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13. ABSTRACT (Maximum 200 Words)

The cad gene is trifunctional and expresses carbamoylphosphate synthetase/aspartate carbamyltransferase/dihydroorotase, which are required for pyrimidine biosynthesis. Treatment of ZR-75 cells with 10 nM 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) resulted in significant downregulation of E2-induced cad mRNA levels. The mechanism of TCDD-induced downregulation was further investigated using constructs containing the growth-responsive -90 to +115 (pCAD1) and -90 to +115 (pCAD2) region of the cad gene promoter. E2 induced reporter gene (luciferase) activity in MCF-7 and ZR-75 cells transfected with pCAD1 and pCAD2 and this transactivation was inhibited by TCDD. Moreover proteosome inhibitor MG132 did not reverse the effects of TCDD on cad gene expression suggesting that limiting levels of ER may not be the factor in the observed downregulation by TCDD. Also treatment of ZR75 cells with PPAR gamma agonist PGJ2 resulted in downregulation of E2-induced cad gene expression suggesting a possible crosstalk between ER and PPAR gamma signaling pathways.

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Introduction

The hormone 17ß-estradiol (E2) and related estrogenic hormones play an important role in several physiological processes, including development of the female and male reproductive tracts as well as bone, vascular, and neuronal function. Estrogens are also risk factors for breast cancer in women and for growth of early stage estrogen receptor (ER)-positive tumors. Breast cancer cell lines have been extensively used as models for understanding the mechanisms associated with mitogen-induced cell growth and for development of antiestrogenic and anticarcinogenic agents for treatment of this disease. The goal of this project was to use MCF7 and ZR75 ER-positive tumor cell lines to understand the mechanism of cad gene expression. Research in this laboratory has demonstrated that the mechanisms of hormonal and growth factor regulation of some genes, including those associated with nucleotide biosynthesis and cell growth, are regulated by a nonclassical DNA-independent mechanism that involves ERα-Sp1 (protein-protein) interactions at E2-responsive GC-rich promoter motifs. This study shows that the trifunctional cad gene is induced by E2 in MCF-7 or ZR-75 breast cancer cells within 3–12 h after treatment, respectively. Analysis of the proximal growth responsive region of the cad gene promoter showed that basal activity was primarily associated with GC-rich motifs and E2 responsiveness was dependent on the same sites which bound ERα/Sp1. Estrogen induced cad gene expression was down regulated by Aryl hydrocarbon receptor (AhR) agonist TCDD and PPAR gamma agonist suggesting a possible cross talk between ER and Ahr signaling and ER and PPAR gamma signaling respectively. Results of this study will increase our understanding of the complex processes underlying the diverse actions of E2 and facilitate strategies for development of mechanism-based drugs for treatment of breast cancer.

BODY

This project has been focused on the mechanism of hormone- induced expression of carbamoyl phosphate synthetase, aspartate transcarbamoylase and dihydroorotase (CAD) in breast cancer cells and inhibition of this response by peroxisome proliferator activated receptor gamma (PPAR γ) agonists. Moreover based on the results of preliminary studies we are also investigating the inhibitory effects of aryl hydrocarbon receptor (AhR) agonists due to the similarly of AhR/PPAR γ agonists. Some of the proposed hormone induced mechanistic studies were completed prior to the receipt of funding for this proposal.
Our results showed that the hormone-responsive region of the CAD gene promoter was primarily associated with the upstream –90/+25 sequence which contains 3 GC-rich elements that bind Sp proteins (Fig. 1)

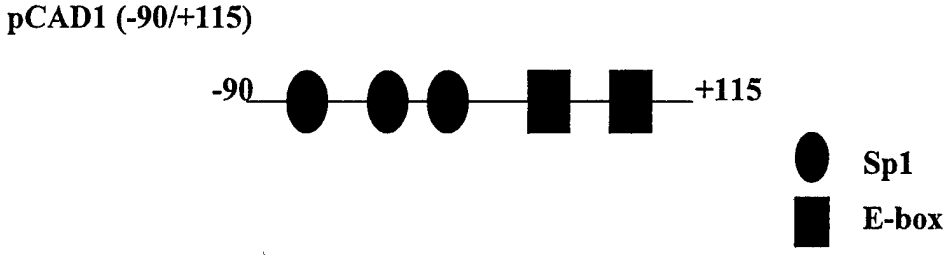

Figure 1. pCAD1 has 3 GC-rich elements and two E-boxes. The hormone responsiveness was found to be associated with two upstream GC-rich sites.

Our results indicated that the hormone-induced trans-activation was associated with ER α/Sp1 interactions with the GC-rich promoters. However recent studies have demonstrated that sp4 is also expressed in MCF7 and ZR75 breast cancer cells and the role of Sp4 in combination with Sp1 in mediating ER α/Sp1- dependent transactivation will be determined in the proposed studies.

Inhibition of hormone-induced transactivation of CAD gene promoter/gene expression by ICI 182780 and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).
Initial studies on inhibition of E2-induced CAD gene expression was investigated in ZR75 (Fig.2) and MCF-7 (Fig. 3) cells transfected with estrogen responsive pCAD constructs containing CAD gene promoter inserts. E2 induced PCAD1 (-90/+115) and pCAD2 (-90/+25) constructs as seen in our previous studies and both TCDD and ICI significantly inhibited estrogen- induced transactivation in ZR75 and MCF7 cells.
Also the effects of TCDD and ICI on Cad mRNA levels were determined in ZR75 cells (Fig 4). ZR75 cells were treated with 10 nM E2 for 1, 3, 6, 12, and 24 h or cotreated with TCDD and E2, TCDD and ICI or TCDD alone and ICI alone for 12 hr and cad mRNA levels were determined at all time points. Significant induction by E2 was observed after 12 h and 24 h and this induction was significantly down regulated by both TCDD and the pure anti-estrogen ICI. These results suggest a possible cross talk between ER and AhR signaling pathways. Previous studies in our lab have shown that TCDD causes

proteosome dependent degradation of ER. Results in Fig 5 shows the effect of proteosome inhibitor MG132 on cad mRNA levels. MG132 did not reverse the effects of TCCD downregulation of E2-induced cad gene mRNA levels suggesting that limiting levels of ER may not be a factor in the observed down regulation of Cad gene expression by TCDD. Further studies are required to elucidate the mechanism of this Ahr/ER cross talk.

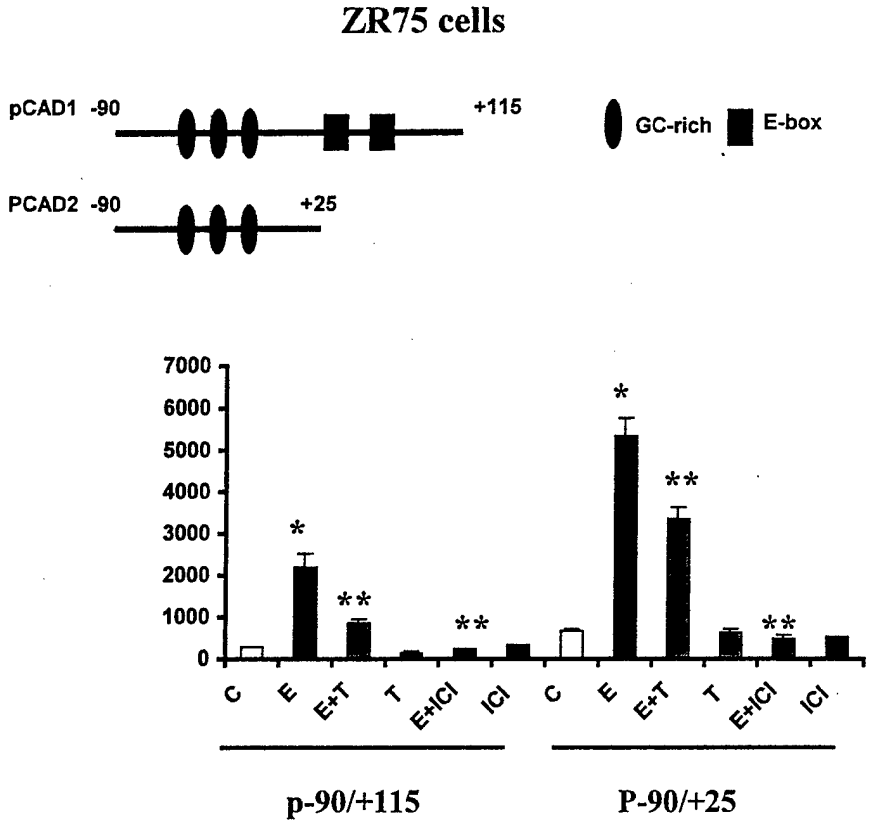

Figure 2. Inhibition of Estrogen induced transactivation of cad gene promoter by TCDD and ICI in ZR75 cells. ZR75 cells were transfected with pCAD1 or pCAD2 constructs, dosed with DMSO (C), 10 nM E2, 10nm TCDD, 1 um ICI or combined treatments and luciferase activities were determined. Results are expressed as means ± SD for at least three replicate determinations for each treatment group and significant ($P < 0.05$) induction is indicated with an *asterisk*.

MCF7 cells

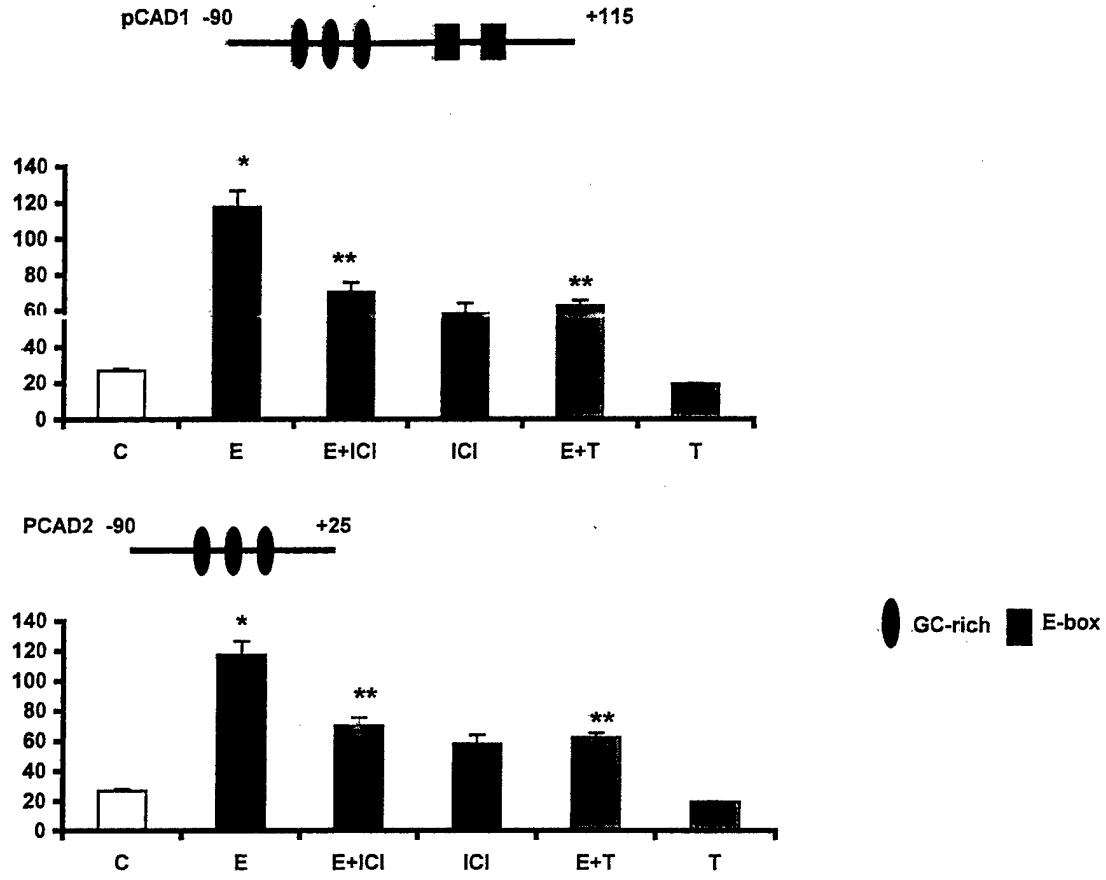

Figure 3. Inhibition of Estrogen induced transactivation of cad gene promoter by 10 nm TCDD and 1 uM ICI in MCF7 cells. MCF7 cells were transfected with pCAD1 or pCAD2 constructs, dosed with different treatments and luciferase activities were determined. Results are expressed as means ± SD for at least three replicate determinations for each treatment group and significant ($P < 0.05$) induction is indicated with an *asterisk.*

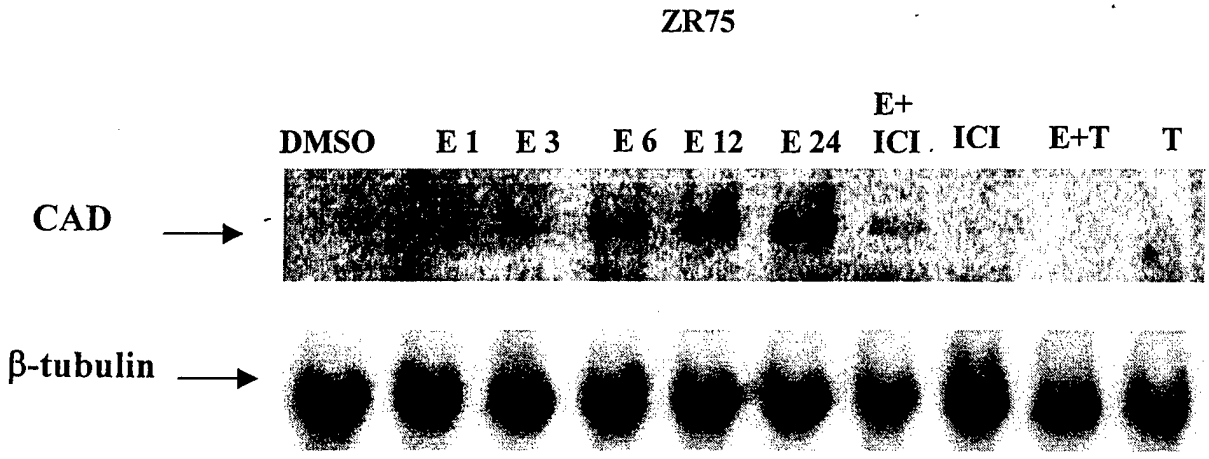

Figure 4. Inhibition of hormone induced activation of cad gene expression by TCDD and ICI. Northern analysis of cad mRNA from ZR-75 cells. The cells were treated with DMSO for 24 h (lane 1) and 10 nM E2 for 1, 3, 6, 12, and 24 h (lanes 2–5, respectively) or co-treated with E2 and ICI (lane 7) or E2 and TCDD (lane 9) or ICI (lane 8) andTCDD

(lane 10) alone for 12 h. Cell extracts were obtained, and total RNA was isolated and subjected to Northern analysis. The intensity values were normalized to the values of ß-tubulin mRNA.

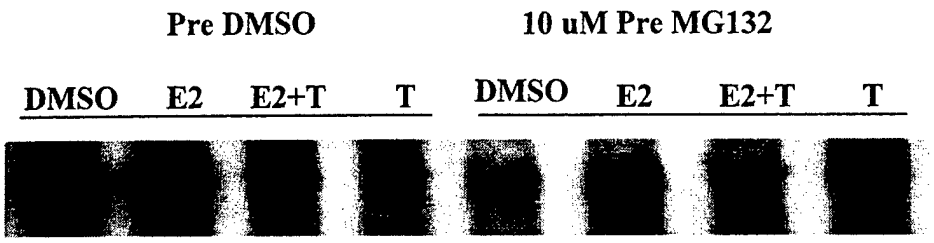

Figure 5. Effect of proteosome inhibitor MG132 on TCDD down-regulation of Cad gene expression in ZR75 cells. ZR75 cells were pretreated with DMSO (lanes 1-4) or 10μm MG132 (lanes 5-8) for 30 minutes before treating with DMSO, 10 nM E2, E2+TCDD or TCDD for 12 hr. Cell extracts were obtained, and total RNA was isolated and subjected to Northern analysis.

PPAR gamma–dependent inhibition of E2-induced CAD gene expression

The effect of PPAR gamma agonist PGJ2 on Cad mRNA levels were determined in ZR75 cells (Fig 6). ZR75 cells were treated with 10nM E2, 10 μM PGJ2, 20 μM PGJ2 or E2+PGJ2 for 12h and mRNA levels were determined. Significant induction by E2 was observed after 12 h and this was significantly down regulated by PGJ2. These results suggest a possible cross talk between ER and PPAR gamma signaling pathways and research is in progress to further characterize these effects.

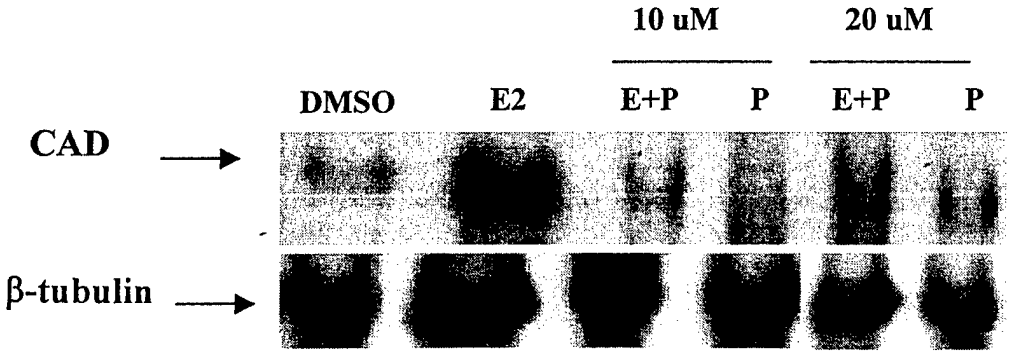

Figure 6. Effect of PPAR gamma agonist PGJ2 on E2-induced Cad gene expression in ZR75 cells. The cells were DMSO, 10nM E2, 10 μM PGJ2 (P)or E2+PGJ2 for 12 h. Cell extracts were obtained, and total RNA was isolated and subjected to Northern analysis. The intensity values were normalized to the values of ß-tubulin mRNA.

Key Research accomplishments

- E2 induced PCAD1 (-90/+115) and pCAD2 (-90/+25) constructs and both TCDD and ICI significantly inhibited estrogen- induced transactivation of cad gene promoter constructs in ZR75 and MCF7 cells.

- Significant induction of cad gene expression by E2 was observed after 12 h and 24 h and this induction was significantly down regulated by both TCDD and the pure anti-estrogen ICI suggesting a possible cross talk between AhR and ER signaling pathways.

- Proteosome inhibitor MG132 did not reverse the effects of TCCD down regulation of E2-induced cad gene mRNA levels suggesting that limiting levels of ER may not be a factor in the observed down regulation of Cad gene expression by TCDD.

- PPAR gamma agonist PGJ2 significantly down regulated E2-induced cad gene mRNA levels suggesting a possible cross talk between PPAR gamma and ER.

Reportable Outcomes

(a) Manuscripts, abstracts, presentations

Khan, S., Abdelrahim, M., Samudio, I. & Safe, S. Estrogen receptor/Sp1 complexes are required for induction of cad gene expression by 17beta-estradiol in breast cancer cells. *Endocrinology.* 144, 2325-2335 (2003).

(b) Patents/licences applied for or issued
None.

(c) Degrees
Ph.D in progress (Shaheen Khan)

(d) Cell lines/serum
No new lines developed.

(e) Informatics
None.

(f) Funding applied
None.

(g) Employment/research opportunities
None.

Conclusions

Results of this study have demonstrated that the reported hormone-dependent increase in cad activity in breast cancer cells is accompanied by induced gene expression that is linked to ERα/Sp1 interactions with GC-rich motifs. Many of the genes regulated by ERα /Sp1 in ER-positive breast cancer cells play a role in purine/pyrimidine biosynthesis (*cad*, thymidylate synthase) and metabolism (adenosine deaminase) and cell proliferation (cyclin D1, *E2F1*, c-*fos*, and *bcl-2*). These observations are consistent with a recent report showing that siRNA for Sp1 inhibits hormone-induced cell cycle progression in MCF-7 cells. Estrogen-induced cad gene expression was down regulated by TCDD suggesting a possible cross talk between ER alpha and AhR signaling. Moreover PPAR gamma agonist PGJ2 also decreased E2-induced cad gene expression suggesting a possible ER and PPAR gamma crosstalk. . Results of this study will increase our understanding of the complex processes underlying the diverse actions of E2 and facilitate strategies for development of mechanism-based drugs for treatment of breast cancer.

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8. Saville, B., Wormke, M., Wang ,F., Nguyen, T., Enmark, E., Kuiper, G., Gustafsson, J-A. & Safe, S. Ligand-, cell- and estrogen receptor subtype (α/ß)-dependent activation at GC-rich (Sp1) promoter elements. *J. Biol. Chem*. 275, 5379–5387 (2000).

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Appendices

None

Statement of Work

Task1. To investigate the effects of PPAR gamma agonists such as PGJ2, Rosiglitazone and a new class of PPAR gamma agonists 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl) methanes on E2-induced CAD gene expression.(12-24 months)

- Perform Northern and Western blot analysis to determine the time-dependent effects of PGJ2, Rosiglitazone and 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl) methanes on CAD mRNA and protein levels in breast cancer cells.
- Analyze regions of the CAD gene promoter required for the inhibitory effects of PPARγ agonists on E2-mediated transactivation using the CAD promoter constructs using transient transfection assays.
- Extensive in vitro gel mobility shift and in vivo chromatin immunoprecipitation (CHIP) assays to determine and confirm the binding of proteins to cad gene promoter in response to PPAR gamma agonists.
- To determine which Sp protein is involved in down regulation using small inhibitory RNA for Sp1, Sp3 and Sp4 in transient transfection assays.

Task 2. To determine the role of Sp4 in E2 -induced transactivation of CAD gene expression and to study the mechanism involved in AhR/ER cross talk using cad gene as the model (24-36 months)

- To determine the role of Sp4 protein in E2-induced transactivation using small inhibitory RNA for Sp4 in transient transfection assays and western Blot Analysis.
- Perform Northern and Western blot analysis to determine the time-dependent effects of TCDD on CAD mRNA and protein levels in breast cancer cells.
- Analyze regions of the CAD gene promoter required for the inhibitory effects of TCDD on E2-mediated transactivation of CAD promoter constructs using transient transfection assays.
- Extensive in vitro gel mobility shift and in vivo chromatin immunoprecipitation (CHIP) assays to determine and confirm the binding of proteins to cad gene promoter in response to TCDD.
- To determine which Sp protein is involved in down regulation using small inhibitory RNA for Sp1, Sp3 and Sp4 in transient transfection assays.