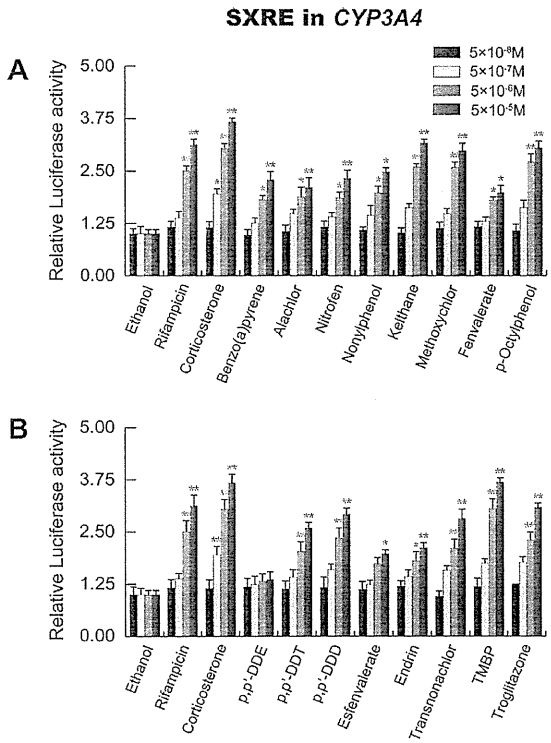

Fig. 3. Dose dependent transactivation of SXRE in *CYP3A4* mediated by SXR bound to chemicals. CV-1 cells were transfected with ptkCYPER6×3luc together with plasmid expressing SXR. Similar protocol was followed as Fig. 2.

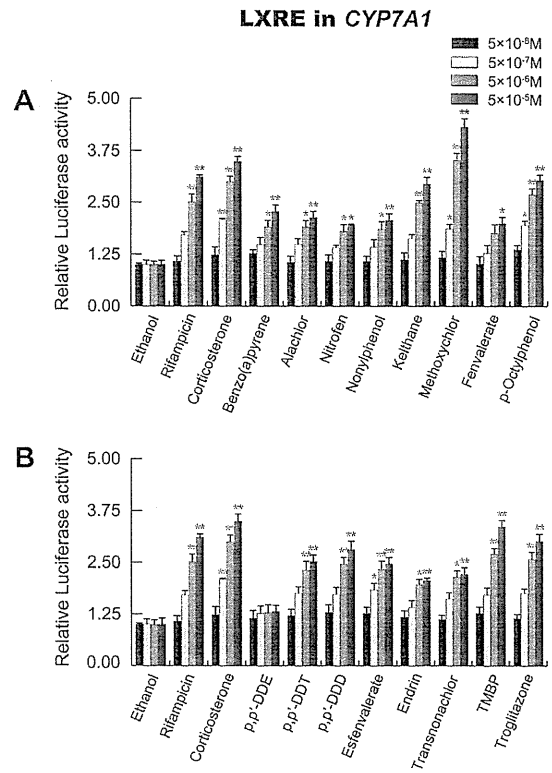

Fig. 4. Dose dependent transactivation of LXRE in rat *CYP7A1* mediated by SXR bound to chemicals. CV-1 cells were transfected with ptkCYPβDR4×3luc together with plasmid expressing SXR. Similar protocol was followed as Fig. 2.

et al., 2008) and prostate cancer (Band et al., 2011). However, different effect of *p,p'*-DDE on PXR in rat was observed by Wyde (Wyde et al., 2003) that *p,p'*-DDE activated PXR and induced expression of *CYP3A1* gene. This is probably due to different sensitivity to *p,p'*-DDE of species specificity between SXR and PXR (Barwick et al., 1996).

We also found that some EDC-bound SXR cross-interacted with LXRE (rat LXRα response element), composed of βDR4 found in rat *CYP7A1* gene promoter region, thus suggesting that EDC-bound SXR may modulate *CYP7A1* transcription. CYP7A1 is a liver-specific P450 enzyme which catalyzes cholesterol 7α-hydroxylation, a metabolic reaction which is the rate-limiting step in catabolism of cholesterol to bile acids (Russell and Setchell, 1992). LXRα is activated by several metabolic products in cholesterol, steroids, and bile acid metabolic pathways including such compounds as mevalonate, and naturally occurring oxysterols (Lehmann et al., 1997; Forman et al., 1997; Janowski et al., 1996). The critical role of LXRα in *CYP7A1* gene induction has been confirmed in mice with a disruption in *LXRα* gene, in which *CYP7A1* induction in response to dietary cholesterol are defective and a very large amount of cholesterol is accumulated in liver (Peet et al., 1998). The regulation of *CYP7A1* gene expression by a variety of nuclear receptors has become elucidated, namely *CYP7A1* is positively regulated by LXRα, and negatively by either FXR or PPARα. However, such gene regulation is very complicated, since these activated receptors are shown not to bind to DNA for transactivation function (Edwards et al., 2002; Hunt et al., 2000; Marrapodi and Chiang, 2000). In order to exclude potential actions of EDC mediated via endogenous PPARα, which is known to bind to many synthetic compounds as a ligand and to be highly expressed in liver (Waxman, 1999), we performed experiments using a heterologous promoter and non-liver cell line, CV-1 cells. Therefore, in our experiments, the transcriptional activation of *CYP7A1* gene by transfected SXR could reflect the direct action of human SXR on LXRE.

Exogenous pharmacological compounds may affect the endogenous steroid metabolism. In our experiment, methoxychlor was the most potent activator of ptkCYPβDR4×3luc, carrying LXRE sequence in rat *CYP7A1*. Animals which have been fed with some EDCs or humans in which the serum EDC levels are increased have been shown to present the elevated serum cholesterol levels, while one fed with other EDCs such as methoxychlor reveals decreased serum cholesterol levels (Dodge et al., 1996). Although another negative regulator of *CYP7A1* gene is FXR, our findings raised the possibility that some EDCs modify endogenous cholesterol metabolism via SXR. Our results further provide evidence for interrelationship between cholesterol metabolism and EDC-bound nuclear receptors, which play an important role in cytochrome P450 regulation, especially in view of SXR, LXRα, and FXR (Zhou et al., 2009).

The activated SXR by benzo(a)pyrene formed fluorescence foci in nucleus of CV-1 cells, similar to the subnuclear localization pattern of SXR activated by positive control (corticosterone or rifampicin). Our results appear to indicate that the subnuclear distribution of activated SXR closely resemble the findings of previous reports (Zhao et al., 2002). Evidence from experiments using chimeric steroid hormone receptors, such as glucocorticoid receptor (GR) or AR fused to fluorescence protein, showed that the activated GR or AR were not distributed homogeneously in nucleus, but instead, produced fluorescence clusters (Presman et al., 2010; Heemers and Tindall, 2009; Goto et al., 2003). Although the exact biological relevance of fluorescence foci in the nucleus remains to be elucidated, this intranuclear fluorescence cluster formation

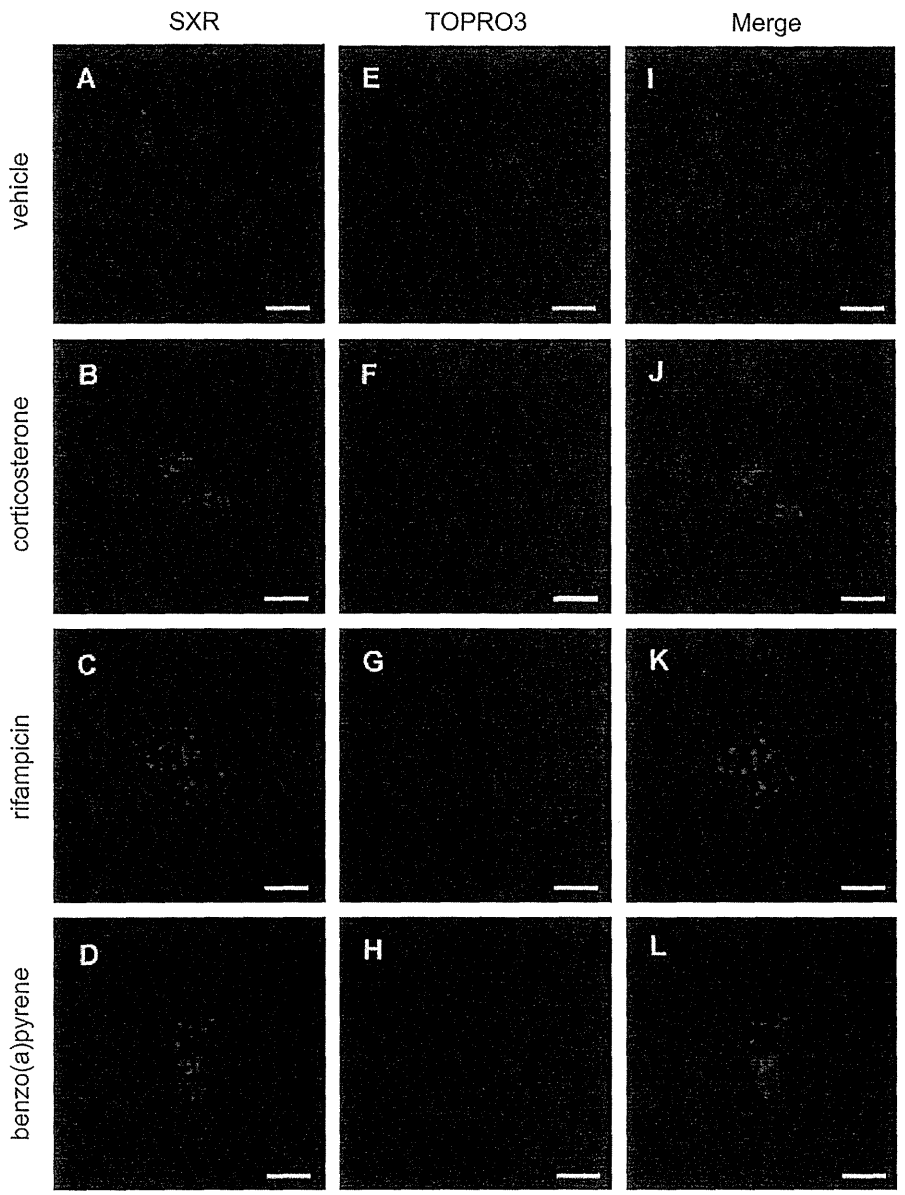

Fig. 5. The subcellular localization of SXR with positive EDC. CV-1 cells were transfected with pECFP-SXR plasmid in the absence or presence of corticosterone, rifampicin and benzo(a)pyrene (5×10^{-6} M). (A) Homogeneous intranuclear distribution of the unliganded SXR (red). (B–D) In the presence of corticosterone, rifampicin or benzo(a)pyrene, intranuclear pECFP-SXR fluorescence foci were produced (red). (E–H) The cells were stained with TOPRO3 to visualized DNA (blue). (I–L) Merged images. Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

may represent activated or transcriptionally active nuclear receptors (Goto et al., 2003).

Our results showed nearly equal effects of 14 positive EDCs on SXR-mediated transactivation of human or rat *CYP3A* and rat *CYP7A1* genes. The molecular structures of the positive EDCs suggest that they do not share obvious similarity. The possible interpretation is that SXR has a large ligand binding domain, and it can be activated by compounds with diverse structure (Watkins et al., 2001).

Among these positive EDCs, two of them are confirmed as carcinogen and four are possible carcinogen according to IARC carcinogens list, U.S. NTP carcinogens list, and so on (Table 3). Cytochrome P450s play a role in metabolism of clinical drugs, leading to resistant to anticancer treatment, and they are also involved in tumorigenesis. CYP3A4 is the major enzyme of P450s, and overexpression of CYP3A4 induced growth of human hepatoma cells (Oguro et al., 2011) and selectively knockdown of CYP3A4 inhibited breast cancer cell proliferation (Mitra et al., 2011). Moreover, induction of CYP3A4 by SXR caused resistance to anticancer drugs (Chen et al., 2009; Chen et al., 2007). These evidences suggest the possible role of EDCs which activate SXR-mediated expression of *CYP3A4* in carcinogenesis and cancer treatment barrier. The mechanisms of EDCs in carcinogenesis and other human health problems still need to be further clarified.

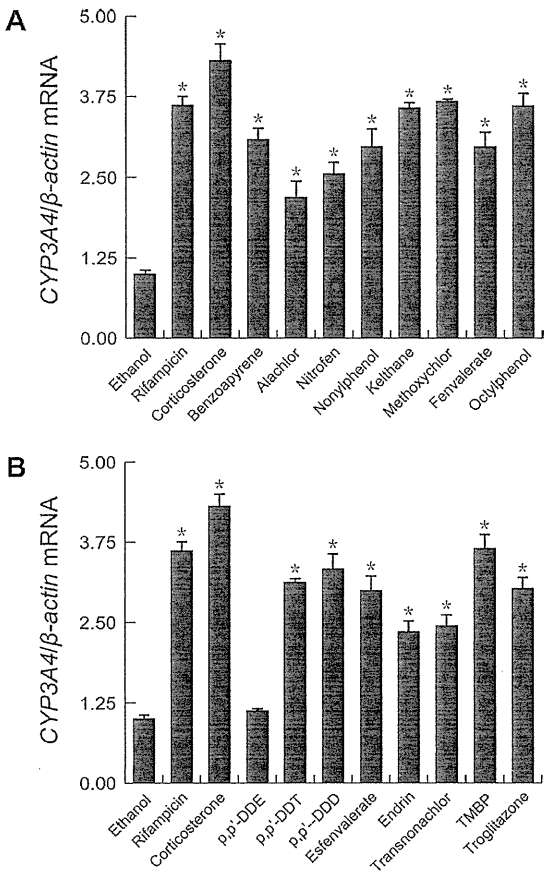

Fig. 6. *CYP3A4* mRNA expression was enhanced by positive EDCs. (A and B) RT-PCR was performed with the total RNA extracted from HepG2 cells after the treatment with EDCs (5×10^{-6} M) as shown in Fig. 1. Both *CYP3A4* and *β-actin* mRNA expression were analyzed, and the gray value representing the induction of *CYP3A4* expression was normalized by *β-actin*. The results are presented as the means ± SD. *$P < 0.01$ (Student's *t* test) as compared with the ethanol control.

Table 3
A list of CAS No., the effect on SXR-induced transactivation, and possibility of carcinogen for 14 positive EDCs. + presents the intensity of the effect of EDCs on SXR-induced transactivation. The carcinogen classification is according to IARC carcinogens list, U.S. NTP carcinogens list, California Prop 65 Known carcinogens list, U.S. EPA carcinogens list and TRI carcinogen list.

Compound	CAS No.	CYP3A2 (DR3)	CYP3A4 (ER6)	CYP7A1 (βDR4)	Carcinogen
Benzo(a)pyrene	50-32-8	+++	+++	++++	Yes
Alachlor	15972-60-8	++	++	++	Possible
Nitrofen	1836-75-5	++	+++	++	Yes
Nonyphenol	25154-52-3	++	+++	++	Not list
Kelthane	115-32-2	+++	+++	+++	Possible
Methoxychlor	72-43-5	+++	++++	+++++	Not list
Fenvalerate	51630-58-1	++	+++	++	Not list
p-Octylphenol	27193-28-8	+++	++++	+++	Not list
p,p'-DDT	50-29-3	+++	+++	+++	Possible
p,p'-DDD	72-54-8	++	+++	+++	Possible
Esfenvalerate	66230-04-4	+++	+++	++	Not list
Endrin	72-20-8	++	++	++	Not list
Transnonachlor	39765-80-5	++	+++	++	Not list
TMBP	140-66-9	++++	++++	++++	Not list

5. Conclusions

EDCs have emerged as a major public health issue because of their potentially disruptive effects on physiological hormonal actions. SXR regulates *CYP3A* gene expression in response to exogenous chemicals, such as EDCs, after binding to the motifs (SXRE).

In this study, we identified 14 EDCs enhanced transactivation of human *CYP3A4* or rat *CYP3A2* gene via SXR. Our data showed that liganded SXR produced fluorescence foci in the nucleus. CYP3A4 is suggested by previous reports to be involved in initiation, progression and chemotherapy resistance of cancer, thus these positive EDCs probably play critical roles in carcinogenesis and anticancer treatment resistance via SXR. In addition, we demonstrated that 14 EDCs activated SXR-mediated transactivation of *CYP7A1* gene, which contributes to catabolism of cholesterol to bile acids. Aberrant activation of CYP7A1 by EDCs may disrupt physiological bile acids homeostasis.

Our data together with previous studies provide evidence for the potential relationship between these positive EDCs in this study and steroid or xenobiotic metabolism homeostasis through SXR. Further studies will be required to address the roles of EDCs in carcinogenesis and other human health problems.

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Dihydrotestosterone Inhibits Lectin-Like Oxidized-LDL Receptor-1 Expression in Aortic Endothelial Cells via a NF-κB/AP-1-Mediated Mechanism

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The mechanisms involved in the antiatherosclerotic effects of androgens are unclear. Although lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in endothelial cells plays critical roles in atherosclerosis, the effects of androgens on endothelial LOX-1 expression has not been examined. Therefore, to investigate the effects of dihydrotestosterone (DHT) on LOX-1 expression in rabbit aortic endothelial cells and cultured human aortic endothelial cells (HAEC), pellets containing DHT or placebo were sc implanted into 26 male New Zealand white rabbits at the time of castration or sham operation. The rabbits were then fed a high-cholesterol diet (HCD) for 2 wk. Microscopic examination of the aortic arch revealed that DHT significantly reduced HCD-induced LOX-1 expression in endothelial cells compared with placebo. In cultured HAEC, DHT at concentrations above 10^{-9} to 10^{-7} mol/liter inhibited TNFα-induced LOX-1 mRNA and protein expression. Deletion and mutation analysis of human LOX-1 promoter-luciferase constructs transfected into HAEC with an androgen receptor (AR) expression plasmid revealed that the 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response element (TRE; nucleotides −60/−53) contributed to the inhibitory effects of DHT on TNFα-induced LOX-1 expression. Chromatin immunoprecipitation (ChIP) and re-ChIP assays revealed that TNFα- and TPA-dependent enrichment of p65 and phosphorylated c-Jun in the TRE chromatin region was inhibited by DHT-AR. Consistent with these results, DHT also suppressed TPA-induced expression of LOX-1. In conclusion, DHT exerts antiatherosclerotic effects by suppressing endothelial LOX-1 expression. This effect is partly mediated by the suppression of nuclear factor-κB- and activator protein 1-dependent activation of the LOX-1 promoter. (***Endocrinology*** **153: 3405–3415, 2012)**

Atherosclerosis is a multifactorial pathological process in which increased plasma levels of low-density lipoprotein (LDL) and its subsequent oxidation to oxidized LDL (ox-LDL) are critical early factors (1). Monocytes, macrophages, vascular endothelial cells, and vascular smooth muscle cells take up ox-LDL, leading to the formation of lipid-laden foam cells, the onset of endothelial dysfunction, and smooth muscle cell proliferation and migration (1). Ox-LDL activates endothelial cells by binding to its receptor, lectin-like ox-LDL receptor-1 (LOX-1) (2), and then up-regulates the expression of LOX-1 through a positive feedback loop. LOX-1 is involved in almost all of the proatherogenic effects of ox-LDL, including the expression of matrix metalloproteinases, induction of apoptosis, expression of adhesion molecules and resultant monocyte adhesion, and reduced endothelial nitric oxide

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Abbreviations: AP, Activator protein; AR, androgen receptor; ChIP, chromatin immunoprecipitation; DHT, dihydrotestosterone; DMSO, dimethylsulfoxide; E2, estradiol; eNOS, endothelial nitric oxide synthase; HDL, high-density lipoprotein; hLOX-1, human LOX-1; IκB, inhibitory κB; LDL, low-density lipoprotein; LOX-1, lectin-like ox-LDL receptor-1; NF, nuclear factor; nt, nucleotide; ox-LDL, oxidized LDL; p-c-Jun, phosphorylated-c-Jun; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRE, TPA response element; VLDL, very-low-density lipoprotein.

synthase (eNOS) generation and nitric oxide (NO) release (1, 3). LOX-1 expression is also transcriptionally up-regulated by many proatherogenic stimuli, including oxidative stress and inflammatory cytokines such as TNFα and IL-1α (1, 3, 4).

It is well established that men have a greater incidence of cardiovascular disease compared with premenopausal women of a similar age. This sex difference contributed to the hypothesis that androgens promote atherosclerosis. However, the most recent clinical findings indicate that androgen deficiency is strongly associated with increased mortality and increased risk of several medical conditions including metabolic syndrome, obesity, diabetes, hypertension, and atherosclerosis (5–8). Serum testosterone levels were also reported to be inversely associated with the progression of carotid artery intima media thickness and plaque area, early markers for atherosclerosis (9). Furthermore, decreased androgen receptor (AR) expression was associated with more extensive coronary artery atherosclerosis in men without known coronary artery disease (10). Interestingly, testosterone was reported to suppress the expression of endogenous inflammatory cytokines, including TNFα and IL-1β (11). In testicular feminized mouse, physiological concentrations of testosterone inhibit aortic fatty streak formation in cholesterol-fed mice, an action that is independent of the classic AR and is at least partly mediated by the conversion of testosterone to 17β-estradiol (12). However, beneficial activities of the AR system in vasodilatation (13) and angiotensin II-induced vascular remodeling (14) was also reported. In another study using male castrated cholesterol-fed rabbits, the suppressive effect of testosterone undecanoate on aortic atherosclerosis was explained only by its effects on lipoproteins (15). Therefore, the antiatherosclerotic mechanisms of androgens seem to be multifactorial.

Dihydrotestosterone (DHT) is the most biologically potent natural androgen and is never aromatized to estrogens. We recently reported that physiological levels of DHT attenuate the development of atherosclerosis in rabbits fed a high-cholesterol diet (HCD) for 8 wk by suppressing intimal foam cell formation of macrophages (16). This was achieved in part by the suppression of LOX-1 expression in the subendothelial region, suggesting direct antiatherosclerotic effects of physiological levels of DHT (16). Surprisingly, we also found that LOX-1 was not expressed in endothelial cells in this relatively advanced atherosclerotic model (16), hindering studies to investigate the effects of DHT on LOX-1 expression in endothelial cells. LOX-1 was reported to be expressed in endothelial cells in early atherosclerotic lesions (17), suggesting that the expression of LOX-1 in atherosclerosis may be stage dependent and cell specific. Therefore, in the present study, to further examine the mechanisms underlying the antiatherosclerotic effects of DHT, we assessed endothelial LOX-1 expression in an early atherosclerotic rabbit model treated with DHT. The observed effects were also examined in cultured human aortic endothelial cells (HAEC). We found that DHT suppressed LOX-1 expression in endothelial cells in early atherosclerotic lesions. This effect was further supported by the finding *in vitro* that TNFα- or 12-O-tetradecanoylphorbol-13-acetate (TPA)-mediated LOX-1 expression in cultured HAEC was suppressed by nuclear factor (NF)-κB/activator protein (AP)-1 signaling.

Materials and Methods

Hypercholesterolemic rabbit model and experimental design

Male New Zealand white rabbits (6–7 wk old, weighing 1.00 ± 0.15 kg) were purchased from Biotek Co., Ltd. (Tosu, Japan) and kept in the animal facility at the Center of Biomedical Research, Faculty of Medical Sciences, Kyushu University. All of the procedures were reviewed and approved by the institutional Animal Care Subcommittee. Twenty-six rabbits were randomly divided into four groups: 1) control group, rabbits that were not castrated and were fed a normal diet (n = 6); 2) sham group, rabbits that underwent sham operation (no castration) and were fed the HCD (n = 6), 3) placebo group, rabbits that were castrated, fed the HCD, and implanted with a sc placebo pellet (n = 7); and 4) DHT group, rabbits that were castrated, fed the HCD, and implanted with a sc 150-mg DHT pellet (n = 7). Castration was performed through a midline abdominal incision after the rabbits were anesthetized by im injection of 10 mg/kg xylazine, followed by iv injection of 50 mg/kg sodium pentobarbital. Subcutaneous implantation of the DHT or placebo pellets (Innovative Research of America, Sarasota, FL) was performed at the time of castration. The pellet hormone delivery system provides a steady and physiological level of the steroid for the duration of implantation (18).

The rabbits received the control diet for 10 d after surgery, after which time they either continued the standard diet or switched to the HCD for 2 wk. The HCD was a cholesterol-enriched chow containing 1% cholesterol and 4% peanut oil (diet no. C30137; Research Diets, New Brunswick, NJ). At the end of the 2-wk dietary intervention, food was withdrawn for 12 h, and the rabbits were weighed and anesthetized with iv sodium pentobarbital (50 mg/kg). Venous blood samples were obtained to measure serum lipid and sex hormone levels. Multiple aortic tissue sections were also collected for immunohistochemical staining.

Serum sampling and analysis

Blood samples were collected at the time of killing. The isolated serum was stored at −20 C until use. Serum total cholesterol, triglyceride, high-density lipoprotein (HDL)-cholesterol, very-low-density lipoprotein (VLDL)-cholesterol, and LDL-cholesterol levels were measured by LipoSEARCH Co. (Tokyo, Japan) using an online dual enzymatic method (19). Serum testos-

terone, DHT, and estradiol (E2) were measured using specific RIA (20–22) by SRL Inc. (Tokyo, Japan). Because antiserum for DHT has 58.2% cross-reactivity to testosterone, the serum DHT level was measured after separation of the DHT fraction using an LH-20 micro-column (21). The detection limits of serum testosterone, DHT, and E2 were 0.1 ng/ml, 0.02 ng/ml, and 10 pg/ml, respectively. The interassay coefficients of variance for testosterone, DHT, and E2 were 6.2, 9.8, and 7.6%, respectively.

Histological study

Aortic samples were fixed in 4% paraformaldehyde, dehydrated with ethanol, embedded in OCT compound, and cut to a thickness of 5–8 μm by a cryostat. The tissue sections were subjected to immunostaining using established methods (16).

For immunofluorescence staining, the aortic cryosections were incubated with monoclonal mouse anti-CD31 antibody (an endothelial cell marker) (diluted to 1:500) and polyclonal rabbit anti-LOX-1 antibody (H-140; Santa Cruz Biotechnology, Santa Cruz, CA) (diluted to 1:200) at 4 C overnight. The sections were then incubated with Alexa Fluor 488-conjugated antimouse IgG antibody or Alexa Fluor 594-conjugated antirabbit IgG antibody. The sections were examined by confocal laser-scanning microscopy (LSM 510 META; Carl Zeiss Co., Ltd., Jena, Germany). The fluorescence intensity for LOX-1 was analyzed using Scion Image software. The number of endothelial cells was counted after staining consecutive slides with 4′,6-diamidino-2-phenylindole (a nuclear marker) (Invitrogen, Carlsbad, CA), and the staining intensity per cell was calculated. The mean value of three sections was taken as the value for each animal.

Plasmid preparation

The 2433-bp human LOX-1 (*hLOX-1*) promoter region (from −2390 to +42) and its 5′-deleted sequences were amplified by PCR from human genomic DNA using appropriate primers. These promoter sequences were then subcloned into the TOPO vector by blunt end ligation. The promoter fragments, isolated from the TOPO vector by *Xba*I and *Bam*HI digestion, were ligated into the PGL 4.19 luciferase reporter vector (Promega, Madison, WI) via *Nhe*I (5′) and *Bgl*II (3′) sites. The name of each promoter deletion mutant (*e.g. hLOX-1 −1821/+42*) indicates the deleted region. The integrity of each construct was verified by sequencing. Primer information is available upon request. We have described the steroid receptor expression vectors, including pCMVhAR and pCMV-AR-C579F, elsewhere (23).

Cell culture, transient transfection, and dual luciferase assay

HAEC were purchased from Applied Cell Biology Research Institute (Kirkland, WA) and were cultured in endothelial basal medium (Clontech, Mountain View, CA). TNFα, TPA, and steroids, including DHT, were purchased from Sigma Chemical Co. (St. Louis, MO). TNFα was dissolved in PBS, TPA was dissolved in dimethylsulfoxide (DMSO), and steroids were dissolved in ethanol.

The *LOX-1* promoter luciferase vector and pCMVhAR were cotransfected using a HCAEC Nucleofector Kit (Lonza, Walkersville, MD). Cells were cotransfected with a renilla luciferase vector pRL-SV40 (Promega) as an internal control for transfection efficiency. Transfection of the constructs to the cells and luciferase assays were performed according to the manufacturer's protocol. The activity of *LOX-1* promoters was expressed as the ratio of *LOX-1* promoter-mediated firefly luciferase activity to renilla luciferase activity (23, 24).

Site-directed mutagenesis

Site-directed mutagenesis within the *hLOX-1* promoter region was performed using the *in vitro* site-directed mutagenesis system (Promega). Mutation was verified by DNA sequencing.

Western blotting

Western blotting analysis of LOX-1 expression in cultured HAEC was performed as previously described (16) using a mouse anti-LOX-1 monoclonal antibody (diluted to 1:1000) kindly provided by Dr. Sawamura (National Cardiovascular Center Research Institute, Osaka, Japan).

RNA preparation and real-time PCR

Total RNA was isolated from cultured HAEC as previously described (25). We performed quantitative analysis of mRNA transcripts by real-time PCR using a LightCycler (Roche Applied Science, Rotkreuz, Switzerland), as described previously (25). Real-time PCR was performed using specific primers for *hLOX-1*, human *AR*, and *β-actin* for 50 cycles. The expression of each transcript was calculated as the relative ratio to β-actin. The primer sequences of each transcript are shown in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed essentially as previously described (25) using ChIP assay kits (Upstate Biotechnology, Lake Placid, NY). After formaldehyde-mediated cross-linking of the cells, the chromatin fragments were sheared by sonication. Immunoprecipitation using the sonicated cell supernatant was performed overnight at 4 C with 3 μg anti-p65 (c-20), anti-c-Fos, or anti-phosphorylated c-Jun (p-c-Jun) antibodies (KM-1) or with normal goat/mouse IgG as negative controls (Santa Cruz Biotechnology). Complexes precipitated with protein A agarose were eluted, and DNA was purified and used for PCR amplification using LOX-1 primers (Supplemental Table 1). Input samples were amplified simultaneously as internal controls. For the re-ChIP assay, solubilized chromatin was first incubated with 3 μg anti-p-c-Jun antibody. Then, 100 μl of the elute was diluted 40-fold with dilution buffer and incubated with 3 μg anti-p65 antibody (26). The other conditions were identical to those used for the ChIP assay.

Statistical analysis

Results are expressed as means ± SD. Statistical significance was analyzed with one-way factorial ANOVA or Student's two-tailed *t* test. A value of $P < 0.05$ was considered statistically significant. All analyses were conducted using SPSS software (SPSS Inc., Chicago, IL).

Results

Serum steroid levels, total body weight, and serum lipid levels

Among the HCD-fed groups, the sham and placebo groups showed marked reductions in serum DHT levels,

whereas the DHT group had normal DHT levels. All three HCD groups (sham, placebo, and DHT) showed marked reductions in testosterone concentrations compared with the control group fed the normal diet (Supplemental Fig. 1). We reported similar results in our earlier study where rabbits were fed the HCD for 8 wk (16). Serum DHT and testosterone were not detectable in the placebo group. DHT, but not testosterone, levels were significantly higher in the DHT group than in the placebo group but did not exceed the level in the control or sham groups, indicating that the DHT pellet provided a sustained near-physiological level of DHT in this experiment. There were no differences in serum E2 levels among the four groups (data not shown).

The body weights of rabbits in the control, sham, placebo, and DHT groups after 2 wk of feeding were 2.11 ± 0.17 kg (mean ± SD) (n = 6), 2.02 ± 0.10 kg (n = 6), 2.05 ± 0.07 kg (n = 7), and 1.99 ± 0.05 kg (n = 7), respectively, and were not significantly different among the four groups. Triglyceride levels were similar in all four groups (Supplemental Fig. 1), whereas serum total cholesterol, LDL-cholesterol, VLDL, and HDL-cholesterol levels were higher in all three HCD-treated groups than in the control group (Supplemental Fig. 1). HDL levels in the sham, placebo, and DHT groups were similar (Supplemental Fig. 1). Administration of 150 mg DHT did not affect HDL levels among the three groups of HCD-fed castrated rabbits.

DHT inhibits the expression of LOX-1 in rabbit aortic endothelial cells

Next, we performed double immunofluorescence staining using a specific antibody against rabbit endothelial cells (CD31) and an anti-LOX-1 antibody, as used previously (16) (Fig. 1A). Extensive LOX-1 staining was observed in the endothelial region of the sham and placebo groups. In both groups, the signals for LOX-1 (green) and CD31 (red) revealed that these proteins were predominantly expressed in the aortic endothelial cells. The merged images of both proteins showed overlapping distribution of the LOX-1-expressing and endothelial cells. Castration (*i.e.* placebo group) increased the expression of LOX-1 compared with that in the control group. In the DHT group, a significant reduction of LOX-1 expression was observed compared with that in the placebo group. These findings were confirmed statistically by quantifying the intensity of LOX-1 fluorescence per cell (Fig. 1B). LOX-1 is expressed in endothelial cells, macrophages, and smooth muscle cells (4, 19, 27, 28). In the sham, placebo, and DHT groups, the CD31-stained endothelial cells (green) were localized to the aortic surface, and the same sections showed no marked staining by normal rabbit/mouse IgG (data not shown).

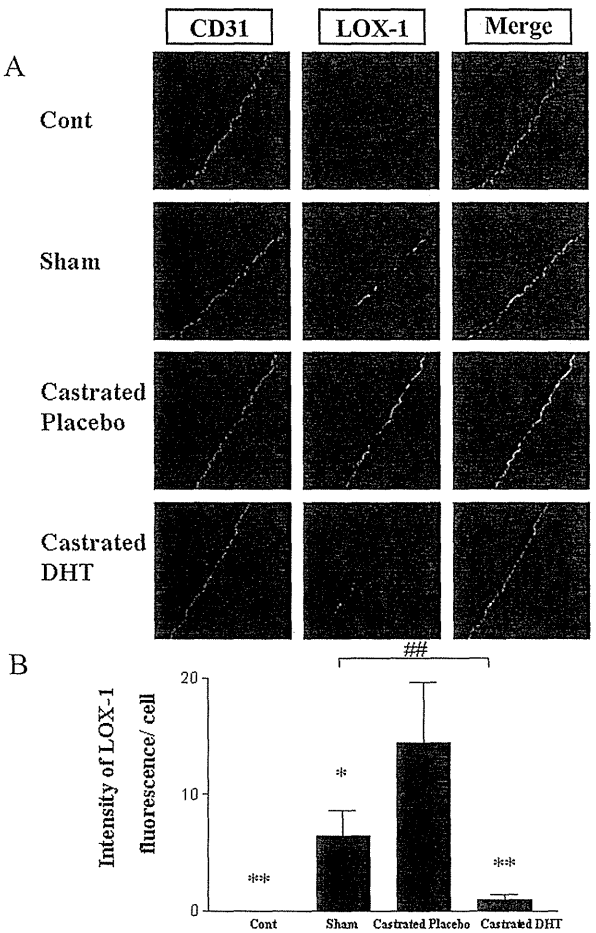

FIG. 1. Immunofluorescence analysis of LOX-1 expression in the aortic arch. A, Representative immunofluorescence images of the aortic arch of rabbits obtained using confocal microscopy. In the sham and placebo groups, the signals for LOX-1 (*green*) and endothelial cells (CD31, *red*) indicate that these proteins are predominantly expressed in the intima region. The merged images indicate overlapping localization of LOX-1 and endothelial cells. *Scale bar*, 50 μm. B, Quantitative analysis of LOX-1 immunofluorescence staining. The mean value of three sections was taken as the value for each animal. Values are means ± SD (n = 5 per group). *, $P < 0.05$; **, $P < 0.01$ *vs.* placebo; ##, $P < 0.05$ for HCD *vs.* DHT. Cont, Control.

DHT inhibited hLOX-1 mRNA and protein expression in HAEC stimulated with TNFα

RT-PCR revealed that HAEC expressed human AR mRNA (Supplemental Fig. 2A). LOX-1 mRNA expression was detected in cultured HAEC, and it was significantly induced by treatment with 10 ng/ml TNFα for 16 h (Fig. 2A), as previously reported (4). Importantly, the induction of LOX-1 mRNA by TNFα was significantly suppressed by DHT. The inhibitory effect of DHT on TNFα-induced LOX-1 mRNA expression was observed at concentrations ranging from 10^{-9} to 10^{-7} mol/liter (Fig. 2A). These changes in LOX-1 mRNA levels were accompanied by similar dose-dependent changes in protein ex-

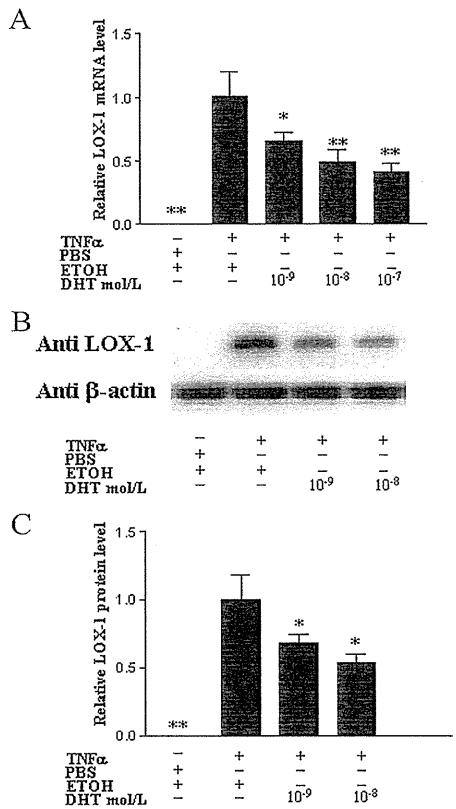

FIG. 2. Effects of DHT on hLOX-1 mRNA and protein expression in cultured HAEC. HAEC were treated with 10 ng/ml TNFα and increasing concentrations of DHT/PBS in ethanol (ETOH) in dextran-coated charcoal serum medium for 16 h. A, The mRNA expression of LOX-1 relative to β-actin was determined by real-time PCR; B, Western blotting of LOX-1 protein expression using the anti-LOX-1 antibody (1:500); C, quantitative analysis of LOX-1 protein expression. Results are means ± SD of three experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* cells treated with TNFα in the absence of DHT.

pression (Fig. 2B). Because basal LOX-1 protein expression was very low, it was difficult to clearly observe any inhibitory effects of DHT on basal expression (data not shown). Interestingly, TNFα decreased the basal expression level of AR mRNA in cultured HAEC. This basal level was, however, restored to the original level by 10^{-8} mol/liter DHT (Supplemental Fig. 2B). A similar finding was reported in a prostate cancer cell line, LNCap (26), suggesting that changes in LOX-1 expression in HAEC are unrelated to changes in endogenous AR expression. Taken together, these data suggest that activation of DHT-AR functionally suppresses TNFα-induced LOX-1 expression at the transcriptional level in HAEC.

DHT inhibited hLOX-1 promoter activity

To investigate the mechanisms involved in the above observations, we examined hLOX-1 promoter activity using HAEC in reporter assays. HAEC were transfected with the PGL4-hLOX-1-luciferase plasmid (hLOX-1 −2390/+42) and the AR expression plasmid and then stimulated with TNFα or vehicle alone for 18 h. Luciferase activity relative to renilla luciferase activity was then measured. The relative luciferase activity increased after stimulation with 10 ng/ml TNFα. Treatment with 10^{-9} to 10^{-7} mol/liter DHT significantly reduced TNFα-stimulated luciferase activity (Fig. 3A). In contrast, the inhibitory effect of DHT on *hLOX-1* promoter activity was not observed in HAEC cells transfected with a mutant AR construct containing a missense mutation in the DNA-binding domain (AR-C579F) (15), indicating that intact AR function is essential for this phenomenon (Supplemental Fig. 3).

Analysis of DHT-AR-mediated hLOX-1 promoter activity by deletion analysis

Because of the absence of a conserved AR responsive element in the hLOX-1 promoter region (30), we hypothesized that AR indirectly inhibits promoter activity. Therefore, we performed deletion analysis of the 2.4-kb region of the *hLOX-1* promoter luciferase construct. Each deletion construct was cotransfected with pCMVhAR into HAEC, and cells were stimulated with 10 ng/ml TNFα in the presence or absence of 10^{-8} mol/liter DHT for 18 h. We found no major differences in the suppressive effects of DHT-AR on TNFα-induced *LOX-1* promoter luciferase activity among the constructs containing different lengths of the *hLOX-1* promoter, from nucleotides (nt) −2390 to −160 (Fig. 3B), suggesting that the proximal region (nt −160/+42) might be critical for the effects reported above. Considering that TNFα induces *LOX-1* expression through NF-κB (31), activation of DHT-AR may affect NF-κB function. Although the NF-κB binding motif (5′-CAGGAGTT-3′) (32) is located at −2153/−2147, deletion analysis demonstrated that this region was not essential. In addition, a *hLOX-1 (−2390/+42)* promoter construct containing a mutant sequence of this site (5′-CATTAGTT-3′) (Fig. 3C) hardly reduced luciferase activity compared with *LOX-1* −2390/+42 (data not shown).

Mutations within the TPA response element (TRE) prevented DHT-AR-mediated inhibition of hLOX-1 promoter activity

The TRE (5′-TTGAGTCA-3′) (−60/−53) within the *LOX-1 −160/+42* promoter region was considered to be the best candidate for mediating transcriptional regulation by DHT-AR (Fig. 3C). The original TRE was mutated to 5′-TTGTTTCA-3′, and *hLOX-1 −160/+42* constructs containing this mutation were transfected into HAEC. Although TNFα significantly increased luciferase activity of *hLOX1-P −160/+42*Luc (wild type), it did not

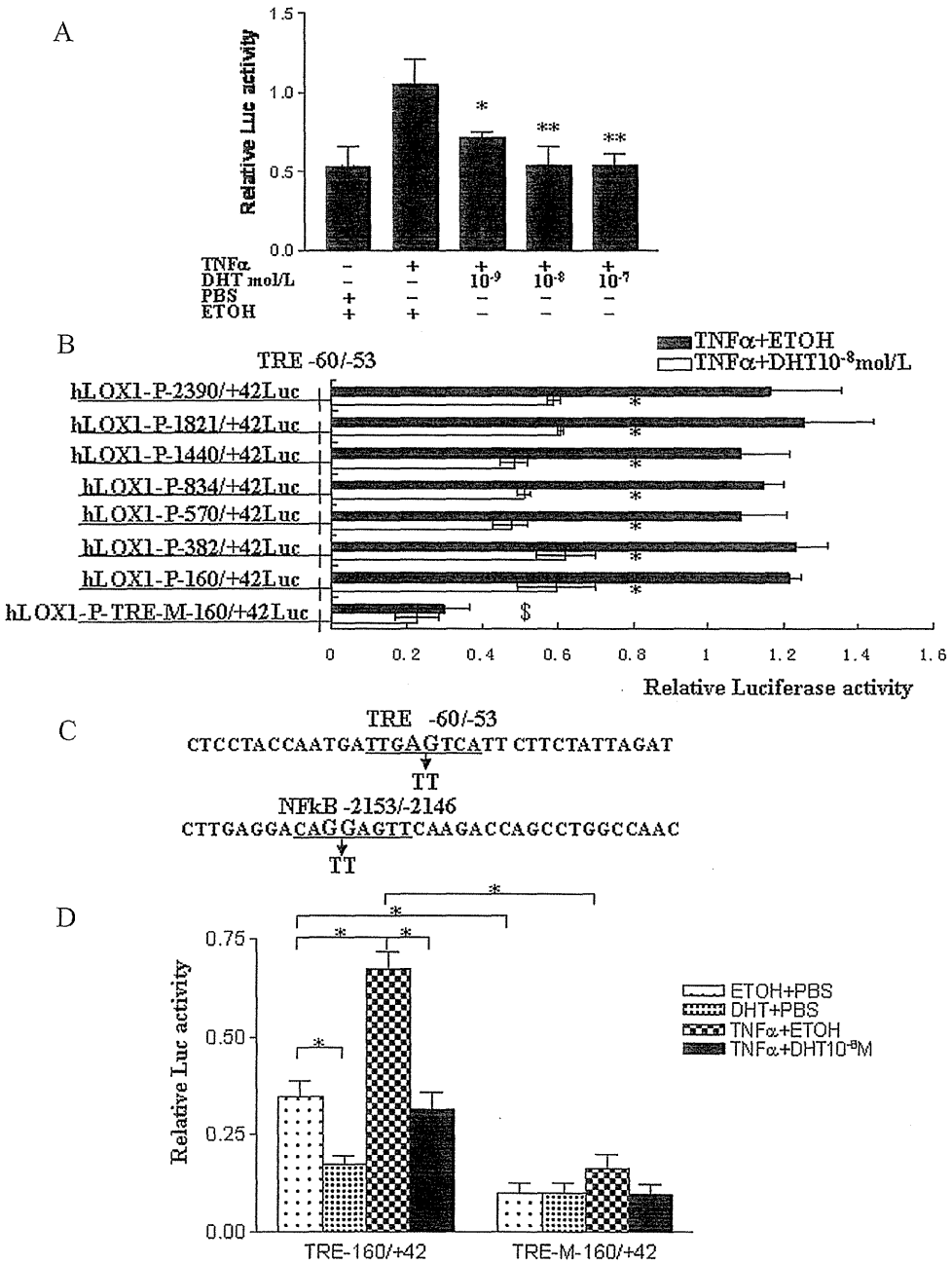

FIG. 3. Effects of DHT-AR on cytokine-mediated *hLOX-1* promoter activity. A, HAEC were cotransfected with 4.29 μg PGL4-*LOX-1 −2390/+42*-LUC, 33 ng pRL-SV40, and 0.7 μg of pCMVhAR for 15 h. Cells were then treated with increasing concentrations of DHT, with or without 10 ng/ml TNFα, for 18 h. Luciferase activity was then measured. **, $P < 0.01$ *vs.* cells treated with TNFα in the absence of DHT. B, Deletion analysis of the *LOX-1* promoter. HAEC were cotransfected with full-length *LOX-1* promoter (LOX-1 −2390/+42) or a series of 5′ deletion promoters and pCMVhAR. The construct *hLOX-1* −161/+42 TRE-M-Luc contains a mutation at the TRE (−60/−53). Cells were treated with 10^{-8} mol/liter DHT or ethanol (ETOH) in the presence of 10 ng/ml TNFα. *, $P < 0.05$ for TNFα *vs.* TNFα+DHT; $, $P < 0.05$ for PGL4-LOX-1-P-TRE-M −160/+42-LUC *vs.* PGL4-*LOX-1 −2390/+42*-LUC. Values are means ± SD of three independent experiments, each performed in triplicate. C, Site-directed mutagenesis of *hLOX-1*, the TRE, and NF-κB binding motifs. The mutations are indicated by *arrows*. D, Effects of the TRE mutation in hLOX-1 −160/+42 in HAEC. Cells were cotransfected with *hLOX-1 −160/+42* or the mutant version [*hLOX-1 −161/+42* (TRE-M)], pRL-SV40, and pCMVhAR and then exposed to PBS or 10 ng/ml TNFα in the presence or absence of 10^{-8} mol/liter DHT for 18 h. Luciferase assays were then performed. Values are means ± SD of three independent experiments, each performed in triplicate. *, $P < 0.05$ between the indicated groups. The absence of markers indicates no statistically significant difference.

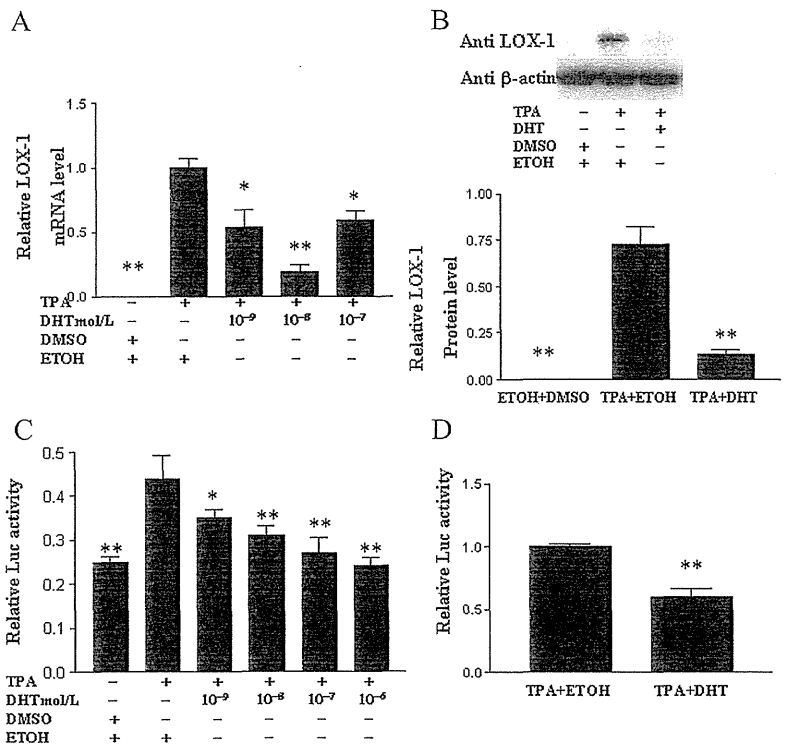

FIG. 4. Effects of DHT on TPA-induced expression of LOX-1. A, Cultured HAEC were treated with increasing concentrations of DHT for 12 h, followed by 10^{-7} mol/liter TPA or DMSO plus ethanol (ETOH) for 12 h. The mRNA expression of LOX-1 relative to that of β-actin was determined by real-time PCR. Values are means ± SD of three experiments. B, HAEC were treated with 10^{-8} mol/liter DHT or ethanol for 12 h, followed by 10^{-7} mol/liter TPA or DMSO for 12 h. The protein expression of LOX-1 and AR was determined by Western blotting using the anti-LOX-1 antibody (1:500) and quantified by densitometry. Values are means ± SD of three experiments. C and D, Cultured HAEC were cotransfected with 4.29 μg *hLOX-1 −2390/+42* or *hLOX-1 −160/+42*, 33 ng pRL-SV40, and 0.7 μg pCMVh*AR* for 15 h. Cells were then treated with increasing concentrations of DHT with or without 10^{-7} mol/liter TPA or DMSO plus ethanol for 18 h. Values are means ± SD of three independent experiments, each performed in triplicate. *, $P < 0.05$; **, $P < 0.01$ *vs.* TPA plus ethanol.

significantly increase the activity of *hLOX-1 −160/+42* containing the TRE mutation (hLOX-1-P-TRE-M−160/+42Luc). Therefore, the suppressive effects of DHT in the presence of TNFα were absent in cells transfected with the mutated construct (Fig. 3, B and D).

DHT inhibited hLOX-1 mRNA and protein expression and reduced TPA-stimulated LOX-1 promoter activity

TPA was reported to up-regulate LOX-1 expression in HAEC (33) and monocytes (34). AP-1, a downstream target of TPA, binds to the TRE (35, 36). Therefore, we examined whether activation of the DHT-AR system can also inhibit TPA-induced LOX-1 expression. As expected, 10^{-8} mol/liter DHT inhibited the mRNA (Fig. 4A) and protein (Fig. 4B) expression of LOX-1 in HAEC treated with 10^{-7} mol/liter TPA. Similar results were observed in luciferase assays using *hLOX-1 −2390/+42* and *hLOX-1 −160/+42* (Fig. 4, C and D).

DHT-AR interfered with the interaction between NF-κB/AP-1 and the hLOX-1 promoter

ChIP assays were performed to determine whether NF-κB could associate with the *hLOX-1* gene promoter (Fig. 5A). HAEC were pretreated with 10^{-8} *mol/liter* DHT for 24 h and then challenged with 10 ng/ml TNFα, 10^{-7} mol/liter TPA, or vehicle (PBS or DMSO) before being subjected to the ChIP assay with an antibody against p65, a component of NF-κB. As shown in Fig. 5B, although a PCR band was weakly detectable after 30 cycles in the absence of the p65 antibody, a small increase in band intensity was observed in the TRE sequence amplified from samples treated with the p65 antibody. This suggests that p65 binds to the TRE in the basal state. The band intensity increased greatly after treatment with TNFα or TPA. However, this increase was suppressed by pretreatment with 10^{-8} mol/liter DHT. These results indicate that DHT-induced activation of AR in HAEC inhibits the recruitment of p65 to the TRE in the hLOX-1 promoter.

We also performed a ChIP assay on TRE using an antibody against p-c-Jun. As shown in Fig. 5C, the intensity of the PCR band for TRE amplified from samples precipitated with the p-c-Jun antibody was increased compared with that of normal mouse IgG. This suggests that p-c-Jun binds specifically to the TRE in the basal state. As expected, TPA greatly increased p-c-Jun binding to the TRE, which was prevented by pretreatment with 10^{-8} mol/liter DHT. On the other hand, treating cells with TNFα increased band intensity, suggesting that TNFα enhances recruitment of p-c-Jun to the TRE. The specific binding of p-c-Jun to the TRE in response to TNFα was suppressed by 10^{-8} mol/liter DHT. Another AP-1 complex, c-Fos, was not recruited to the TRE in response to either TPA or TNFα (data not shown).

These results suggest that stimulation with either TNFα or TPA can induce p65 and p-c-Jun to form a complex that stimulates *LOX-1* promoter activity through the TRE. To confirm this possibility, a re-ChIP assay was performed. In this assay, the chromatin fragments immunoprecipitated

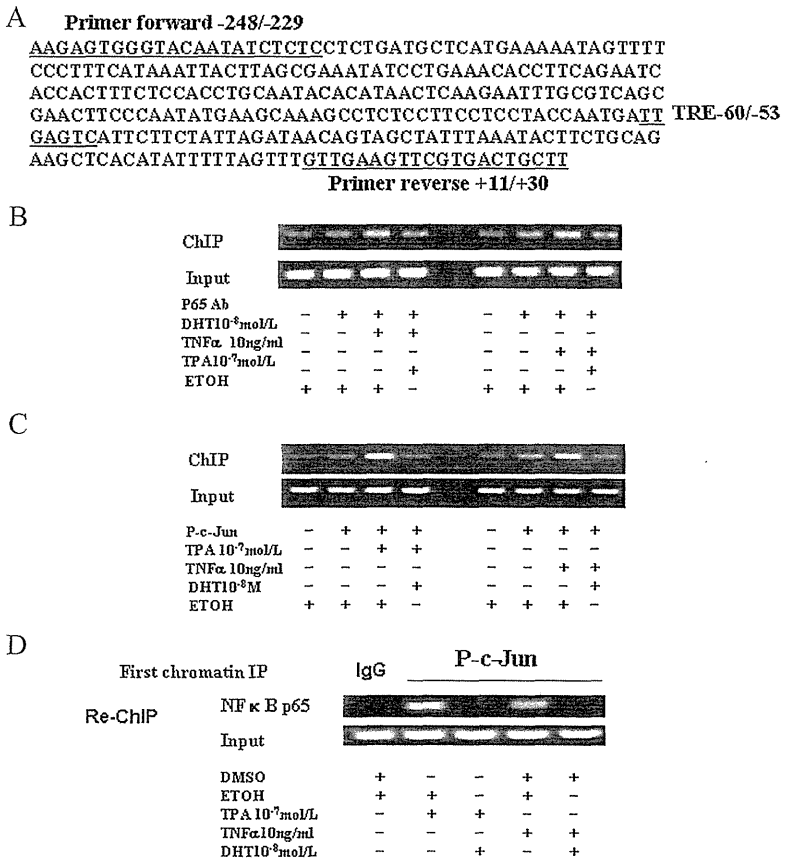

FIG. 5. ChIP and re-ChIP assays of p65 and of p-c-Jun binding to the hLOX-1 TRE motif. HAEC were pretreated with 10^{-8} mol/liter DHT or ethanol (ETOH) for 24 h, followed by treatment with 10 ng/ml TNFα, 10^{-7} mol/liter TPA, ethanol, PBS, or DMSO. A, The locations of primer sequences and TRE site in the *LOX-1* promoter. B, ChIP assay performed with an anti-p65 antibody or normal goat IgG as a negative control; *upper panel*, PCR amplification of the *hLOX-1* promoter; *lower panel*, PCR amplification of the hLOX-1 promoter for input controls. C, ChIP assay performed with an anti-p-c-Jun antibody or normal mouse IgG as a negative control. Conditions are the same as those in B. D, Two-step ChIP showing concurrent occupancy of p65 and p-c-Jun on the same genomic *LOX-1-TRE* chromatin fragment. DHT-AR inhibited NF-κB p65/p-c-Jun concurrent occupancy of the LOX-1 promoter TRE. HAEC were pretreated with 10^{-8} mol/liter DHT for 24 h, followed by 10 ng/ml TNFα or 10^{-7} mol/liter TPA and 10^{-8} mol/liter DHT for 2 h and processed for two-step ChIP. Solubilized chromatin was first incubated with 3 μg anti-p-c-Jun antibody. Then, 100 μl of the eluate was diluted 40-fold and incubated with 3 μg anti-p65 antibody. The final precipitation was used for PCR amplification of the hLOX-1 promoter.

using the anti-p-c-Jun antibody were re-immunoprecipitated using the anti-p65 antibody. This two-step ChIP showed concurrent occupancy of p65 and p-c-Jun at the TRE, suggesting that p65 and p-c-Jun form a complex at the TRE. DHT inhibited concurrent occupancy of this complex at the TRE, suggesting that DHT-AR inhibits the recruitment of the complex to DNA sequences within the TRE (Fig. 5D).

Discussion

Recent findings indicate that endogenous androgens may be antiatherosclerotic, rather than proatherosclerotic (5–16). Various beneficial effects of endogenous androgens on lipids, obesity, inflammation, and the vascular system have been reported (5, 7–15), conferring protection against atherosclerosis. In this study, we demonstrated that the androgen DHT suppresses LOX-1 expression in rabbit aortic endothelial cells of HCD-induced initial atherosclerosis *in vivo*. *In vitro*, we found that DHT suppressed TNFα- or TPA-mediated LOX-1 expression in cultured HAEC via a TRE motif in the *hLOX-1* proximal promoter, providing a new mechanism for the antiatherosclerotic effects of androgens. Because DHT, unlike testosterone, is never aromatized to produce estrogens, our data clearly indicate the importance of DHT-mediated activation of AR in these effects. This was also evidenced by the fact that functionally defective AR were unable to suppress *hLOX-1* promoter activity. Although the mechanism has not been elucidated, it is notable that some of the atheroprotective effects of androgens might be mediated via AR-independent mechanisms (12). The effective concentration of DHT required to suppress hLOX-1 expression was 10^{-9} to 10^{-8} mol/liter, which is similar to the physiological serum DHT level (60–120 ng/dl), approximately $2–4 \times 10^{-9}$ M in males aged 30–59 yr (37).

DHT reduced TNFα secretion from lipopolysaccharide-treated splenic macrophages but induced the secretion of both TNFα and IL-6 from Kupffer cells (38). DHT also stimulated NO production via AR-dependent activation of eNOS in HAEC (13). Meanwhile, DHT decreased TNFα and lipopolysaccharide-induced inflammatory responses in HAEC, and DHT-bound AR exerts antiinflammatory effects by suppressing the NF-κB pathway (39). Interestingly, testosterone was reported to suppress circulating TNFα and IL-1β levels in men (11), which may contribute to the suppression of LOX-1 expression in vascular lesions.

NF-κB belongs to the Rel family of proteins, which form homodimers or heterodimers (40). The most commonly found combinations are p50-p65 heterodimers and p50 homodimers. NF-κB is normally present in the cytoplasm in an inactive form, where it is bound to inhibitory

κB (IκB). Several extracellular stimuli, including TNFα, IL-1α (41, 42), and angiotensin II (32), activate the NF-κB signaling pathway, leading to the activation of IκB kinase, which phosphorylates IκB. Phosphorylated IκB is degraded by *26S* proteasome, leading to the release of NF-κB, which translocates to the nucleus and initiates transcription of its target genes (40), including LOX-1 (32). TNFα also activates the protein kinase C (PKC)-c-Jun N-terminal protein kinase pathway, which ultimately activates AP-1 (42). TPA is a typical PKC activator, and activated PKC can stimulate several transcription factors, including AP-1 and NF-κB (43). TPA activates AP-1 and NF-κB and, hence, increases LOX-1 expression in human monocytes (34).

We proposed that DHT-AR activation suppresses LOX-1 expression, at least in part, by suppressing the NF-κB/p65 and AP-1/c-Jun-dependent pathways, via the TRE located in the *hLOX-1* promoter region at nt −60/−53. Although there is a consensus NF-κB site at nt −2153/−2146 and an AP-1 site at nt −1597/−1588, our deletion and mutation analyses suggest that these sites are not essential for the effects of DHT-AR. A transcription factor, octamer-1, acting on a potential binding site at nt −1599/−1494 was reported to be involved in the mechanism by which ox-LDL induced hLOX-1 expression (44). Interestingly, although ox-LDL can activate NF-κB, no correlation was found between NF-κB- and ox-LDL-induced *hLOX-1* promoter activation (44). On the other hand, NF-κB activation through the NF-κB site at nt −2153/−2146 seems to be involved in angiotensin II-induced LOX-1 expression (32). However, no studies have documented the role of the TRE in the regulation of *hLOX-1*.

Consistent with our present data, the suppressive effects of androgens on NF-κB signaling have been reported by other researchers using prostate cancer cells. For example, DHT-AR was reported to suppress NF-κB activity by maintaining IκBa levels, resulting in the repression of the IL-6 promoter (45). It was also reported that testosterone inhibits NF-κB nuclear translocation (46). In fact, a weak protein-protein association between AR and p65 has already been documented, although this interaction did not significantly influence the DNA-binding activity of either protein (47).

On the other hand, AP-1 belongs to a transcription factor family that includes c-Jun, c-Fos, Jun B, and Jun D (35, 36). The TRE, a binding element of AP-1 target genes, is recognized by c-Jun homodimers and by c-Jun-c-Fos heterodimers (35, 36). Interestingly, AR and c-Jun can inhibit each other's binding to their respective DNA-binding site (48) through an interaction between the DNA- and ligand-binding domains of AR and the leucine zipper region of c-Jun. Our results of the ChIP and re-ChIP assays suggest that TNFα or TPA induced p-c-Jun to bind to specific DNA sequences within the TRE, possibly as a complex with p65. It was reported that p65 and c-Jun cooperate to stimulate AP-1-dependent promoter activity and that a p65-c-Jun complex enhanced DNA binding and target biological functions via AP-1 response elements (49). This is because the leucine zipper region of c-Jun can physically interact with the Rel homology domain of p65 (29, 49). These reports suggest that the p65-c-Jun complex is recruited to the TRE. In accordance with this concept, our results indicate that the negative regulation of hLOX-1 expression by DHT-AR is mediated by transcriptional regulation, based on several lines of evidence. First, TNFα-/TPA-induced LOX-1 promoter luciferase activity was suppressed by DHT-AR. Second, TNFα/TPA stimulated p-c-Jun to specifically bind to DNA sequences within the TRE, and the ChIP and re-ChIP assay results suggested that a p65-p-c-Jun complex bound to spe-

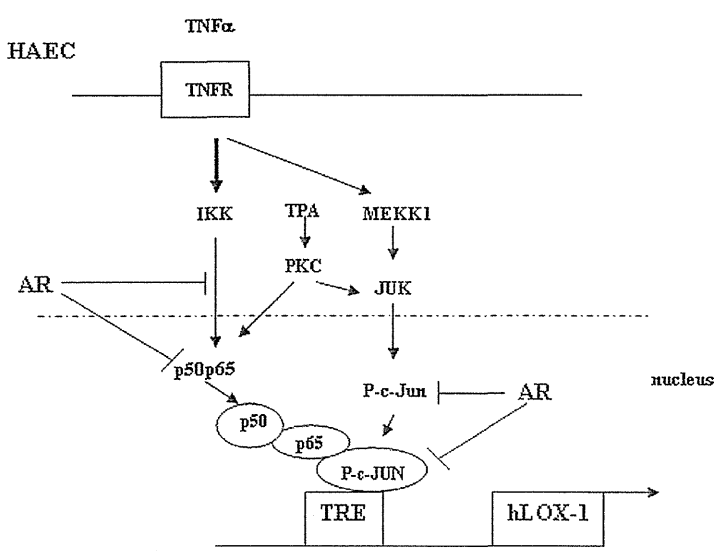

FIG. 6. Schematic diagram of DHT-AR-mediated suppression of cytokine-induced LOX-1 expression. Based on our results and the results of other studies, we propose the following scheme. First, TNFα and TPA activate NF-κB and AP-1 signaling (34, 40–43), which recruits p65 and p-c-Jun to the TRE in the hLOX-1 promoter. Then, p65 and p-c-Jun colocalize on the TRE, and p65 physically associates with p-c-Jun to form a p65-p-c-Jun complex (29, 49). DHT-AR inhibits NF-κB signaling and inhibits recruitment of the p65-p-c-Jun complex to the TRE chromatin region (42). In addition, NF-κB inhibits human AR gene transcription (26). Ikk, IκB kinase; JUK, c-Jun NH_2-terminal kinase; MEKK1, Mitogen-activated protein kinase.

cific DNA sequences within the TRE. Third, DHT-AR inhibited the recruitment of p65-p-c-Jun to DNA sequences within the TRE after TNFα/TPA stimulation. This proposed interaction between DHT-AR and NF-κB/AP-1 signaling is summarized in Fig. 6.

In summary, this study showed that DHT-AR suppresses LOX-1 expression in endothelial cells, at least in part, by suppressing NF-κB- and AP-1-dependent activation via the TRE in the *hLOX-1* promoter located at nt −60/−53. These results reveal a new mechanism for the antiatherosclerotic effects of androgens.

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Lifestyle Changes Through the Use of Delivered Meals and Dietary Counseling in a Single-Blind Study

– The STYLIST Study –

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Background: Dietary habits are associated with obesity, and both are important contributing factors to lifestyle-related diseases. The STYLIST study examined the effects of dietary counseling by registered dietitians and the delivery of proper calorie-controlled meals (UMIN Registration No: 000006582).

Methods and Results: Two-hundred adult patients with hypertension and/or diabetes mellitus were randomly divided into 2 groups with/without dietary counseling and consumed an ordinary diet for 4 weeks. Each group was then subdivided into 2 groups with/without dietary counseling and received calorie-controlled lunch and dinner boxes for the next 4 weeks. The calories in the delivered meals were based on the subject's ideal body weight (BW) and physical activity level. BW, waist circumference, blood pressure, and laboratory data, including glycoalbumin, were measured at 0, 4, and 8 weeks. BW and the other parameters were significantly reduced during the study period in patients who received diet counseling in the ordinary diet period and/or delivered meal period but not in patients without dietary counseling, as assessed by linear mixed models for longitudinal data.

Conclusions: The combination of dietary counseling by dietitians and delivery of calorie-controlled meals was effective in reducing BW, as well as blood pressure and glycoalbumin, in patients with hypertension and/or diabetes mellitus. (*Circ J* 2012; **76:** 1335–1344)

Key Words: Body weight; Delivered meals; Dietary counseling; Registered dietitians; Single-blind study

Recently, the percentage of the population that could be considered obese has increased, in both developed countries,[1] including Japan,[2] and developing countries. Because obesity is related to the cardiovascular disease (CVD) burden and other metabolic disorders, including hypertension (HT), dyslipidemia, type 2 diabetes mellitus (DM), metabolic syndrome,[3–5] etc, body weight (BW) reduction entails both medical and economic considerations.

Editorial p1322

Although in many previous trials, calorie-restricted diets or formula food for the treatment of obesity, type 2 DM and HT were used with small to large populations[6–10] and dietary intervention (including dietary counseling) and exercise have been shown to lower the risk of CVD, there have been only a few reports on the use of delivered meals with/without dietary counseling in subjects with HT or type 2 DM.[11–13] In those

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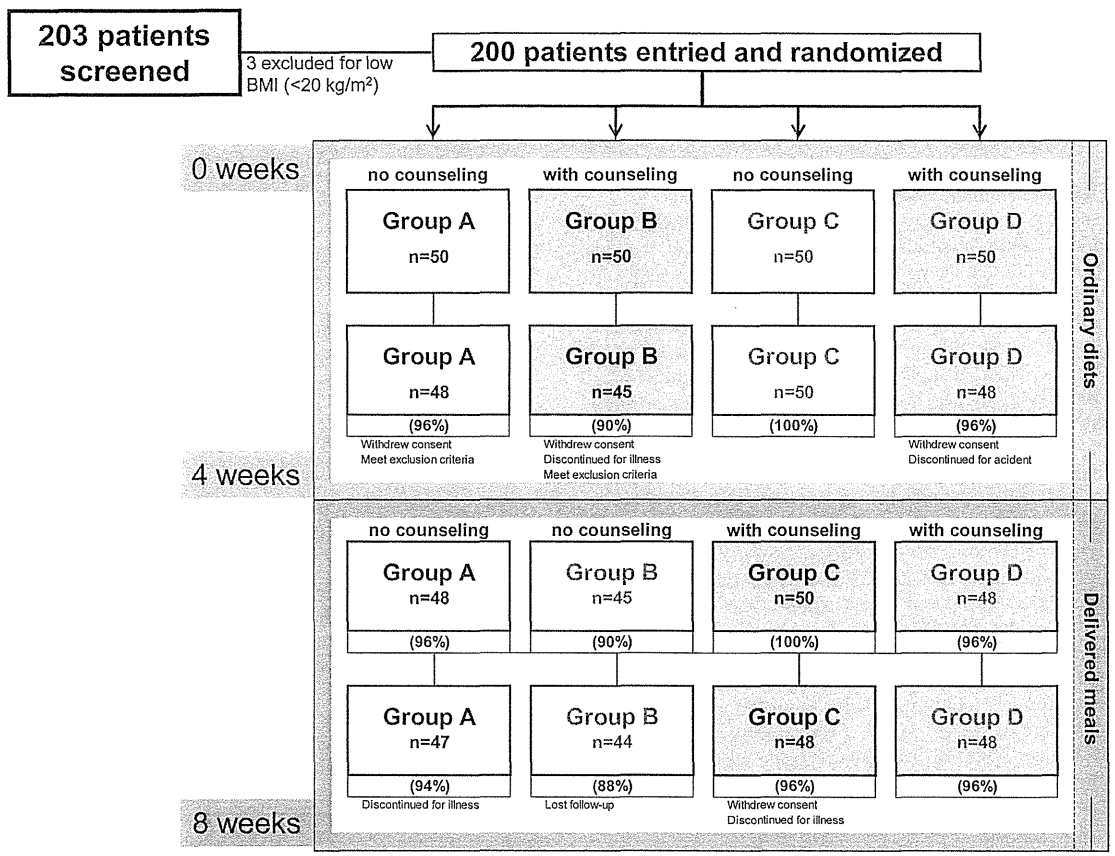

Figure 1. Flow chart of the participants from screening to the end of the study.

studies, meals were not delivered daily to the individual's home,[11,12] and no remarkable changes in BW were observed,[13] despite a long study period of 1 year. In addition, no clinical data were provided.[11,12] Our intension is to verify the effects of a combination of delivered meals with dietary counseling within a short period from a cost-benefit perspective. Therefore, our hypothesis was that the more frequent delivery of proper calorie-controlled meals for lunch and dinner in conjunction with dietary counseling could be effective for reducing BW in patients with HT/DM within a short period. This is the first, registered, multicenter, randomized, single-blind study from Japan of the use of dietary counseling together with delivered proper calorie-controlled meals in patients with HT/DM to note changes in BW.

Methods

Patients

The study subjects were recruited from among outpatients at Fukuoka University Hospital and Yuai Hospital in Fukuoka, Japan. The protocol was approved by the Independent Review Board (IRB) of Fukuoka University Hospital [No. 11-9-9], and registered under UMIN000006582. At the beginning of the trial, the Fukuoka University Extension Center contacted residents of Fukuoka City (Nanakuma area) to describe the research protocol, and the details of the trial. Of the 203 applicants that included residents of Fukuoka City, 3 were excluded due to low body mass index (BMI) (**Figure 1**). Each subject signed an informed consent form after the protocol was explained in detail. A subject was eligible for inclusion if all of the following criteria were met: type 2 DM, including impaired glucose tolerance and/or essential HT; aged ≥20 years, and able to eat meals regularly 3 times a day. A subject was not eligible for inclusion if any of the following exclusion criteria were met: allergic to common food; stroke or myocardial infarction within the past 3 months; on hemodialysis with end-stage renal disease; cancer or under cancer treatment; inability to ingest or digest, secondary obesity; HbA_{1c} >12% as defined by the Japan Diabetes Society (JDS) scale (because the regularly delivered meals would not be suitable for such patients); BMI<20 (lean individuals were excluded); height >1.8 m; patients who planned to change their lifestyle habits or reduce their BW during the study period; patients who planned to change their smoking habit during the study period; and women who were pregnant or lactating. The reason why we excluded patients with a height >1.8 m was that the maximum number of calories in the delivered meals was 1,800 kcal/day. More than this and the caloric deficit would have to be supplemented by additional ordinary diet, which we wanted to avoid. Finally, 200 subjects (97 males, 103 females, age 22–72 years)

were enrolled in this trial. In group A (see Protocol), 2 subjects dropped out at 4 weeks and 1 dropped out at 8 weeks; in group B, 5 dropped out at 4 weeks and 1 dropped out at 8 weeks; in group C, 2 subjects dropped out at 8 weeks; in group D, 2 subjects dropped out at 4 weeks (**Figure 1**). The participants withdrew because of adverse events that were not related to the delivered meals.

The Research Consortium

The present trial was supported by the Japanese Ministry of Economy, Trade and Industry and the Japan Research Institute, Ltd (Tokyo), together with a consortium of Nissin Healthcare Food Service Co Ltd (Tokyo), Kyudenko Co Ltd (Fukuoka), Yuai Hospital (Fukuoka, Japan), and the AIG Collaborative Research Institute of Cardiovascular Medicine, Fukuoka University, and included approximately 200 patients with HT and/or type 2 DM. The main focus of this consortium is the creation of a new service industry associated with medical care.

Protocol

The 2-by-2 protocol design is shown in **Figure 1**. After informed consent was given, physicians completed the screening forms. According to the information on the forms, the Fukuoka University Hospital Clinical Research Assist Center (CRAC, where computed randomization was performed, independent of the research consortium) randomly assigned the participants to 4 groups at the beginning of the ordinary diet period (0 week) as group A (no counseling during either study period), group B (counseling with the ordinary diet, but not with delivered meals), group C (counseling with delivered meals, but not with the ordinary diet), and group D (counseling for both study periods) (**Figure 1**). The ordinary diet was given for 4±1 weeks, and meals were delivered for an additional 4±1 weeks.

The calories in the delivered meals were based on each patient's ideal BW and physical activity. The ideal BW was the square of height (m)×22. The optimal number of calories/day was the ideal BW multiplied by physical activity. Physical activity (life intensity) was assigned 1 of 4 levels based on lifestyle and work-related strength.[14,15] For levels 1, 2, 3, and 4, the rates were 25–29.9, 30–34.9, 35–39.9, ≥40, respectively. Physical activity level was evaluated by physicians during a face-to-face interview regarding the intensity of daily activities at 0 weeks.[14,15] Three different delivered meals were prepared by Nissin Healthcare Food Service Co Ltd (Tokyo) to contain 400, 533 and 600 kcal/lunch or dinner, and, together with an ordinary breakfast, gave an estimated total daily caloric intake of 1,200, 1,600, and 1,800 kcal, respectively. The meals that each subject received were selected according to the calculated optimal number of calories/day. If the calculated optimal calories/day was more than 1,800, the caloric deficit was supplemented by additional ordinary diet. Each meal contained less than 3 g of salt. The chilled lunch and dinner boxes were delivered to the subjects or obtained by the subjects themselves from a convenience store (Family Mart. Co Ltd, Fukuoka, Japan) near their workplace or home from Monday to Friday, depending on their choice. On Saturday and Sunday, all subjects consumed an ordinary diet. The meals were heated before eating and no leftovers were stored.

Physicians

The physicians in charge of this trial were either in general practice, cardiologists, or diabetologists. They promoted a healthy lifestyle to the patients, as usual, but instructed the participants not to change their lifestyle throughout the study period. Changing their lifestyle was an exclusion criterion, no one was excluded from the study for this reason. The physicians were blinded to the randomization of dietary counseling and BW, but not blood pressure (BP).

Nutritional Counseling

All participants were interviewed by registered dietitians (RDs) to assess their dietary habits and exercise habits using a food and exercise frequency questionnaire (FEFQ) at week 0 before randomization. The FEFQ is a self-administered tool based on educational material regarding dietary counseling from the Ministry of Health Labour and Welfare of Japan.[16] The FEFQ comprises 28 food groups for staple food, side dish, oils, salts, sugar, and alcohol categories, and 4 exercise frequency groups (walking, jogging, gym, and other exercise). Food groups comprise 35 food items, including 10 quantity items (rice, bread, other staple food, vegetables, cooking sugar, sake, beer, shochu, whisky, and other alcohols), 3 preference items (fish: oily/usual/white; meat: high-fat/usual/low-fat; and flavor: strong/usual/thin), and 22 frequency items (tubers and roots, fruit, fish, meat, eggs, soybean and soybean products, milk, dairy products, seaweed, fried food, mayonnaise and dressing, pickles, salted food, processed food, instant food, miso soup, soup, noodle soup, coffee and black tea, fruit juice, confectionaries, and hol drinking). The FEFQ was completed by each patient under the supervision of a dietitian, who then checked the questionnaire before randomizing the patient into group A, B, C, or D; each patient completed a FEFQ before randomization. The participants in the counseling groups received individual face-to-face dietary counseling sessions for 30–60 min based on information from the FEFQ at the beginning of each study period with the ordinary diet for groups B and D, and the delivered meals for groups C and D. Counseling focused on principles of good nutrition and advice on meal planning, dietary calories, and alcohol consumption. In addition, a 10–20-min telephone counseling session was performed in the middle of each period, and the RDs checked dietary performance and advised the participant. Thus, the maximum number of counseling sessions was 0 for group A, 2 for groups B and C, and 4 for group D.

Nutritional Intervention and Adherence to the Intervention

Based on information from the FEFQ, dietary advice on the correct amounts of rice and bread (staple food), salt, vegetables, fruits, fish, meat, eggs, soy proteins, fiber, etc, during the ordinary diet period was given based on the Food Substitution Table for Diabetes Mellitus Diet Therapy 6th version of the Japan Diabetes Society.[17] During the study period with delivered meals, advice focused on breakfast and the RDs ensured that the delivered lunches and dinners were being consumed correctly. If the volume of staple food in the delivered meals exceeded the individual's usual volume, the subjects were advised that they did not need to eat it all. RDs also gathered and relayed information about the participants' tastes to the company responsible for meal production and delivery, and checked whether the subjects were complying with the dietary counseling. The RDs recorded anthropometric measurements, except BP, and coordinated the follow-up appointments. The participants documented all relevant information on daily diet report sheets at 4 and 8 weeks; for example, eating lunch or dinner at a restaurant (eating out), failure to eat a delivered meal, or the consumption of additional snacks, meals or alcohol, and the RDs could then estimate any unusual caloric intake for each participant.

Table 1. Baseline Characteristics of Patients

	Group A (n=50)	Group B (n=50)	Group C (n=50)	Group D (n=50)	P value**
Age, years	65.6±11.4	64.0±12.5	65.9±12.7	64.5±10.6	0.84
Sex, n, (%)					
Female	22 (44%)	29 (58%)	21 (42%)	31 (62%)	0.11
Male	28 (56%)	21 (42%)	29 (58%)	19 (38%)	0.11
Height, m	1.60±0.08	1.60±0.07	1.61±0.09	1.59±0.08	0.68
BMI (kg/m²)	25.9±3.4	27.3±4.8	26.0±3.6	25.6±3.5	0.17
Components of MetS, n (%)					
High WC	37 (74%)	38 (76%)	35 (70%)	34 (68%)	0.71
High TG	18 (36%)	12 (24%)	19 (38%)	16 (32%)	0.49
Low HDL-C	4 (8%)	1 (2%)	9 (18%)	4 (8%)	0.09
High BP	30 (60%)	32 (64%)	25 (50%)	34 (68%)	0.59
High blood glucose	23 (46%)	14 (28%)	21 (42%)	26 (52%)	0.18
Risk factors of CHD, n (%)					
HT	41 (82%)	41 (82%)	37 (74%)	38 (76%)	0.68
Type 2 DM	28 (56%)	21 (42%)	26 (52%)	22 (44%)	0.45
Smoking	6 (12%)	0 (0%)	6 (12%)	3 (6%)	0.06
Dyslipidemia, n (%)	28 (56%)	17 (34%)	25 (50%)	19 (38%)	0.09
Complications, n (%)					
CHD	15 (30%)	15 (30%)	12 (24%)	9 (18%)	0.46
Cerebrovascular disease	6 (12%)	3 (6%)	3 (6%)	4 (8%)	0.65

**Category and continuous variables were compared among groups by chi-square analysis and analysis of variance, respectively.
BMI, body mass index; MetS, metabolic syndrome; WC, waist circumference; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; BP, blood pressure; CHD, coronary heart disease; HT, hypertension; DM, diabetes mellitus.

Primary and Secondary Endpoints

The primary endpoint was a change in BW during the ordinary diet period and delivered meals period with/without dietary counseling. Secondary endpoints were changes in waist circumference (WC), BP, blood sugar, glycoalbumin, HbA_{1c}, and serum lipids, with/without dietary counseling. The BW and WC of the participants were measured at each visit, every 4 weeks during the trial period. WC was measured halfway between the lower rib and the iliac crest at the level of the navel. BP and pulse rate were measured every 4 weeks during the trial period.

Other Measurements

Blood cell counts, urinalysis and serum levels of triglycerides, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), glucose, HbA_{1c}, AST, ALT, LDH, γ-GTP, creatinine, uric acid, sodium, potassium, chloride, calcium, glycoalbumin, and adiponectin were measured at the beginning (0 week) and end of each study period (at 4 and 8 weeks) by a central clinical laboratory (BML Corporation, Fukuoka, Japan), and the participants were requested to visit an out-patient clinic after overnight fasting at those times. After the physician ordered clinical laboratory tests, blood was drawn from the patient by technical staff in the Department of Clinical Laboratory, and sent to BML for measurement after separation.

Statistical Analysis

All the data analyses were performed using SAS (Statistical Analysis System Ver. 9.2, SAS Institute Inc, Cary, NC, USA) at Fukuoka University (Fukuoka, Japan), as described previously.[18,19] Baseline characteristics of patients were compared among groups for category and continuous variables by chi-square analysis and analysis of variance, respectively. Continuous variables during the study periods were presented as least-square means and standard error. Significant changes of continuous variables vs. baseline were examined by analysis of variance using linear mixed model, based on the intention-to-treat (ITT) principle, and differences among groups at baseline and at 4 and 8 weeks were examined by analysis of covariance after adjusting for stable variables including age, sex, HT, type 2 DM, smoking, and dyslipidemia. The combined effects of delivered meals and diet counseling on anthropometric measurements and blood glucose concentrations were examined by likelihood-based ignorable analyses using linear mixed models for longitudinal data. Type 3 tests of fixed effects are shown for group and group by period interaction and solutions for fixed effects are shown for group by period interaction. Sample size was calculated using SAS/STAT Power and Sample Size Application. To detect a mean difference of 1.5 kg BW change between patients with and without diet counseling, assuming an unequal standard deviation of 2.5 and 5 kg, a sample size of 174 was calculated with a 1-sided significance level of 0.05 and a power of 0.8. Therefore, 200 patients were recruited. The significance level was considered to be less than 0.05 unless indicated otherwise.

Results

Baseline Characteristics and Lifestyle of Participants

Table 1 shows the baseline characteristics of groups A, B, C, and D: there were no significant differences in age, sex, BMI, WC, or the prevalence of HT and DM, or risk factors, among the 4 groups at week 0. Among all of the patients, the prevalence of HT and type 2 DM were 78.5% (n=157) and 48.5% (n=97), respectively, and 27% (n=54) of the patients had both HT and type 2 DM. Components of the metabolic syndrome were categorized based on the criteria in Japan,[20] and there

Table 2. Lifestyles of Patients and Lifestyle Changes During the Periods of Ordinary Diets and Delivered Meals

	Group A (n=50)	Group B (n=50)	Group C (n=50)	Group D (n=50)	P value**
Lifestyle					
Physical activity level, n (%)					
I	31 (62%)	37 (74%)	33 (66%)	26 (52%)	0.14
II	18 (36%)	13 (26%)	17 (34%)	23 (46%)	0.22
III	1 (2%)	0 (0%)	0 (0%)	1 (2%)	0.57
Proper daily calorie intake range, kcal					
Lower calorie intake limit	1,534±231	1,481±199	1,525±219	1,537±247	0.57
Higher calorie intake limit	1,812±253	1,756±220	1,805±245	1,810±271	0.64
Estimated salt intake, n (%)					
>10 g/day	42 (84%)	42 (84%)	41 (82%)	41 (82%)	0.99
Alcohol drinking, n (%)	12 (24%)	24 (48%)	13 (26%)	15 (30%)	0.04
<3days/week	4 (8%)	13 (26%)	4 (8%)	6 (12%)	0.02
≥3days/week	8 (16%)	11 (22%)	9 (18%)	9 (18%)	0.89
Exercise habit, n (%)	17 (34%)	15 (30%)	17 (34%)	15 (30%)	0.95
Walking	14 (28%)	11 (22%)	16 (32%)	12 (24%)	0.68
Jogging	2 (4%)	0 (0%)	0 (0%)	2 (4%)	0.25
Sport gym exercise	1 (2%)	5 (10%)	1 (2%)	1 (2%)	0.10
Target daily calorie control with delivered meals					
1,200 calories, n (%)	7 (14%)	6 (12%)	5 (10%)	8 (16%)	0.83
1,600 calories, n (%)	23 (46%)	31 (62%)	32 (64%)	21 (42%)	0.06
1,800 calories, n (%)	20 (40%)	13 (26%)	13 (26%)	21 (42%)	0.16
Average calories, kcal	1,624±196	1,604±174	1,612±164	1,620±207	0.95
Delivered meal intake rate, %	62±13%	66±12%	69±11%	64±13%	0.04
Lifestyle change					
During ordinary diet period, day/4 weeks					
Eating out	8±7	6±5	6±8	10±9	0.04
Snack	8±9	8±7	7±9	9±8	0.73
Drink less than usual	10±9	8±6	4±1	5±8	0.43
Drink more than usual	4±3	3±2	4±3	2±2	0.55
Exercise more than usual	5±5	3±2	7±7	6±6	0.30
Exercise less than usual	6±6	5±4	7±4	4±3	0.29
During delivered meal period, day/4 weeks					
Eating out	4±3*	3±3*	3±3*	4±3*	0.22
Snack	7±8	7±7	5±6	7±7	0.46
Drink less than usual	10±8	6±7	14±8	8±9	0.34
Drink more than usual	4±2	2±2	4±3	4±3	0.31
Exercise more than usual	7±7	4±3	4±3	7±9	0.63
Exercise less than usual	8±6	6±6	7±4	4±1	0.37

*P<0.001, delivered meal period vs. ordinary diet period, assessed by Wilcoxon signed rank test.
**Category and continuous variables were compared among Groups by chi-square analysis and analysis of variance, respectively.

were no significant differences among the 4 groups.

Table 2 shows the lifestyles of Groups A, B, C, and D: there were no significant difference in physical activity level, salt intake, and exercise habit among the 4 groups. Estimated salt intake >10 g/day was 84%, 84%, 82%, and 82%, in groups A, B, C, and D, respectively. The proportion of patients having an alcohol drinking habit for more than 3 days/week was not significantly different among the 4 groups (range, 16–22%), although group B patients had a higher proportion of patients with an alcohol drinking habit of less than 3 days/week (26%) compared to the other groups of patients (8–12%). In total, 68% of the patients did not have an exercise habit, and 26.5% patients walked for more than 30 min/day on more than 4 days/week.

Patients were recommended not to change their lifestyle during the study period. The number of days of alcohol drinking and exercising more than and less than usual were not significantly changed during the ordinary diet and delivered meal periods (Table 2). However, because meals were provided during the delivered meal period, the number of days of eating out was significantly reduced compared to the ordinary diet period (Table 2).

Primary and Secondary Endpoints

At baseline (week 0), BW, WC, BP, blood glucose concentrations, and serum lipid concentrations were not significantly different among the 4 groups, as assessed by analysis of covariance after adjusting for age, sex, HT, type 2 DM, smoking, and dyslipidemia (Table 3). During the study period, a significant reduction in BW at 8 weeks was observed in group B, C, and