candidates, including most cytokines. The present study suggests that the factor in question is PGE_2. PGE_2 has been reported to be present at high levels in malignant human breast tumors and is synthesized by several human breast cancer cell lines (20,21). It is also synthesized by tumor fibroblasts (21) and adipose stromal cells (22) as well as by macrophages which have been shown to infiltrate many breast tumor sites (7). Thus, breast tumor sites provide a potentially rich source of PGE_2 which can stimulate aromatase expression both in the tumor itself and in the surrounding adipose tissue. The resulting increased estrogen biosynthesis in local sites in turn may result in increased growth and development of the tumor.

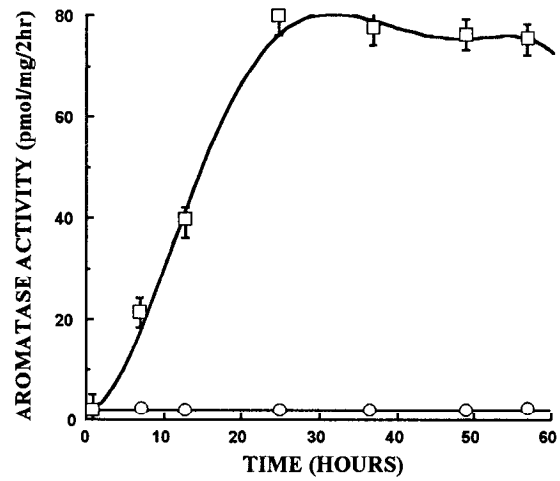

Fig. 3. Time course of induction of aromatase activity of adipose stromal cells by PGE_2. Adipose stromal cells in confluent monolayer culture in serum-free medium were maintained in the presence (open squares) or absence (open circles) of PGE_2 (10^{-6}M), for the times indicated. Aromatase activity was then assayed as described in the legend to Fig. 1.

These considerations are consistent with recent evidence which suggests that polyunsaturated fatty acids stimulate the growth and development of mammary tumors (23). It is also consistent with epidemiological evidence from a recent study which examined the association of non-steroidal anti-inflammatory drugs and breast cancer risk in a case-controlled study of 511 breast cancer patients and 1534 population control subjects (24). The results indicated that the nonsteroidal anti-inflammatory drugs may have chemo-preventive potential against the development of breast cancer. The present study points to at least one mechanism whereby this could occur, and suggests that PGE_2 produced by breast tumors or by infiltrating macrophages can indirectly increase the growth and development of breast tumors, namely by stimulating the production of estrogen in local sites within the breast as a consequence of paracrine epithelial-mesenchymal interactions.

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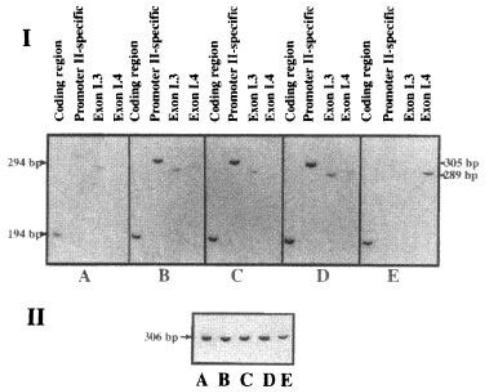

Fig. 4 (I) Amplification of specific exons I (promoter-specific exons) of *CYP19* gene transcripts in cDNA from 1 μg RNA isolated from adipose stromal cells maintained under different culture conditions: (A) control; (B) PGE_2 (10^{-8}M); (C) forskolin (25μM); (D) Bt_2 cAMP plus PMA; (E) dexamethasone plus IL-11. RT-PCR was performed according to a recently standardized competitive method which we developed for this purpose. Specific primers used to amplify the various exons I and the coding region corresponded to sequence of the 5'-ends of specific exons I and the coding region of exon II respectively. The 3'-end antisense primer corresponded to a region of exon III (coding region) and was the same in all reactions. A trace amount of [^{32}P]dCTP was added to each of the PCR reactions. The reaction products were analyzed on 4% non-denaturing polyacrylamide gels and radioactivity on the gels was visualized by exposure to x-ray film. PCR-amplified products were of the expected sizes. (II) To check the integrity and comparative quantity of RNA used in amplification of *CYP19* gene transcripts, transcripts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, were amplified by the RT-PCR method as described earlier.

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