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 N C W 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 17 β -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 $\pm$ 1.5% reduction at 1 μ M 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 μ g E₂ for 7 days decreased apo AI protein and hepatic mRNA by 72 $\pm$ 21% and 68 $\pm$ 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 postmenopausal 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.* 1978, 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 17 β -oestradiol (*E*₂) on apo AI promoter activity in males. The following studies show that *E*₂ inhibits *apo AI* gene transcription via a *cis*-acting promoter motif designated site B. In addition, *E*₂ 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 (gatccGCAGCCCCCGCAGCTTCCTGTTg, 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 (gatccAGGCTAGGCAAACAGAGTGGGCAAACAGGAAC), 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-ACCGaatt) were purchased from Integrated DNA Technologies, Inc., Coralville, IA, USA, for use in electrophoretic mobility shift assays (EMSA). Human recombinant oestrogen receptor protein (ER α) was purchased from Affinity Bioreagents, Inc., Golden, CO, USA. The pCMV-ER was a kind gift from Dr R Misicek, University of New York.

Cell culture and transfection

Male human fetal hepatoma HuH-7 cells were cultured in serum-free RPMI-ISE medium, as previously described (Nakabyashi *et al.* 1989). This medium contains 1.4×10^{-5} M phenol red but, to observe the effect of exogenous *E*₂, 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 *E*₂ studies, HuH-7 cells were subcultured to 25 cm² flasks for 48 h in RPMI-ISE 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 *E*₂ 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.*

1996b). The CAT activities were corrected for protein concentration, β -galactosidase activity and assay duration.

Electrophoretic mobility shift assay

The ERE, site S and site B oligomers were labelled with [³²P] α -dCTP or [³²P] α -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 5×10^{-6} M E₂. 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 *ad libitum*, and housed under conditions of 12-h light–12-h darkness. 17 β -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 μ g/100 μ 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 E₂ (up to 200 μ 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 g for 10 min at 4 °C and stored frozen at –80 °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 *in vitro* transcription and nuclear proteins for EMSA studies.

Northern analysis

Total hepatic RNA (10–40 μ 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 *in vitro* transcription assay were as described previously (Taylor *et al.* 1996a). Each labelling reaction contained $1.3\text{--}4.8 \times 10^7$ nuclei. Blots were hybridized at 65 °C with $3.3\text{--}4.3 \times 10^6$ 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 °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 E₂

To determine whether E₂ 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 E₂ caused a dose-dependent decrease in promoter activity with a maximal 91% reduction at a hormone concentration of 5×10^{-6} M (Fig. 1).

To locate the motif that mediated the effects of E₂, we assayed the activity of deletional constructs

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 E_2 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 $\pm$ 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 E_2 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 $\pm$ 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.

containing smaller amounts of the promoter (schematic map; Fig. 2A) in the presence of pCMV-ER and 10^{-6} M E_2 . Removal of the DNA between -474 to -187 bp did not significantly alter the inhibitory actions of E_2 (pAI.186.CAT, Fig. 2B). However, most of the inhibitory effect of E_2 was lost in constructs lacking -474 to -170 bp or -144 bp ($60 \pm 7\%$ and $25 \pm 2\%$ 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 E_2 , 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 E_2 (pAI.46.CAT; Fig. 2). These observations suggest that the inhibitory effect of E_2 is mediated by site B.

Next we examined the ability of site B to transmit the inhibitory actions of E_2 . For these studies we created a template, p5'B.CAT, containing duplicate B sites placed in front of the heterologous

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 $49 \pm 13\%$ (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 72.4% (Fig. 2B). This level of repression was more pronounced than the sum of that of either site S (pS1.CAT; 2.3% reduction) or site B alone (pAI.170.CAT; 58.4% 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 ER α (hER α) to [³²P]ERE. Purified hER α bound to [³²P]ERE (lane 1, Fig. 3). The addition of 100-fold M excess unlabelled ERE blocked the formation of the ER–ERE complex, but excess site S or B (lanes 2–4 respectively, Fig. 3A) had no effect. These findings indicate that hER α 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 pAI.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/ μ g protein/h (Fig. 4B). An additional 45% reduction was evident in the presence of exogenous *E₂* (from 0.85% to 0.473% CAT conversion/ μ g 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 pAI.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/ μ g 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, pAI.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 pAI.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 pAI.158.CAT – a template that lacks the 5' HNF-3 site – had very low basal CAT activity (0.47% CAT conversion/ μ g 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/10⁶ 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

FIGURE 3. EMSA of ER binding to ERE but not to apo AI sites S or B. (A) Purified human recombinant ERα protein was incubated with [³²P]-labelled ERE for 30 min in EMSA binding buffer (lane 1) supplemented with 5×10^{-5} M E₂. A 400-fold molar excess of unlabelled ERE (lane 2), site S (lane 3), or site B (lane 4) was added to the basic reaction mixture. Bound and free radioactivity were visualized as described in Materials and Methods. The autoradiogram is a representative of four separate experiments. (B) E₂ decreases binding to site B but not site S. Nuclear extracts from ER-containing HuH-7 cells treated with or without E₂ were incubated with either [³²P]-labelled sites S (lanes 2 and 3 respectively) or B (lanes 5 and 6, and 7 and 8 respectively) for 30 min. DNA-protein complexes that were reduced after treatment with E₂ are indicated by asterisks and an arrow. Non-specific complexes are indicated by asterisks or the dot. The autoradiogram is representative of three separate experiments. (C) Competition analysis. Site B binding activity in extracts from control cells is bound to radiolabeled DNA (lane 1) in the presence of 100-fold M excess unlabeled authentic HNF-3β (lane 2) or site B (lane 3) binding motif. Addition of excess non-specific DNA had no effect on intensity of the complexes (data not shown). The only specific complex is indicated by an arrow.

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 E₂. Cells co-transfected with pCMV-ER were treated with vehicle and 10^{-6} M E₂ for 24 h. Cellular proteins were assayed for CAT activity and the data presented relative to pAI.BCD.CAT. Values are shown as means $\pm$ s.d. from two experiments performed in triplicate. The insert shows levels of ER α 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.

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 5. E_2 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 E_2 -treated ($n=4$) female, and (C) control ($n=12$) and E_2 -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 $\pm$ S.D.; * $P<0.05$ (ANOVA).

FIGURE 6. E_2 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 E_2 . (B) Densitometric quantitation of the signal from the control ($n=12$), males treated with 20 µg E_2 ($n=6$) and those treated with 200 µg E_2 ($n=9$) shown in panel A. Data are the mean $\pm$ S.D.; * $P<0.05$; ** $P<0.01$ (Student's unpaired t -test). (C) Hepatic nuclei from control and E_2 -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 $\pm$ S.D. from three separate experiments per group. * $P<0.05$, Student's t -test.

AI promoter. The clinical observation that E_2 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 E_2 decreases apo AI promoter activity in males.

In the initial transfection experiment with pAI.474.CAT and E_2 , an inhibitory effect of E_2 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),

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 L-tri-iodothyronine 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 hER α binds to TRE (data not shown, and Klinge *et al.* 1997), but clearly ER 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 hER α to either site S or site B. As expected, hER α 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 hER α from the radiolabelled probe. In addition, the converse experiment of hER α added to radiolabelled site S or site B produced no visible protein–DNA complexes (data not shown). These observations indicate that hER α 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 17 β -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/ μ g 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 17 β -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, Iafrati *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 hypercholesterolaemic component of CVD. For example,

because E₂ decreases male apo AI, a specific antioestrogen 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

- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA & Struhl K (Eds) 1995 Unit 12.1 DNA–protein Interactions. In *Current Protocols in Molecular Biology*, pp 12.1.1–12.1.3. New York: John Wiley & Sons.
- Barrett-Connor E & Bush TL 1991 Estrogen and coronary heart disease in women. *Journal of the American Medical Association* **265** 1861–1867.
- Barter PJ & Rye KA 1996 High density lipoproteins and coronary heart disease. *Atherosclerosis* **121** 1–12.
- Bindal RD, Carlson KE, Katzenellenbogen BS & Katzenellenbogen JA 1988 Lipophilic impurities, not phenolsulfonphthalein, account for the estrogenic activity in commercial preparations of phenol red. *Journal of Steroid Biochemistry* **31** 287–293.
- Bland M 1990 *An Introduction to Medical Statistics*. Oxford: Oxford University Press.
- Bush TL 1990 The epidemiology of cardiovascular disease in postmenopausal women. *Annals of the New York Academy of Sciences* **592** 263–271.
- Bush TL, Barrett-Connor E, Cowan LD, Criqui MH, Wallace RB, Suchindran CM, Tyroler HA & Rifkind BM 1987 Cardiovascular mortality and noncontraceptive use of estrogen in women: results from the Lipid Research Clinics Program Follow-up Study. *Circulation* **75** 1102–1109.
- Cerillo G, Rees A, Manchanda N, Reilly C, Brogan I, White A & Needham M 1998 The estrogen receptor regulates NF κ B and AP-1 activity in a cell-specific manner. *Journal of Steroid Biochemistry and Molecular Biology* **67** 79–88.
- Chan J, Nakabyashi H & Wong NCW 1993 HNF-4 increases activity of the rat apo AI gene. *Nucleic Acids Research* **21** 1205–1211.
- Chang WC, Nakao J, Orimo H & Murato SI 1980 Stimulation of prostacyclin biosynthetic activity by estradiol in rat aorta smooth muscle cell in culture. *Biochimica et Biophysica Acta* **619** 107–118.
- 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.
- Dellovade TL, Zhu YS, Krey L & Pfaff DW 1996 Thyroid hormone and estrogen interact to regulate behaviour. *PNAS* **93** 12581–12586.
- Fangman RJ & Hellwig CA 1947 Histology of coronary arteries in newborn infants. *American Journal of Pathology* **23** 901–902.

- Farhat MY, Lavigne MC & Ramwell PW 1996 The vascular protective effects of estrogen. *FASEB Journal* **10** 615–624.
- Fortin CJ, Klein T, Messmore HL & O'Connell JB 1984 Myocardial infarction and severe thromboembolic complications. As seen in an estrogen-dependent transsexual. *Archives of Internal Medicine* **144** 1082–1083.
- Gisclard V, Miller VM & Vanhoutte PM 1988 Effect of 17 beta-estradiol on endothelium-dependent responses in the rabbit. *Journal of Pharmacology and Experimental Therapeutics* **224** 19–22.
- Glazer G 1991 Atherogenic effects of anabolic steroids on serum lipid levels: a literature review. *Archives of Internal Medicine* **151** 1925–1933.
- Hargrove GM, Junco A & Wong NCW 1999 Hormonal regulation of apolipoprotein AI. *Journal of Molecular Endocrinology* **22** 103–111.
- Harnish DC, Malik S & Karathanasis SK 1994 Activation of apolipoprotein A-I gene transcription by the liver-enriched factor HNF-3. *Journal of Biological Chemistry* **269** 28220–28226.
- Harnish DC, Evans MJ, Scicchitano MS, Bhat RA & Karathanasis SK 1998 Estrogen regulation of the apolipoprotein AI gene promoter through transcription cofactor sharing. *Journal of Biological Chemistry* **273** 9270–9278.
- Henriksson P, Linde B & Edhag O 1987 Deleterious effects of low-dose oestrogen therapy on coronary status in patients with prostatic cancer. *European Heart Journal* **8** 779–784.
- Iafrati MD, Karas RH, Aronovitz M, Kim S, Sullivan TR Jr, Lubahn DB, O'Donnell TF Jr, Korach KS & Mendelsohn ME 1997 Estrogen inhibits the vascular injury response in estrogen receptor-deficient mice. *Nature Medicine* **3** 545–548.
- Jacquemin P, Lannoy VJ, Rousseau GG & Lemaigre FP 1999 OC-2 a novel mammal member of the ONECUT class of homeodomain transcription factors whose function in liver partially overlaps with that of hepatocyte nuclear factor-6. *Journal of Biological Chemistry* **274** 2665–2671.
- Kalin MF & Zumoff B 1990 Sex hormones and coronary disease: a review of the clinical studies. *Steroids* **55** 330–352.
- Kashyap ML 1998 Mechanistic studies of high-density lipoproteins. *American Journal of Cardiology* **82** 42U–48U.
- Kilbourne EJ, Widom R, Harnish DC, Malik S & Karathanasis SK 1995 Involvement of early growth response factor Egr-1 in apolipoprotein A-I gene transcription. *Journal of Biological Chemistry* **270** 7004–7010.
- Klaiber EL, Broverman DM & Dalen JE 1984 Serum estradiol levels in male cigarette smokers. *American Journal of Medicine* **77** 858–862.
- Klinge CM, Bodenner DL, Desai D, Niles RM & Traish AM 1997 Binding of type II nuclear receptors and estrogen receptor to full and half-site estrogen response elements *in vitro*. *Nucleic Acids Research* **25** 1903–1912.
- Lahuna O, Fernandez L, Karlsson H, Maiter D, Lemaigre FP, Rousseau GG, Gustafsson J-A & Mode A 1997 Expression of hepatocyte nuclear factor 6 in rat liver is sex-dependent and regulated by growth hormone. *PNAS* **94** 12309–12313.
- Lai E, Clark KL, Burley SK & Darnell JE Jr 1993 Hepatocyte nuclear factor 3/forkhead or 'winged helix' proteins: a family of transcription factors of diverse biologic function. *PNAS* **90** 10421–10423.
- Landschulz KT, Pathak RK, Rigotti A, Kreiger M & Hobbs HH 1996 Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. *Journal of Clinical Investigation* **98** 984–995.
- Lockwood CJ, Krikun G, Hausknecht VA, Papp C & Schatz F 1998 Matrix metalloproteinase and matrix metalloproteinase inhibitor expression in endometrial stromal cells during progestin-initiated decidualization and menstruation-related progestin withdrawal. *Endocrinology* **139** 4607–4613.
- Ma L, Benson GV, Lim H, Dey SK & Maas RL 1998 Abdominal B (AbdB) Hoxa genes: regulation in adult uterus by estrogen and progesterone and repression in müllerian duct by the synthetic estrogen diethylstilbestrol (DES). *Developmental Biology* **197** 141–154.
- McCarthy TL, Ji C, Shu H, Casinghino S, Crothers K, Rotwein P & Centrella M 1997 17β-Estradiol potently suppresses cAMP-induced insulin-like growth factor-1 gene activation in primary rat osteoblast cultures. *Journal of Biological Chemistry* **272** 18132–18139.
- Maniatis T, Fritsch EF & Sambrooke J 1983 *Molecular Cloning: a Laboratory Manual*, pp 464–465. New York: Cold Spring Harbor Laboratory Press.
- Marmorston J, Moore FJ, Kuzma OT, Magidson O & Weiner J 1960 Effect of Premarin on survival of men with myocardial infarction. *Proceedings of the Society for Experimental Biology and Medicine* **110** 400–408.
- Morgan MY 1982 Sex and alcohol. *British Medical Bulletin* **38** 43–52.
- Nakabyashi H, Watanabe K, Saito A, Otsuru O, Sawadaishi K & Tamaoki T 1989 Transcriptional regulation of alpha-fetoprotein expression by dexamethasone in human hepatoma cells. *Journal of Biological Chemistry* **264** 266–271.
- Nerlov C, De Cesare D, Pergola F, Caracciolo A, Blasi F, Johnsen M & Verde P 1992 A regulatory element that mediates co-operation between a PEA3-AP-1 element and an AP-1 site is required for phorbol ester induction of urokinase enhancer activity in HepG2 hepatoma cells. *EMBO Journal* **11** 4573–4582.
- Neufeld HN, Wagenvoort CA & Edwards JE 1962 Coronary arteries in fetuses, infants, juveniles and young adults. *Laboratory Investigations* **11** 827–844.
- O'Kane MJ, Lynch PL, Callender ME & Trimble ER 1997 Abnormalities of serum apo AI containing lipoprotein particles in patients with primary biliary cirrhosis. *Atherosclerosis* **131** 203–210.
- Phillips GB 1984 Evidence for hyperestrogenemia as the link between diabetes mellitus and myocardial infarction. *American Journal of Medicine* **76** 1041–1048.
- Pratt MA, Satkunaratnam A & Novosad DM 1998 Estrogen activates raf-1 kinase and induces expression of Egr-1 in MCF7 breast cancer cells. *Molecular and Cellular Biochemistry* **189** 119–125.
- Reese JC & Katzenellenbogen BS 1991 Differential DNA-binding abilities of estrogen receptor occupied with two classes of antiestrogens: studies using human estrogen receptor overexpressed in mammalian cells. *Nucleic Acids Research* **19** 6595–6602.
- Romney JS, Chan J, Carr FE, Mooradian AD & Wong NCW 1992 Identification of the thyroid hormone-responsive messenger RNA spot 11 as apolipoprotein-AI messenger RNA and effects of the hormone on the promoter. *Molecular Endocrinology* **6** 943–950.
- Ruebner BH, Miyai K & Abbey H 1961 The low incidence of myocardial infarction in hepatic cirrhosis: a statistical artifact? *Lancet* **2** 1435–1436.
- Sakurai T, Tosaka A, Yamada T, Kuwahara T & Masubuchi K 1978 Childhood coronary sclerosis. *Acta Pathologica Japonica* **28** 41–52.
- Sambrooke J, Fritsch EF & Maniatis T 1989 *Molecular Cloning: a Laboratory Manual*, edn 2, pp 7:37–7:52. Plainview, NY: Cold Spring Harbor Laboratory Press.
- Sanders MM & McKnight GS 1988 Positive and negative regulatory elements control the steroid-responsive ovalbumin promoter. *Biochemistry* **27** 6550–6557.

- Seishima M, Bisgaier CL, Davies SL & Glickman RM 1991 Regulation of hepatic apolipoprotein synthesis in the 17 α -ethinyl estradiol-treated rat. *Journal of Lipid Research* **32** 941–951.
- Shewmon DA 1994 Lipids, atherosclerosis, and the postmenopausal woman: a clinical perspective. *Obstetrics and Gynecology Clinics of North America* **21** 337–355.
- Song W, Chen J & Redinger RN 1998 Apolipoprotein A-I complexed with phospholipid promotes hepatic lipoprotein and apolipoprotein secretion in the perfused hamster liver. *Journal of Investigative Medicine* **46** 460–469.
- Sonnenschein C & Soto AM 1998 An updated review of environmental estrogen and androgen mimics and antagonists. *Journal of Steroid Biochemistry and Molecular Biology* **65** 143–150.
- Srivastava RAK, Baumann D & Schonfeld G 1993 *In vivo* regulation of low-density lipoprotein receptors by estrogen differs at the post-transcriptional level in rat and mouse. *European Journal of Biochemistry* **216** 527–538.
- Srivastava RAK, Krul ES, Lin RC & Schonfeld G 1997 Regulation of lipoprotein metabolism by estrogen in inbred strains of mice occurs primarily by posttranscriptional mechanisms. *Molecular and Cellular Biochemistry* **173** 161–168.
- Staels B & Auwerx J 1998 Regulation of apo A-I gene expression by fibrates. *Atherosclerosis* **137** S19–S23.
- Stampfer MJ & Colditz GA 1991 Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiologic evidence. *Preventative Medicine* **20** 47–63.
- Sullivan JM, van der Zwaag R, Hughes JP, Maddock V, Kroetz FW, Ramanathan KB & Mirvis DM 1990 Estrogen replacement and coronary heart disease: effect of survival in post menopausal women. *Archives of Internal Medicine* **150** 2557–2562.
- Sylvester JE, Whiteman DA, Podolsky R, Pozsgay JM, Respass J & Schmickel RD 1986 The human ribosomal RNA genes: structure and organization of the complete repeating unit. *Human Genetics* **73** 193–198.
- Taylor AH, Raymond J, Dionne JM, Romney J, Chan J, Lawless DE, Wanke IE & Wong NCW 1996a Glucocorticoid increases rat apolipoprotein A-I promoter activity. *Journal of Lipid Research* **37** 2232–2243.
- Taylor AH, Wishart P, Lawless DE, Raymond J & Wong NCW 1996b Identification of functional positive and negative thyroid hormone-responsive elements in the rat apolipoprotein A-I promoter. *Biochemistry* **35** 8281–8288.
- Vu-Dac N, Gervois P, Grotzinger T, De Vos P, Schoonjans K, Fruchart J-C, Auwerx J, Mariani J, Tedgui A & Staels B 1997 Transcriptional regulation of apolipoprotein A-I gene expression by the nuclear receptor ROR α . *Journal of Biological Chemistry* **272** 22401–22404.
- Walsh BW, Schiff I, Rosner B, Greenberg L, Ravnkar V & Sacks FM 1991 Effects of postmenopausal estrogen replacement on the concentrations and metabolism of plasma lipoproteins. *New England Journal of Medicine* **325** 1196–1204.
- Weinstein I, Wilcox HG & Heimberg M 1986 Effects of high-dose ethinyl estradiol on serum concentrations and hepatic secretion of the very-low-density lipoprotein, triacylglycerol, cholesterol, and apolipoprotein A-I in the rat. *Biochimica et Biophysica Acta* **876** 450–459.
- Xie W, Duan R & Safe S 1999 Estrogen induces adenosine deaminase gene expression in MCF-7 human breast cancer cells: role of estrogen receptor–Sp1 interactions. *Endocrinology* **140** 219–227.
- Zou K & Ing NH 1998 Oestradiol up-regulates oestrogen receptor, cyclophilin, 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