Oestradiol decreases rat apolipoprotein AI transcription via promoter site B

A H Taylor, A E Fox-Robichaud1, C Egan1, J Dionne1, D E Lawless1, J Raymond1, J Romney1 and N C W Wong1

Gynaecology Research Group, Department of Obstetrics and Gynaecology, Faculty of Medicine and Biological Sciences, University of Leicester, PO Box 65, Leicester LE2 7LX, UK

1Endocrine Research Group, Departments of Medicine and Biochemistry & Molecular Biology, the Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

(Requests for offprints should be addressed to NCW Wong, Faculty of Medicine, Departments of Medicine and Biochemistry & Molecular Biology, Health Sciences Center, 3330 Hospital Drive NW, Calgary, Alberta, Canada, T2N 4N1; Email: ncwwong@ucalgary.ca)

ABSTRACT

Oestrogens protect against ischaemic heart disease in the post-menopausal female by increasing serum concentrations of apolipoprotein (apo) AI and the abundance of high-density lipoprotein particles. In men and experimental male animals, the administration of oestrogen has variable effects on apo AI expression. As the major mode of oestrogen action on target genes involves regulating promoter activity and hence transcription, oestrogen is expected to alter transcription of the apo AI gene. To test this hypothesis, the effect of 17β-oestradiol (E2), on rat apo AI promoter activity in male hepatoma HuH-7 cells, was tested by co-transfecting a reporter template, pAI.474.CAT containing −474 to −7 of the rat apo AI promoter and an oestrogen receptor (ER) expression vector, pCMV-ER. Transfected cells exposed to E2 showed a dose-dependent decrease in chloramphenicol acetyltransferase (CAT)-activity, with a maximum 91.5% reduction at 1 µM E2. Deletional analysis of the promoter localized the inhibitory effect of ER and E2 to site B (−170 to −144) with an adjacent 5′ contiguous motif, site S (−186 to −171) acting as an amplifier. HuH-7 cell nuclear extracts showed binding activities with both sites S and B, but recombinant human ER did not. Furthermore, nuclear extracts from E2-treated HuH-7 cells showed weaker binding activity to site B, but not to site S. In summary, the inhibitory effect of ER and E2 on rat apo AI gene activity is mediated by a promoter element, site B. This inhibitory effect arises from a mechanism that does not involve direct ER binding to the B-element. The conclusion that E2 inhibits apo AI transcription was confirmed in vivo. Treatment of male adult Sprague–Dawley rats with up to 200 µg E2 for 7 days decreased apo AI protein and hepatic mRNA by 72±21% and 68±1.4% respectively. Results of ‘run-on’ transcription of the apo AI gene in isolated hepatic nuclei showed a 55% decrease in hormone-treated male rats. These findings suggest that E2 exerts primarily an inhibitory effect within male hepatic nuclei.

Journal of Molecular Endocrinology (2000) 25, 207–219

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of premature death of men and women in developed countries. Although death rates from CVD are similar, at 46% and 52% in men and women, the major difference is that the peak incidence in women occurs 10 years later than in men (Barter & Rye 1996). The protection afforded to premenopausal women is in large part due to the beneficial effects of endogenous oestrogens. Oestrogen deficiency, caused by surgical or natural menopause, increases the risk of CVD in women by threefold (Barrett-Connor & Bush 1991). Furthermore, epidemiological studies suggest that oestrogen replacement therapy in postmenopausal women reduces the risk of CVD by up to 50% (Bush 1990, Stampfer & Colditz 1991). In women with prior myocardial infarction, the benefit of hormone replacement may be as much as a 90% reduction.
in CVD risk (Sullivan et al. 1990). The precise mechanisms underlying the cardioprotective action of oestrogens are unknown. Although the mechanism is most probably multifactorial (Shewmon 1994), a major reduction in CVD risk is attributed to the effects of oestrogen on the metabolism of apolipoprotein (apo) AI and high-density lipoprotein (HDL) and low-density lipoprotein (LDL) particles (Bush et al. 1987).

Previous studies demonstrated that pre- or post-menopausal women treated with exogenous oestrogens have an increased abundance of apo AI and HDL (Walsh et al. 1991). However, oestrogen does not appear to augment the concentrations of apo AI and HDL in males and thus does not appear to protect individuals from CVD. Studies detailing the effect of oestrogens in males have shown contradictory findings. For example, male patients with prostate cancer or those undergoing sex-change procedures who are treated with exogenous oestrogen have a greater risk of developing new myocardial infarctions (Fortin et al. 1984, Henriksson et al. 1987). Similarly, hyperoestrogenaemia in males attributed to increased hepatic conversion of testosterone as a result of cigarette smoking, alcohol intake, diabetes, or drug abuse carried a greater risk of CVD, in some (Morgan 1982, Klaiber et al. 1984, Phillips 1984, Glazer 1991), but not all studies (Ruebner et al. 1961). In contrast, other data suggest that oestrogens reduce the risk of CVD in males (Marmorston et al. 1960, Farhat et al. 1996). In addition, newborn animals, pre-pubertal boys and men with cirrhosis of the liver—that is, those males with high concentrations of oestrogens but low testosterone—have reduced prevalence of CVD (Fangman & Hellwig 1947, Neufeld et al. 1962, Sukurai et al. 1961, O’Kane et al. 1997). In experimental animals the data are equally confusing, various studies showing decreased (Weinstein et al. 1986, Srivastava et al. 1993, Zou & Ing 1998), increased (Seishima et al. 1991) and no (Srivastava et al. 1997) oestrogenic effect on apo AI expression in the male.

The preceding data portray an unclear picture of the cardioprotective role of oestrogen in normal intact males. As oestrogens and other steroid hormones increase apo AI expression in the female through activation of the apo AI promoter, we examined the effect of 17β-oestradiol (E2) on apo AI promoter activity in males. The following studies show that E2 inhibits apo AI gene transcription via a cis-acting promoter motif designated site B. In addition, E2 increases female, but decreases male, rat serum apo AI protein concentrations.

MATERIALS AND METHODS

Materials

The rat apo AI cDNA, rat apo AI antibody, wild-type, mutant and site-specific pAI.CAT constructs have been described previously (Romney et al. 1992, Taylor et al. 1996a). Both the human ribosomal RNA (Sylvester et al. 1986) and pRSV-β-galactosidase plasmids were gifts (Chan et al. 1993). The p5’S.CAT, p5’S2.CAT and p5’S3.CAT constructs were created by ligating one, two or three copies, respectively of site S (gatccGAGCCCCGCAGCTTCCTGTTg, Gibco BRL, Burlington, Ontario, Canada) into the SmaI site of pGEM-CAT.5’ (Romney et al. 1992). The SB.CAT construct was created by annealing the antisense strand of site S with site B (gatccAGGCTAGGCAAAACAGATGGCAGAC–ACAGGAAc), followed by a ‘fill in’ reaction with the Klenow fragment of DNA polymerase I. The product was then cloned into the SmaI site of pGEM-CAT.5’ in the correct orientation as a monomer. Complementary strands of both sites S and B with protruding EcoRI ends and an oestrogen response element (ERE; aattCGGTCACTGTG–ACCGAa) were purchased from Integrated DNA Technologies, Inc., Coralville, IA, USA, for use in electrophoretic mobility shift assays (EMSA). Human recombinant oestrogen receptor protein (ERα) was purchased from Affinity Bioreagents, Inc., Golden, CO, USA. The pCMV-ER was a kind gift from Dr R Mischeck, University of New York.

Cell culture and transfection

Male human fetal hepatoma HuH-7 cells were cultured in serum-free RPMI-1640 medium, as previously described (Nakabayashi et al. 1989). This medium contains 1·4 × 10⁻⁵ M phenol red but, to observe the effect of exogenous E₂, cells are cultured in medium that lacks phenol red because this compound is weakly oestrogenic (Bindal et al. 1988). In the absence of phenol red, HuH-7 cells rapidly die (authors unpublished observations). Therefore, for E₂ studies, HuH-7 cells were subcultured to 25 cm² flasks for 48 h in RPMI-1640 medium and then transferred to medium containing a lower concentration of phenol red (7 × 10⁻⁷ M) for a further 24 h before transfection in the same medium. After transfection (Romney et al. 1992), cells were exposed to E₂ for 24 h and then harvested (Taylor et al. 1996a). Cell lysate was assayed for β-galactosidase and chloramphenicol acetyltransferase (CAT) activities as described elsewhere (Taylor et al.
The CAT activities were corrected for protein concentration, \( \beta \)-galactosidase activity and assay duration.

**Electrophoretic mobility shift assay**

The ERE, site S and site B oligomers were labelled with \(^{32}\)P\(\alpha\)-dCTP or \(^{32}\)P\(\alpha\)-dATP in the presence of the Klenow fragment of DNA polymerase I and the radiolabelled oligomer purified by size-exclusion chromatography (Maniatis et al. 1983). Nuclear extracts from control HuH-7 cells or those transfected with pCMV-ER (Reese & Katzenellenbogen 1991) were prepared using an established method (Ausubel et al. 1995). The EMSA studies were performed as described previously (Taylor et al. 1996a), but with the method modified so that concentrations of radiolabelled oligomer, nuclear protein, polydI.dC and time of electrophoresis were optimized for each probe. Reactions to test the binding of ER to ERE contained 5 \( \times 10^{-6} \) M E\( _2 \). Protein–DNA complexes were visualized by autoradiography using Kodak Ektascan EMC-1 or Biomax MR films. Competition analysis was performed in the presence of 100-fold molar excess of specific or non-specific oligonucleotides.

**Animals, serum protein and RNA preparation**

Sprague-Dawley rats (150–200 g) from the Charles-River Breeding Company, St Constance, Quebec, Canada were fed a diet of rat chow (Purina), with water available \textit{ad libitum}, and housed under conditions of 12-h light–12-h darkness. 17\( \beta \)-Oestradiol (Sigma-Aldrich, Oakville, Ontario, Canada) was dissolved in 95% ethanol before further dilution with propan-1,2-diol to give a final concentration of up to 200 \( \mu \)g/100 \( \mu \)l. Control animals received 0·9% saline and diluent control animals were injected with vehicle (15% ethanol and 85% propan-1,2-diol) alone. The animals were then injected subcutaneously with saline, diluent or E\( _2 \) (up to 200 \( \mu \)g/day) for 7 days. On the eighth day, the rats were killed under light ether anaesthesia (Chan et al. 1993) and the sera prepared by centrifugation at 800 \( \times \) g for 10 min at 4 \( ^\circ \)C and stored frozen at \(-80\ ^\circ\)C until required for western analyses. Approximately 500 mg freshly excised liver were snap-frozen for RNA extraction using the guanidinium isothiocyanate–phenol–chloroform method (Promega, Madison, WI, USA). The remainder of the fresh liver was used to prepare intact nuclei for \textit{in vitro} transcription and nuclear proteins for EMSA studies.

**Northern analysis**

Total hepatic RNA (10–40 \( \mu \)g) was analysed by northern blot analysis (Sambrooke et al. 1989). The 28S and 18S rRNA were visualized by staining with ethidium bromide to ensure that the RNA was intact, and equal amounts were loaded. Contents of the gel were blotted to Zetabind membrane (AMF Cuno Inc., Meriden, CT, USA) and probed with radiolabelled rat apo AI cDNA (Taylor et al. 1996a). Apo AI mRNA abundance was determined by video-assisted densitometry and normalized to the density of the 18S RNA obtained from a photographic negative of the ethidium-bromide-stained gel.

**Preparation of nuclei and nuclear ‘run-on’**

Preparation of rat liver nuclei and their use in the \textit{in vitro} transcription assay were as described previously (Taylor et al. 1996a). Each labelling reaction contained 1·3–4·8 \( \times 10^7 \) nuclei. Blots were hybridized at 65 \( ^\circ \)C with 3·3–4·3 \( \times 10^6 \) c.p.m. ‘input’ RNA. Membranes were washed at high stringency and signal detected by autoradiography using Kodak XAR-5 film for 2–4 days at \(-80\ ^\circ\)C. The hybridization signal was assessed by video-assisted densitometry. These values were expressed relative to rRNA standards and then normalized for both ‘input’ RNA per hybridization and the number of nuclei per assay.

**Statistical analysis**

Data are presented as mean \( \pm \) s.d. and significance determined by either unpaired Student’s \( t \)-test or one-way analysis of variance (ANOVA) using the least significance difference test provided in the Clinstat software package (Bland 1990).

**RESULTS**

Site B mediates the repressive effects of E\( _2 \)

To determine whether E\( _2 \) inhibits apo AI promoter activity, we measured activity of a construct, pAI.474.CAT containing the \(-474\) to \(-7\)bp fragment of rat apo AI DNA in a male human hepatoma cell line, HuH-7, co-transfected with an ER expression vector, pCMV-ER. Subsequent exposure of these cells to E\( _2 \) caused a dose-dependent decrease in promoter activity with a maximal 91% reduction at a hormone concentration of 5 \( \times 10^{-6} \) M (Fig. 1).

To locate the motif that mediated the effects of E\( _2 \), we assayed the activity of deletional constructs

1996b).
containing smaller amounts of the promoter (schematic map; Fig. 2A) in the presence of pCMV-ER and 10⁻⁶ M E₂. Removal of the DNA between −474 to −187 bp did not significantly alter the inhibitory actions of E₂ (pAI.186.CAT, Fig. 2B). However, most of the inhibitory effect of E₂ was lost in constructs lacking −474 to −170 bp or −144 bp (60 ± 7% and 25 ± 2% respectively) compared with control. Together these findings show that the cis-acting element spanning −170 to −145 bp mediates a dominant role in the inhibitory actions of E₂, and that an adjacent element, −186 to −171 bp, has a minor role. These two elements correspond to two motifs, B and S, that we described previously (Taylor et al. 1996a). A minimal promoter, −46 to −7 bp, was non-responsive to E₂ (pAI.46.CAT; Fig. 2). These observations suggest that the inhibitory effect of E₂ is mediated by site B.

Next we examined the ability of site B to transmit the inhibitory actions of E₂. For these studies we created a template, p5'B.CAT, containing duplicate B sites placed in front of the heterologous

---

**Figure 1.** Dose-dependent decrease in apo AI transcription by oestrogen. HuH-7 cells were co-transfected with pAI.474.CAT, β-galactosidase, and pCMV-ER and treated with the indicated doses of E₂ for 24 h. CAT activity in the cells was normalized against β-galactosidase activity. The data are expressed relative to untreated cells. Values shown are means ± s.d. for at least six experiments performed in duplicate.

**Figure 2.** Apo AI site B contains the oestrogen-responsive sequence. (A) Schematic diagram showing the rat apo AI promoter–CAT constructs used in the transfection studies. The various geometric shapes represent the cis-acting elements of the rat apo AI promoter. The boxes labelled SV40 and CAT are the heterologous SV40 promoter sequence and the CAT reporter gene respectively. Numbering is relative to the transcription start site. (B) HuH-7 cells were transfected with the constructs noted on top, β-galactosidase, and pCMV-ER and treated with 10⁻⁶ M E₂ for 24 h. Cellular proteins were assayed for CAT activity and corrected for β-galactosidase activity. The data are expressed relative to cells containing the same deletional construct without pCMV-ER (the abscissa). Values are means ± s.d. for at least three experiments performed in triplicate. *P<0·05, **P<0·01, ***P<0·001, one-way ANOVA compared with untreated control.

*Journal of Molecular Endocrinology (2000) 25, 207–219*
SV40 promoter that is fused to the *CAT*-gene (Fig. 2A). CAT activity in E2-treated HuH-7 cells co-transfected with p5′B.CAT and pCMV-ER was decreased by 49 ± 13% (p5′B.CAT, Fig. 2). The specificity of this effect to site B was demonstrated in the study of similar constructs, pS1.CAT, pS2.CAT, pS3.CAT and p5′A.CAT, that contained one, two or three copies of site S and duplicate site A respectively. E2 had no effect on activities of these constructs (Fig. 2).

**Potential roles of site S**

To examine the potential roles of site S (−186 to −171 bp), we created pSB.CAT, which contains sites S and B fused in tandem to the SV40 promoter 5′ to the *CAT*-gene (Fig. 2A). E2 inhibited the activity of this construct by 72% (Fig. 2B). This level of repression was more pronounced than the sum of that of either site S (pS1.CAT; 23% reduction) or site B alone (pA1.170.CAT; 58% reduction). Together, these data suggest that E2 acts through site B and that site S amplifies or augments the inhibitory actions of E2 through site B.

**E2 receptor does not bind to sites S and B**

In an attempt to define the mechanism by which E2 inhibits the transcription of the rat apo AI promoter, EMSA were performed with purified human recombinant ERα (hERα) to [32P]ERE. Purified hERα bound to [32P]ERE (lane 1, Fig. 3). The addition of 100-fold M excess unlabelled ERE blocked the formation of the ERE–ERE complex, but excess site S or B (lanes 2–4 respectively, Fig. 3A) had no effect. These findings indicate that hERα binds to the ERE, but not to sites S or B.

**Nuclear protein binding to sites S and B**

Next we examined the ability of HuH-7 nuclear proteins to bind sites S and B (Fig. 3B). Incubation of nuclear extracts with radiolabelled site S revealed the presence of at least three binding activities (lane 2, Fig. 3B). These activities were unaffected by the treatment of cells with E2 (lane 3, Fig. 3B). In contrast, the pattern of nuclear protein binding to site B DNA was more complex (lanes 4–8, Fig. 3B). Nuclear proteins from HuH-7 cells had at least five site B binding activities. The intensities of the three complexes with the slowest mobilities (asterisks and arrow) were less abundant in E2-treated cells transfected with ER (compare lanes 5 and 6 with 7 and 8, Fig. 3B). In addition, there was a slight increase in intensity of the second most mobile band in extracts from E2-treated cells (Fig. 3B), but this complex represented non-specific binding. Only a single complex was specific in competition analysis (arrow, Fig. 3C). The abundance of this complex was decreased in response to E2. The most mobile band (denoted by a dot in Fig. 3B) remained unchanged. These data show that E2 decreases specific site B binding activity.

**Mutation of HNF3 elements in site B abolish actions of ER**

Site B in the apo AI promoter is comprised of direct repeats, with two HNF-3 motifs of 9 bp separated by a 4 bp spacer (Fig. 4A). To determine whether this element mediates the repressive actions of ER and E2, we used a reporter template, BCD.CAT (Fig. 4A). The activity of this template was similar to that of pA1.170.CAT in the absence or presence of pCMV-ER and E2 (compare Fig. 4B and Fig. 2). CAT activity in cells transfected with BCD.CAT alone was not significantly different from control after treatment with E2. However, in the presence of ER there was a 89% reduction in CAT activity from 7.85% to 0.85% CAT conversion/µg protein/h (Fig. 4B). An additional 45% reduction was evident in the presence of exogenous E2 (from 0.85% to 0.473% CAT conversion/µg protein/h). The inset to Fig. 4B shows a western blot analysis of lysate from control cells or those transfected with pCMV-ER. ER was not detected in control cells, but is clearly evident in those containing the expression vector. This result supports the finding that E2 suppression of the reporter requires ER.

Next, we used three templates containing mutations to the HNF-3 sites. First, we found that pA1.Bm1.CAT, which contained a mutation of the HNF-3 motif at the 5′ end of site B, had a dramatic reduction in basal activity from 7.85% to 0.17% CAT conversion/µg protein/h (Fig. 4B). Despite this low expression, the activity of this construct was further reduced by 25% in the presence of ER (Fig. 4C). However, no further reduction was evident after the addition of E2. Another template, pA1.Bm2.CAT, containing a mutation of the HNF-3 motif at the 3′ end of site B, showed a similar reduction in basal activity and, like pA1.Bm1.CAT, it was no longer responsive to E2 (Fig. 4C). These data confirm that the inhibitory action of ER requires an intact site B. In addition, the construct pA1.158.CAT – a template that lacks the 5′ HNF-3 site – had very low basal CAT activity (0.47% CAT conversion/µg protein/h) compared with BCD.CAT (Fig. 4B). However, the activity of this construct, which retains an unmodified 3′ HNF-3 site, was reduced by 28% in the presence of
ER (Fig. 4C). Together, these mutants and deletion analyses show that intact HNF-3 sites are required for E2 to exert its inhibitory actions in the presence of ER and the 3′ HNF-3 motif alone appears capable of mediating the repressive actions of ER.

These data suggest that the HNF-3 sites have a critical role in mediating the actions of E2.

E2 decreases apo AI abundance, expression and transcription

As expected, administration of E2 to female rats produced a significant 2.9-fold up-regulation of serum apo AI concentrations (Fig. 5A, B). However, administration of E2 to male rats had the opposite effect on apo AI expression: male animals injected with E2 for 7 days had 72% less apo AI protein in serum (Fig. 5C, D) and a 68% lower hepatic mRNA content (Fig. 6A, B). Similarly, E2 also reduced the 'run-on' transcription rate of the gene by 55%, from 12.9 ± 3.1 to 5.8 ± 0.77 ng RNA/10^6 nuclei/h (Fig. 6). However, the rate of albumin gene transcription was not significantly affected by hormone administration (Fig. 6D). These data suggest that E2 reduced male apo AI serum protein and hepatic mRNA levels via a reduction in transcription of the gene.

DISCUSSION

The major economic and social impact of CVD on members of our society makes this condition an important topic for biomedical research. Apo AI...
and HDL act as protectors against CVD by amelioration of hypercholesterolaemia via several mechanisms that decrease cholesterol serum concentrations (Kashyap 1998). Therefore, it seems reasonable to postulate that to increase rates of synthesis of serum apo AI would have beneficial effects on health. In order to work towards this goal, we have embarked on a detailed analysis of the apo

* Figure 4. Mutation of HNF-3 sites in site B abolishes oestrogen responsiveness. (A) Schematic diagram and site B sequences showing the rat apo AI promoter–CAT constructs used in the transfection studies. HNF-3 sites are represented by the arrows and mutations are underlined. Numbering is relative to the transcription start site. (B) HuH-7 cells were transfected with wild-type (pAI.BCD.CAT), mutated (pAI.Bm1.CAT, pAI.Bm2.CAT), or deleted (pAI.158.CAT) pAI–CAT constructs. Parallel cultures were grown and treated with 10^{-6} M E_2. Cells co-transfected with pCMV-ER were treated with vehicle and 10^{-6} M E_2 for 24 h. Cellular proteins were assayed for CAT activity and the data presented relative to pAI.BCD.CAT. Values are shown as means ± s.d. from two experiments performed in triplicate. The insert shows levels of ERα as detected by western blot of lysates from cells that were non-transfected or transfected with pCMV-ER. *P<0.05, **P<0.01; ANOVA with least significant difference test.
AI promoter. The clinical observation that E2 in women reduces their risk of developing CVD prompted us to investigate whether this beneficial effect can be extended to males, by examining effects of the hormone on the apo AI promoter. Results of these studies show that E2 decreases apo AI promoter activity in males.

In the initial transfection experiment with pAI.474.CAT and E2, an inhibitory effect of E2 was demonstrated and shown to be dependent on the dose of hormone in HuH-7 cells, which are derived from a male liver (Fig. 1). Similar studies performed on the human apo AI promoter in HepG2 cells (also derived from a human male liver),

**Figure 5.** E2 increases female and decreases male apo AI serum protein concentrations. Representative western blot analysis of 1 µg per lane total serum proteins from (A) control (n=4) and E2-treated (n=4) female, and (C) control (n=12) and E2-treated (n=18) male Sprague-Dawley rats. (B, D) Densitometric quantitation of the female and male data presented in (A) and (C) respectively. Data are presented as the mean ± s.d.; *P<0.05 (ANOVA).

**Figure 6.** E2 decreases hepatic apo AI mRNA levels and ‘run-on’ transcription of rat apo AI gene. (A) Apo AI northern blot analysis of total hepatic RNA (10 µg) from control (lanes 1–3) Sprague-Dawley male rats and male rats treated with either 20 µg (lanes 4–6) or 200 µg (lanes 7–9) of E2. (B) Densitometric quantitation of the signal from the control (n=12), males treated with 20 µg E2 (n=6) and those treated with 200 µg E2 (n=9) shown in panel A. Data are the mean ± s.d.; *P<0.05; **P<0.01 (Student’s unpaired t-test). (C) Hepatic nuclei from control and E2-treated (200 µg) male rats were incubated in the presence of [32P]UTP. RNA harvested from these nuclei was hybridized to mouse rRNA gene (1–8 ng), rat apo AI (1 µg) and rat albumin (1 µg) cDNAs and EcoRI fragment of pBS-KS+. Hybridized RNA was detected by autoradiography. Apo AI and albumin transcription rates were determined by video-assisted densitometry and then normalized to the rRNA standard curve signal values. The data were further corrected for ‘input’ RNA and the values shown are means ± s.d. from three separate experiments per group. *P<0.05, Student’s t-test.
co-transfected with a similar ER expression plasmid, showed similar dose-dependent decreases in apo AI promoter activity (Harnish et al. 1998). Maximal inhibition of reporter transcription from the human apo AI promoter in HepG2 cells was an order of magnitude lower than in the results presented here using a rat apo AI promoter construct (5 × 10⁻⁸ M compared with 5 × 10⁻⁷ M in HuH-7 cells). The difference in extent of inhibition by E₂ in these two sets of data may be due to differences in the nature of the human and rat promoters. Although these promoters share greater than 90% homology, there is a significant difference in their responses to many transcription factors, including ROR and PPAR (Vu-Dac 1997, Staels & Auwerx 1998). However, although the human and rat apo AI promoter differ in response to some factors, in both HepG2 cells and HuH-7 cells, the apo AI promoter activity is inhibited by E₂.

The expression of oestrogen-responsive genes such as ovalbumin and IGF-1 are also regulated by E₂ at the transcriptional level. In these cases, liganded ER interacts with ERE motifs in the promoter to decrease gene expression (Sanders & McKnight 1988, McCarthy et al. 1997). Other genes such as the EGF receptor, the progesterone receptor and ER itself are up-regulated at the transcriptional level via promoter-specific ERE motifs. However, an examination of the apo AI promoter DNA for consensus ERE motifs (GGT-CAnnnTGACC), shows none to be present. As some E₂-dependent genes lack consensus EREs, E₂ action may also be mediated via non-genomic mechanisms or via other cis-acting elements. For example, E₂ regulates gene transcription via AP-1 and SP-1 sites (Cerillo et al. 1998, Xie et al. 1999) in which the liganded ER does not contact the DNA.

The lack of a consensus ERE in the rat apo AI gene led us to perform deletional analysis of the rat apo AI promoter. The element that mediates the inhibitory actions of E₂ is site B. This conclusion comes from the fact that removing DNA spanning -186 to -145 bp caused the inhibitory effect of E₂ on promoter–CAT activity to lessen from 85% to 20%. There are two cis-acting sites, which we have designated site S and site B, in this fragment of the promoter. Removal of site S (-186 to -171) caused a 25% change in E₂ effectiveness from 85% to 60%, indicating that this portion of the fragment has a minor role in the actions of E₂. Further removal of site B (-170 to -145) caused a 40% change in E₂ effectiveness, indicating that site B is the major mediator of E₂ inhibition. This idea was confirmed by the observation that E₂-inhibited the CAT activity of the construct, p5'B.CAT, but not that of p5'A.CAT, p5'S.CAT, p5'S2.CAT or p5'S3.CAT. As expected, the control template, p5'A.CAT containing site A, a thyroid hormone receptor (TR) and/or a retinoic acid responsive element (Taylor et al. 1996b) was not affected by E₂. Although ER can bind to 1,3,5-triiodothyronine response elements (TREs) and TRs bind to EREs, they do not transactivate the genes involved (Dellovade et al. 1996). Indeed, bacterially-expressed TR1 binds to ERE and hERα binds to TRE (data not shown, and Klinge et al. 1997), but clearly E₂ does not affect the activity of the A-site (p5'A.CAT, Fig. 2).

Our data also show that site S is not an ERE, because E₂ failed to affect the CAT activity of constructs containing a monomer, dimer or trimer of site S (Fig. 2). In addition, site S must be present for site B to be fully active, as the activity of a construct containing only the B-element, p5'B.CAT, was inhibited to a lesser extent than that of pSB.CAT, which contained both sites S and B (Fig. 2). These data add further support to the idea that site S is an amplifier sequence, as previously reported in the response of apo AI to glucocorticoid (Taylor et al. 1996a). At present, the trans-acting factors that may possibly bind to site S include Egr-1 and PEA-3 (Nerlov et al. 1992, Kilbourne et al. 1995) – developmental genes that are regulated by E₂ in vitro (Lockwood et al. 1998, Pratt et al. 1998). There are other developmental genes, such as the homeobox gene family member proteins Hox a10 and Hox a11, which are also regulated in an E₂-dependent manner (Ma et al. 1998). Although the responsive elements for the Hox family of transcription factors are not known, it is possible that Hox genes might interact with the apo AI promoter, and we speculate that a cascade of transcriptional factors and elements are involved in some of the actions of E₂ on rat apo AI promoter repression. Furthermore, trans-acting factors that bind to adjacent cis-acting elements – HNF-4, ARP-1, and others – may play a role in the inhibitory effect of E₂ through protein–protein interactions (Harnish et al. 1998, Hargrove et al. 1999).

The results of the EMSA studies suggest that the inhibitory effect of E₂ on apo AI transcription does not require binding of hERα to either site S or site B. As expected, hERα binds to radiolabelled ERE (Fig. 3) and the formation of this complex is inhibited by unlabelled ERE. However, neither excess site S nor site B sequences are effective in displacing hERα from the radiolabelled probe. In addition, the converse experiment of hERα added to radiolabelled site S or site B produced no visible protein–DNA complexes (data not shown). These observations indicate that hERα does not directly
bind to site S or site B and ER probably decreases apo AI transcription via an indirect mechanism.

The preceding observations prompted us to search for a potential mechanism using EMSA. If ER does not transmit the inhibitory effects of E₂ directly by binding to apo AI site B, then what does? Nuclear extracts from E₂-treated HuH-7 cells had no effect on protein binding to radiolabelled site S, but did decrease nucleoprotein binding to site B (Fig. 3). This finding suggests that loss of hepatonuclear protein binding to apo AI site B is responsible for the observed decreased in apo AI transcription.

Site B contains two HNF-3 motifs (Fig. 4) that bind HNF-3 (Harnish et al. 1994). Constructs containing a mutation or deletion of either HNF-3 motif were no longer responsive to E₂ (Fig. 4). These data are qualitatively similar to those from previous studies in which removal of the HNF-3 motifs from site B caused the loss of basal promoter activity (Harnish et al. 1994) and stimulated response to dexamethasone (Taylor et al. 1996a). The present observations show that both HNF-3 motifs are required by the promoter to maintain full responsiveness to ER and E₂. It is conceivable that E₂ influences the activity, abundance, or both, of HNF-3 isoforms (Lai et al. 1993, Harnish et al. 1998) or HNF-3-like proteins (Jacquemin et al. 1999) to transmit its effect on site B. This hypothesis also suggests that the HNF-3 isoforms or HNF-3-like proteins would have opposing activities on apo AI transcription via site B. Indeed, HNF-6 (an HNF-3-like transcription factor) has recently been shown to inhibit HNF-3 trans-activation in a gender-specific manner (Lahuna et al. 1997).

The literature provides a confusing picture of the effect of E₂ in experimental animals when compared with that in humans, with the species and strain of experimental animal being of utmost importance. Our studies on serum protein concentrations showed that administration of E₂ to female and male Sprague–Dawley rats increased and decreased serum apo AI concentrations respectively (Fig. 5). These observations negate our original hypothesis that the beneficial effect of E₂ on apo AI serum concentrations in females would also be observed in males. In physiological doses, E₂ had no significant effect on serum apo AI concentrations in male rats (data not shown). These data are consistent with the findings of a number of studies, showing either that male rats do not respond to administration of E₂, or that it decreases their apo AI secretion rate (Weinstein et al. 1986, Srivastava et al. 1993, 1997, Zou & Ing 1998).

In some, but not all, studies transcription of apo AI was shown to be unaffected, and the main effect of E₂ occurred through gene translation (Srivastava et al. 1997). However, these data are not consistent with other data showing that 17β-ethinyl oestradiol in male rats increased apo AI concentrations (Seishima et al. 1991). In the present study, we used intact animals to measure serum apo AI and used high doses of E₂. Perhaps this combination could explain the discrepancy between the findings presented here and those of others that involved castrated animals, smaller doses of E₂, measured plasma apo AI concentrations or used alternative E₂ formulations or routes of administration. As reporter assays may be more sensitive than in vivo studies because of ligand availability, we decided to increase the dose of E₂ to a pharmacological value that simulates the concentrations of E₂ in a pregnant woman (Check 1999). Our data suggest that supraphysiological doses of E₂ in the male rat decrease the transcription of apo AI, in keeping with the findings of some animal studies (Weinstein et al. 1986, Srivastava et al. 1993, Zou & Ing 1998). If decreased translation of apo AI mRNA is the main mechanism of decreased apo AI serum protein concentrations in rats (Srivastava et al. 1997), transcriptional rates and steady-state-levels of apo AI mRNA would presumably remain constant.

It is clear from our western and northern blot analyses of apo AI protein and hepatic RNA that E₂ decreases both the abundance of apo AI protein in serum and mRNA in the liver of treated male rats. Furthermore, nuclear ‘run-on’ experiments show that the effects of E₂ on apo AI gene expression arise from its ability to inhibit transcription of the gene in hepatic nuclei of hormone-treated male rats. The 55% reduction in transcription rate is similar to the 70% reduction in mRNA and 60% reduction in serum protein concentrations. Although we have observed a good correlation between decreased apo AI transcription rates, steady-state mRNA and secreted protein concentrations, we cannot rule out that E₂ could still exert a major effect on apo AI translation. Indeed, this may explain the differential effect of E₂ in the male and female rats used in the present study. First, the relative concentrations of apo AI in male and female serum were different: males had significantly greater amounts of apo AI/µg total serum protein. Secondly, in male rat hepatocytes, high doses of E₂ decrease apo AI transcription, but the hormone may also alter the expression of other proteins involved in the secretion or processing of apo AI. In the female rat hepatocyte, high doses of E₂ also decrease apo AI transcription, but the hormone may increase the activity of proteins involved in apo AI secretion and processing. This might lead to the observed gender differences in response to high-dose E₂. In addition,
E₂ increases the expression of hepatic LDL receptor and lipoprotein lipase, which could increase the availability of re-circulated apo AI within the hepatocyte, and in turn may have an inhibitory effect on apo AI transcription via altered translation, as a result of apo AI/lipoprotein recycling events (Song et al. 1998).

The findings summarized above show that, in contrast to females, male rats treated with low-dose E₂ do not have the expected increases in the serum concentration of apo AI; furthermore, males treated with high doses of E₂ show decreased serum apo AI concentrations. These results support the idea that E₂ has a detrimental effect on lipid metabolism in the male (Kalin & Zumoff 1990) and are consistent with a recent report in which male rats treated with 17β-ethinyl oestradiol had undetectable apo AI (Landschultz et al. 1996). This information helps to explain the observations made in clinical studies (Fortin et al. 1984, Henriksson et al. 1987), in which the use of exogenous E₂ to prevent further myocardial infarctions had the opposite effect, and actually enhanced CVD risk in males. A decrease in apo AI expression may not be the only factor involved in the increased CVD risk in E₂-treated males, because E₂ has a number of additional effects on the progression of CVD. For example, E₂ has potent thrombotic activity, and increases prostacyclin and prostaglandin secretion in the vasculature, but decreases intima thickening (Chang et al. 1980, Gisclard et al. 1988, Iafriati et al. 1997).

One could postulate that, as E₂ decreases apo AI in male Sprague–Dawley rats, the hormone would have a similar effect in the human male and, therefore, the use of E₂ in men would be contraindicated. It could be argued that male exposure to E₂ is easily avoided, but oestrogens are commonly used in two clinical settings: the treatment of prostate cancer, and feminization during sex-change procedures. In addition, men are constantly exposed to environmental oestrogens that are believed to cause male infertility (Sonnenschein & Soto 1998). The information arising from the current study is therefore of significant clinical relevance.

Our findings support previous reports that E₂ has a detrimental effect on male health and actually enhances the risk of CVD, possibly by decreasing the concentrations of apo AI. Although the mechanism by which E₂ increases apo AI concentrations in females but decreases it in males is not yet known, the data presented here help us to understand a fundamental aspect of apo AI gene expression. These data will be useful in the development of strategies to reduce the hyper-cholesterolaemic component of CVD. For example, because E₂ decreases male apo AI, a specific anti-oestrogen treatment that inhibits the effect of E₂ on hepatic apo AI expression might have a beneficial effect on CVD risk in the male.

ACKNOWLEDGEMENTS

Funding for this project was provided by the Heart and Stroke Foundation of Canada and Medical Research Council of Canada (MRC). N C W W is the recipient of Scientist awards from the MRC and Alberta Heritage Foundation for Medical Research.

The authors wish to thank Mrs J Forden for excellent technical assistance and Dr C K Krekowski, University of Calgary, for the gift of human rRNA cDNA.

REFERENCES


Check JH 1999 Low and high responders – at what levels of serum estradiol do things start to get fuzzy? Fertility and Sterility 71 582–583.

Delovade TL, Zhu YS, Krey L & Pfaff DW 1996 Thyroid hormone and estrogen interact to regulate behaviour. PNAS 93 12581–12586.


www.endocrinology.org


Phillips GB 1984 Evidence for hyperestrogenemia as the link between diabetes mellitus and myocardial infarction. *American Journal of Medicine* 76 1041–1048.


Zou K & Ing NH 1998 Oestradiol up-regulates oestrogen receptor, cyclophillin, and glyceraldehyde phosphate dehydrogenase copy 1 mRNA concentrations in endometrium, but down-regulates them in the liver. *Journal of Steroid Biochemistry and Molecular Biology* **64** 231–237.

**REVISED MANUSCRIPT RECEIVED 26 April 2000**