Diethylstilbestrol decreased adrenal cholesterol and corticosterone in rats

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Abstract

The synthetic oestrogen diethylstilbestrol (DES), which is known to bind oestrogen receptors (ERs), has been reported to have adverse effects on endocrine homeostasis; however, the molecular mechanisms underlying these effects are poorly understood. In this study, we treated rats with DES and found high levels of this compound in the liver, adrenal glands and pituitary gland, as compared with other tissues. We have also detected early adverse effects of DES in the adrenal glands. The adrenal glands of rats treated with DES (340 µg/kg body weight every 2 days) for 2 weeks showed increased weight and size and a decreased fat droplet size. Following 1 week of treatment with DES, the blood and adrenal corticosterone levels were substantially decreased without any histological alterations. The levels of the precursors for corticosteroid biosynthesis in the adrenal glands were also decreased, as determined using mass spectroscopy. Cholesterol, the principal material of corticosteroid biosynthesis, decreased substantially in the adrenal glands after only 1 week of treatment with DES. In conclusion, cholesterol insufficiency results in a reduction in adrenal corticosterone biosynthesis, which may lead to endocrine dysfunction, such as reproductive toxicity.

Key Words

- ▶ diethylstilbestrol
- ▶ endocrine disruptor
- ▶ corticosterone
- ▶ adrenal insufficiency
- ▶ cholesterol

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Introduction

Concerns have been raised regarding human health and the potential adverse effects of environmental chemicals, such as endocrine disruptors, which mimic the effects of oestrogen in reproduction and development. Endocrine disruptors are known to exert reproductive toxicity via a non-genomic oestrogen receptor (ER)-initiated pathway (Wong & Cheng 2011), although there is increasing evidence that environmental toxins can affect the reproductive system via multiple pathways and various mechanisms (Watson *et al.* 2011). One of the most

well-studied endocrine disruptors, diethylstilbestrol (DES), is a synthetic non-steroidal oestrogen that was used in the USA and Europe from the mid-1940s to the 1970s for the prevention of spontaneous abortion and premature delivery. The offspring of these types of pregnancies have been shown to be at risk for numerous medical conditions, including vaginal adenocarcinoma and malformation of the testes (Schrager & Potter 2004, Newbold *et al.* 2006, Martin *et al.* 2008, Palmer *et al.* 2009, Shin *et al.* 2009, Ünüvar & Büyükgebiz 2012). In one large

cohort, the median total dose of DES administered to pregnant mothers ranged from 1625 to 10 424 mg (~ 23 mg to 150 mg/kg of body weight; Giusti *et al.* 1995). Although the detailed mechanism of DES action and its pharmacokinetics are not well understood, DES has been shown to bind to the ERs (Korach & McLachlan 1985, Kuiper *et al.* 1997, Henley & Korach 2006). While DES is no longer used clinically for the prevention of miscarriage, it remains a useful compound for investigating the mechanism of action of exogenous oestrogens (Kaludjerovic & Ward 2012). DES has been extensively used as a treatment for prostate cancer in humans. Higher doses of DES-diphosphate (500–1000 mg/day for 2–5 weeks) were found to suppress the serum level of testosterone for the treatment of prostate cancer patients (Kitahara *et al.* 1999). DES, a synthetic non-steroidal oestrogen, binds to ERs and exhibits strong oestrogenic toxicities, including adverse reproductive effects on male animals (Hillered & Ernster 1983, Dean *et al.* 1997). It is now accepted that oestrogens play a role in male fertility and that exposure to exogenous oestrogens during foetal and neonatal life can lead to reproductive disorders in males. However, the ER-mediated processes involved in the regulation of male reproduction during foetal and neonatal development remain largely unclear. It is also important to keep the reproductive organs of adult men and male animals healthy by preventing adverse effects from environmental pollutants and chemicals that also possess oestrogenic activity. Further investigation of the detailed molecular mechanisms of the deleterious effects on adult males is necessary. It is important to identify the initial targets and the early adverse effects of DES by following reproductive abnormalities in adult males. Results of the DES studies are currently being used to better characterise the biological effects of other oestrogenic compounds (Kaludjerovic & Ward 2012). Owing to the complexity of the processes involving DES, it is difficult to determine the molecular mechanism until a specific toxic effect is observed. As described in Kaludjerovic & Ward (2012), it would be valuable to elucidate the mechanism and to identify the multiple pathways involved in DES toxicity. The triggers that elicit the aforementioned toxic effects have yet to be identified.

Endocrine-disrupting chemicals, including polychlorinated biphenyls, dioxin, lindab and others, can affect the synthesis of adrenal steroids; however, only a few studies investigating their effects on the adrenal glands and adrenal steroidogenesis have been carried out, and no studies of the adrenal P450s, which are involved in corticosterone biosynthesis, have been

reported (Diamanti-Kandarakis *et al.* 2009). Zimmerman *et al.* (1991) is the only research paper to report an increase in adrenal weight and decrease in steroid hormone levels. Corticosterone, which is the principal glucocorticoid in rodents and is synthesised in the zona fasciculata (ZF), regulates metabolism, the stress response and immune function. In addition, adrenal steroids play important roles in foetal development and delivery (Chida *et al.* 2011, Huang *et al.* 2012). Therefore, the objective of this study is to investigate the interactions between endocrine disruptors and adrenal corticosterone synthesis suppression.

In this study, we directly demonstrated the tissue distribution of DES and found that the adverse effects of DES involve corticosteroid biosynthesis in the adrenal glands as an initial step of its mechanism of toxicity, which may lead to the disruption of various biosynthetic pathways.

Materials and methods

Treatment of animals

Male Sprague–Dawley rats (weight, 280 ± 20 g and age, 8–10 weeks) were fed, housed and allowed to adapt to their environments for 1 week before the experiments. The rats received an oral dose of 0.1 mg of DES dissolved in 1 ml of olive oil (~ 340 μ g/kg of body weight) to study the distribution of DES. The same dose (~ 340 μ g/kg of body weight) was administered every 2 days for a total of four doses over 1 week or eight doses over 2 weeks to study the effects of DES. Control rats received 1 ml of olive oil. Blood was collected from the abdominal aorta of the animals via exsanguination under pentobarbital anaesthesia. After dissection, the organs were excised post-mortem, weighed and then immediately frozen and stored at -25°C . All animals were treated according to the Laboratory Animal Control Guidelines of Rakuno Gakuen University, which conform to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA (NIH 1985).

Morphological analysis

Rats (three per group) were anaesthetised with Nembutal (pentobarbital; Abbott Japan Co., Tokyo, Japan). First Ringer's solution and then a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 10 min were perfused through the aortae. Following fixation, tissue blocks (3 mm thick) of the adrenal gland were dehydrated in an ascending ethanol series, cleared in

xylene and embedded in paraffin wax. The sections were cut to a thickness of 5 µm and stained with haematoxylin and eosin (HE) before examination under a light microscope. For analysis by transmission electron microscopy, adrenal glands perfused with 4% paraformaldehyde were cut into 1-mm cubes and fixed via immersion in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PB for 2 h. The tissue blocks were rinsed in 0.1 M PB and post-fixed in 1.0% osmium tetroxide in 0.1 M PB for 1 h. After dehydration in a graded ethanol series, the specimens were embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Ultrathin sections (80–100 nm-thick) were prepared and stained with 2% methanolic uranyl acetate and 1% lead citrate and were subsequently observed using a transmission electron microscope (JEM-1220; JEOL, Tokyo, Japan). Cryosections (7 µm-thick) of snap-frozen adrenal glands were prepared on a cryostat microtome (CR-502; Yamamoto Kohki Industrial, Saitama, Japan). The cryosections were routinely stained with haematoxylin for nucleic acid visualisation and Oil Red O (Sigma) for lipid visualisation.

Preparation of samples and assay method for MS analysis

The samples were prepared, and the MS analysis was carried out according to our previously reported method (Duncan *et al.* 2009, Maeda *et al.* 2013a). Briefly, the samples (rat blood and adrenal glands) were homogenised with acetonitrile, and the supernatants were purified using two columns. For the identification of the steroids and conjugates in the samples, the various compounds present in the blood and adrenal gland preparations were separated using HPLC (UFLC Nexera; Shimadzu, Kyoto, Japan) and an L-column 2 (C18, 2.1 × 150 mm, 2 mm particle size; Chemicals Evaluation and Research Institute, Tokyo, Japan) at a flow rate of 200 µl/min (40 °C). The chemical formula for each steroid was calculated and identified via LC-TOF MS (microTOF-QII; Bruker Daltonics, Bremen, Germany) using mass error and SigmaFit, as previously reported (Maeda *et al.* 2013a). Finally, the steroid levels were quantified with LC-MS/MS (LC: Ultimate 3000, MS: TSQ Quantum Ultra; Thermo Fisher Scientific, San Jose, CA, USA) as described in our previous report (Maeda *et al.* 2013a).

Adrenocorticotrophic hormone determination

Adrenocorticotrophic hormone (ACTH) peptides digested with trypsin were separated and identified using nanoLC-Captivespray-microTOFQII (Bruker Daltonics) and then quantified with LC-MS/MS analysis as shown in

Supplementary Fig. 1-1, see section on supplementary data given at the end of this article by a method based on techniques described in previous reports (Kuzyk *et al.* 2009, Harbourt *et al.* 2012). The concentration of the serum peptide ACTH was determined by the standard addition procedure (Stuber & Reemtsma 2004) using the highest peak '1013.5/505' shown in Supplementary Fig. 1-2, and the calibration curve was obtained accurately, as shown in Supplementary Fig. 1-3.

Quantitative RT-PCR

The quantitative expression of the *StAR* (F primer, 5'-ggaaggctggaagaaggaaa-3'; R primer, 5'-acacctggcaccacctact-3'), peripheral benzodiazepine receptor (*PBR* (*ACBD3*); F primer, 5'-gaaaccctctctggcatcc-3'; R primer, 5'-tccagatatgtaggagccatacc-3') and cytochrome P450scc (*P450SCC* (*CYP11A1*); F primer, 5'-acatttcgcgtttgctt-3'; R primer, 5'-cacgatctctccaacatcc-3') mRNAs in the adrenal gland and *POMC* mRNA (F primer, 5'-aggacctcaccacgaaag-3'; R primer, 5'-ccgagaggtcgagtctgc-3') in the pituitary gland were investigated using real-time RT-PCR with the QuantiTect SYBR Green PCR Kit (Qiagen) and the iQ5/MyiQ Single-Color System (Bio-Rad Laboratories, Inc.). β -actin (F primer, 5'-ctaaggccaaccgtgaaaag-3'; R primer, 5'-gcctggatggctacgtaca-3') was used as the internal standard. The copy number of each transporter gene was divided by that of β -actin for normalisation. The quantitative values are the mean $\pm$ S.E.M. of four amplifications.

Western blot analysis

The adrenal gland tissue was gently homogenised in a 0.25 M sucrose solution (200 µl) at 4 °C and then centrifuged at 900 *g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 5000 *g* for 10 min at 4 °C. The final supernatant was used as the cell lysate, and the precipitate was used as the mitochondrial fraction. The protein concentration of the cell lysate and the mitochondrial fraction were estimated using the Lowry protein assay (Lowry *et al.* 1951). The cell lysate for the detection of LDL receptor (LDLR) and scavenger receptor type B1 (SR-B1) and the mitochondrial fraction for the P450scc were separated via SDS-PAGE and then transferred to PVDF membranes (ATTO, Tokyo, Japan) for western blot analysis. The membranes were probed with the following primary antibodies as described previously (Sakaguchi *et al.* 2013): rabbit polyclonal antibody against rat P450scc (purchased from Millipore Corp. (Billerica, MA, USA), AB1244) as described previously (Maeda *et al.* 2013b), SR-B1 (Sigma-Aldrich), chicken polyclonal antibody

against LDLR (Sigma–Aldrich) and rabbit polyclonal antibody against β -actin (Abcam, Cambridge, UK). The expression levels of each protein were analysed densitometrically using CS Analyzer ver 3.0 (ATTO).

Enzymatic activity assay of cholesterol side chain cleavage reaction

Enzymatic activities were assayed as described previously (Maeda *et al.* 2013b) using a 20-hydroxycholesterol as a substrate, and the reaction product, pregnenolone, was identified as shown in Supplementary Fig. 2, see section on supplementary data given at the end of this article, and determined using MS analysis (Maeda *et al.* 2013a).

Cholesterol analysis

The total cholesterol level in the adrenal gland was assayed using the Cholesterol E Kit (Wako, Osaka, Japan) with cholesterol oxidase and cholesterol esterase. Free cholesterol was determined using the same procedure without cholesterol esterase. The cholesterol ester level was calculated using the total and free cholesterol levels. The adrenal gland tissue was gently homogenised in a 0.25 M sucrose solution (200 μ l), followed by centrifugation at 900 *g* for 10 min. The amount of total cholesterol in the resulting supernatant was assayed according to the manufacturer's protocol. The total cellular cholesterol and free cholesterol were determined after disrupting the organelle membranes with a 0.25 M sucrose solution containing 0.1% CHAPS and 0.1% SDS.

Statistical analysis

Results are expressed as the mean $\pm$ S.E.M. of three to five independent experiments. Statistical analysis was carried out using the *F*-test and Student's *t*-test.

Results

Tissue distribution of DES

A single dose of DES was orally administered to adult male rats (0.1 mg DES/rat). After 1 h of treatment, the DES concentration in various organs was determined using an accurate LC–MS-based method that was previously developed by our group (Maeda *et al.* 2013a), and the results are shown in Fig. 1. Interestingly, a high level of DES was observed in the liver, adrenal glands and pituitary gland (Fig. 1).

Figure 1

Distribution of DES after 1 h of treatment. A single dose of DES was orally administered to adult male rats (0.1 mg/rat), and the amount of DES in various organs was determined via LC–MS analysis using our previously reported protocol (Maeda *et al.* 2013a). One gland or organ of five rats was used for the experiments. The data for each point represent the mean $\pm$ S.E.M. of five of each organ.

Weight of the adrenal glands and other organs

Larger adrenal glands were observed in rats treated with DES for 2 weeks compared with rats that had undergone the control treatment, as shown in Fig. 2A. In addition, the dry weight and the total protein content of the adrenal gland were higher in the rats treated with DES compared with the control rats (Fig. 2B and C). The body and organ weights of the adult male rats that were orally administered DES (0.1 mg/rat every 2 days) for 2 weeks were measured, and the body and testis weights were found to be decreased as a result of DES administration (Fig. 2D). However, the only weight increase was observed for the adrenal gland, which was $\sim$ 1.3-fold heavier after only 1 week of DES exposure (Fig. 2D).

Histological observation of the adrenal glands

Adrenal gland tissue sections from the rats that were administered DES for 2 weeks were stained with HE (Fig. 3A, B, C, D, E and F). Adrenal hypertrophy was observed in the zona fasciculata (ZF) and zona reticularis (ZR) of DES-treated rats (Fig. 3A, B, C and D). In the adrenal cortex, the droplet size was smaller as a result of DES administration, as shown in Fig. 3E and F.

Levels of corticosterone, ACTH and POMC

The corticosterone levels in the blood of rats treated with DES for 1 or 2 weeks were assayed according to a previously reported protocol (Maeda *et al.* 2013a). The control

Figure 2

Effects of diethylstilbestrol (DES) treatment on the gross morphology of the adrenal gland. DES was orally administered to male rats (0.1 mg/rat every 2 days) for 1 and 2 weeks. Rats treated only with olive oil were used as controls (Cont). Photographs (A) of an adrenal gland of a rat treated with DES for 2 weeks and an adrenal gland from a control rat. The adrenal gland was dried using a vacuum dryer, and the dry weight is shown (B). The total protein content in the 900 g supernatant of the adrenal homogenate is also shown (C). The body and organ weights are shown in (D). The data for each group represent the mean $\pm$ s.e.m. of three to four rats. * P <0.05 and ** P <0.01 compared with controls.

levels showing ~ 100 ng/ml of blood were corroborated with data obtained previously via RIA (Henning 1978, Besedovsky *et al.* 1981). The corticosterone levels in the blood showed a clear decrease after only 1 week of treatment, as shown in Fig. 4A. The levels of serum ACTH and pituitary *Pomc* mRNA were also assayed, and the results are shown in Fig. 4B and C. Unexpectedly, the ACTH concentration was unchanged, and the expression of *Pomc* mRNA, which encodes the protein that produces the ACTH peptide in the pituitary gland, was also not induced. Other serum factors (glucose, creatinine and electrolytes) were observed to display no alterations (data not shown).

Levels of corticosteroids and their precursors in the adrenal glands

The levels of corticosterone, aldosterone and the biosynthetic corticosterone precursors in the organs of the DES-treated rats were assayed, and the levels in the adrenal glands are shown in Fig. 5. Corticosterone and all of the corticosterone precursors except for deoxycorticosterone decreased significantly after 1 week of DES treatment. Aldosterone and 18-hydroxycorticosterone also decreased after 1 week of treatment, as shown in Fig. 5.

Rate-limiting factors in adrenal steroidogenesis and levels of SR-B1 and LDLR

The transport of cholesterol into the mitochondria, which is mediated by *Star* and *Pbr*, is the rate-limiting process of steroidogenesis (Hauet *et al.* 2002, Miller 2013). The mRNA levels of the proteins that mediate these rate-limiting reactions were assayed using RT-PCR, and the results are shown in Fig. 6. In the adrenal glands of the rats treated with DES for 1 week, the relative gene expression levels of *Star* and *Pbr* tended to increase (Fig. 6A and B). Recently, we have found that an initial target of DES in testicular steroidogenesis dysfunction is the reduction in the expression and the enzyme activity of P450scc (Maeda *et al.* 2013b). In the case of the adrenal, the mRNA expression level of *P450scc* tended to increase after a 1-week of treatment with DES (Fig. 6C). The P450scc protein expression level and the enzymatic activity of the cholesterol side chain cleavage reaction were also slightly increased after 1 week of treatment with DES, as shown in Fig. 6D and E. The expression of LDLR and SR-B1 (HDL receptor (HDLR)) was examined using western blot analysis with specific antibodies, and the results are shown in Fig. 7A. LDLR and SR-B1 were induced twofold

Figure 3

Histological analysis of the adrenal gland in rats treated with DES for 2 weeks. Haematoxylin and eosin staining of the adrenal gland in control rats treated with only olive oil (A, C and E) and rats treated with DES for

2 weeks (B, D and F) is shown. Adrenal cortex divided into zona glomerulosa (ZG), zona fasciculata (ZF) and zona reticularis (ZR) is shown (C and D). Each group (control and DES) contained three to four rats.

and three- to fourfold in the adrenal glands of the DES-treated rats, respectively, as shown in Fig. 7B.

Cholesterol in the adrenal glands

The results of the Oil Red O staining of the adrenal cortex are shown in Fig. 8A, B, C, D, E and F. The fat droplets, which are dyed bright red, clearly decreased in the adrenal

cortex as a result of the administration of DES for 2 weeks (Fig. 8B, D and F). To examine the tissue and cellular changes in more detail, we carried out a histopathological analysis using an electron microscope and verified that the droplets were smaller in the adrenal cortex following the 2-week treatment (Fig. 8G and H). The total cholesterol and free cholesterol in the adrenal glands were measured following DES administration for 1 or 2 weeks (Fig. 8I).

Figure 4

Levels of corticosterone and ACTH in the serum. Diethylstilbestrol (DES) was orally administered to adult male rats (0.1 mg/rat every 2 days) for 1 and 2 weeks (DES). Rats treated only with olive oil were used as controls (Cont). The corticosterone levels (A) in the blood and serum ACTH levels (B) were determined via LC–MS analysis as described previously (Maeda *et al.* 2013a) and as described in the 'Materials and methods' section and Supplementary Figs 1 and 2. Pituitary *Pomc* mRNA (C) was analysed as described in the 'Materials and methods' section. The data for each group represent the mean $\pm$ S.E.M. of three to four rats. ** $P < 0.01$ compared with controls.

As shown in Fig. 8A, B, C, D, E, F, G, H and I, the free cholesterol level and the cholesterol ester level calculated from the levels of total and free cholesterol significantly decreased in the adrenal droplets of DES-treated rats.

Discussion

Our dose of ~ 0.34 mg/kg of body weight (0.1 mg/rat) every 2 days for 1 or 2 weeks is based on results from our previous study where we had examined the effects of two doses of DES (0.1 mg/rat every 2 days and 1.0 mg/rat every 2 days) on adult male rats. We found adverse effects on the testicular proteins at the lower dose (0.1 mg/rat every 2 days; Li *et al.* 2011, Maeda *et al.* 2013b). In this study, we found adrenal abnormalities only after 1 week of treatment with DES (0.1 mg/rat every 2 days), and revealed that the endocrine disruptor DES disrupts corticosteroid biosynthesis via cholesterol insufficiency. We have recently developed a method for the accurate determination of steroids and chemicals in organs using LC–MS analysis (Maeda *et al.* 2013a). In this study, we determined the tissue distribution of DES, and this is the first report, to our knowledge, of its direct detection in organs.

Figure 5

Levels of adrenal corticosterone, aldosterone and the corticosterone precursors pregnenolone, progesterone, deoxycorticosterone and 18-hydroxycorticosterone. Rats were treated with diethylstilbestrol (DES) (0.1 mg/rat every 2 days) for 1 or 2 weeks (DES). Rats treated only with olive oil were used as controls (Cont). The steroid levels were determined via LC–MS analysis as described previously (Maeda *et al.* 2013a) and as described in the 'Materials and methods' section. The data for each group represent the mean $\pm$ S.E.M. of three to four rats. * $P < 0.05$ and ** $P < 0.01$ compared with controls.

Figure 6

The expression of *P450scc*, *Star* and peripheral benzodiazepine receptor (*Pbr*) in the adrenal gland. The adrenal mRNA levels of *Star*, *Pbr* and cytochrome *P450scc* (*P450scc*) in the DES-treated rats were determined via RT-PCR as described in the 'Materials and methods' section. The rats were treated with DES (0.1 mg/rat every 2 days) for 1 and 2 weeks (DES). Rats treated only with olive oil were used as controls (Cont). The data are the relative amount of each mRNA normalised to β -actin (A, B and C). The protein expression levels of mitochondrial *P450scc* were analysed densitometrically using a CS Analyzer (ATTO), and enzymatic activities of *P450scc* were analysed using the methods described in the 'Materials and methods' section. The data are shown in (D) and (E) respectively. The data for each group represent the mean $\pm$ S.E.M. of three to four rats. * $P < 0.05$ compared with controls.

Our results showed that DES was distributed throughout the body and was present at high levels in the liver, adrenal glands and pituitary gland. The reasons for this specific distribution are unknown, but it may lead to early direct damage in these organs by orally administered DES. We observed early adverse effects of DES on the adrenal glands, as shown in Figs 2, 3, 4, 5, 6, 7 and 8. Our data will contribute to the elucidation of the mechanism of DES toxicity. Although *in vitro* experiments are useful, *in vivo* data indicate that an interaction exists among these organs on the basis of the DES distribution data.

We found that DES preferentially accumulates in the adrenal and pituitary glands rather than in the testis, where DES toxicity has been reported in previous studies. The increase in adrenal weight and the decrease in the vacuolisation of the ZF cells observed in all high-dose male groups have also been reported for oestradiol (Biegel *et al.* 1998) and ethinylestradiol (Andrews *et al.* 2002). This

evidence indicates that the oestrogenic activity of these compounds and DES may reflect a change in adrenal steroid metabolism, leading to the increase in adrenal weight.

It has been recently demonstrated that aldosterone induced dose-dependent increases in testosterone production (Ge *et al.* 2005). Part of the testicular reproductive toxicity of DES may be indirectly induced upon the decrease in aldosterone level in the DES-treated male rat, as shown in Fig. 5. The adrenal and pituitary glands express high levels of ER α but not ER β (Kuiper *et al.* 1997) and DES can bind to both ER α and ER β with equal affinity (Kuiper *et al.* 1997). However, the reproductive toxicity of DES acts in an ER α -dependent manner in mice (Henley & Korach 2006), indicating that DES has a higher affinity for ER α -expressing organs such as the adrenal and pituitary glands. The adrenal glands may be the principal target of DES. In addition, in this study, the dry weight of the adrenal glands completely agreed with their wet weight (Fig. 2), and the total protein content in the 900 g supernatants of the adrenal homogenates were also consistent with the wet weight data, indicating that the increase in the adrenal weight as a result

Figure 7

Levels of SR-B1 and LDL receptor (LDLR). The protein expression levels of the HDL receptor (SR-B1) and the LDLR were assayed via western blot analysis using specific antibodies as described in the 'Materials and methods' section (A). (B) Each band for the control rats (Cont; three to four animals) and for the DES-treated rats (DES; three to five animals) was analysed densitometrically using a CS Analyzer (ATTO), and the expression levels of LDLR and SR-B1 were induced approximately twofold (LDLR) and 3.4- to 3.9-fold (SR-B1) respectively. The data for each group represent the mean $\pm$ S.E.M. of three to four rats. * $P < 0.05$ and ** $P < 0.01$ compared with the control.

of DES treatment was due to the hypertrophy of adrenal tissue, as demonstrated by our histological observations (Fig. 3). The adverse effects of DES described earlier were observed in adult male rats that were administered DES (0.1 mg/rat every 2 days) for 2 weeks. Some studies have reported that lipid accumulation results in adrenal hyperplasia (Cummins *et al.* 2006, Miller & Bose 2011). Uniquely, the enlarged adrenal glands in rats treated with DES contained lower levels of cholesterol. In addition, the levels

of adrenal corticosterone and its precursors decreased after 1 week of DES administration without any morphological abnormalities being observed (data not shown). These results indicate that hypertrophy of the adrenal glands may have resulted from the ACTH feedback induced by low plasma corticosterone levels. However, the serum ACTH levels and pituitary *Pomc* mRNA were not increased by DES treatment in this study. It has been reported that DES-diphosphate (500 or 1000 mg/day for 2–5 weeks) suppressed the serum level of testosterone for the treatment of prostate cancer patients without significant effect on serum levels of ACTH (Kitahara *et al.* 1999). Recently another factor for adrenal development has been reported. The expression level of the transcription factor *Sf1* (steroidogenic factor 1) was stimulated in the ovaries of neonatally DES-treated 3-month-old mice (Kakuta *et al.* 2012). *SF1* is essential for normal development and function of the adrenal glands, and overexpression of *Sf1* results in adrenal hyperplasia (Gardiner *et al.* 2012). Further study is required to elucidate the mechanism underlying the hypertrophy of adrenal glands induced by DES.

Congenital lipid adrenal hyperplasia, which is characterised by severe deficiencies in steroidogenesis, is known to result from *Star* and *P450scc* mutations (Miller & Bose 2011). Cholesterol, which has a 17-carbon steroid nucleus, is the starting material for adrenal steroidogenesis, which produces corticosterone, aldosterone and adrenal androgens. For adrenal steroidogenesis, cytosolic cholesterol

Figure 8

Cholesterol in the adrenal gland. Cryosections of the adrenal cortex were stained with Oil Red O for neutral lipids and counterstained with haematoxylin for nuclei. (A, B, C, D, E and F). Electron microscopic images of the cells of the zona fasciculata in adrenal glands from control (G) and DES-treated (H) rats are also provided. The amount of total cholesterol and free cholesterol in the adrenal glands of rats treated with DES for 1 and 2 weeks were also determined (I). The free cholesterol (gray columns) was determined using cholesterol oxidase, and the total cholesterol (whole columns) was determined using the oxidase and cholesterol esterase after the destruction of the organelle membranes in the presence of 0.1% CHAPS and 0.1% SDS, as described in the 'Materials and methods' section. The free cholesterol levels (gray columns) were $\sim 10.36 \pm 0.37$ μg/mg of protein in control rats after 1 week, 5.43 ± 0.46 μg/mg of protein in rats treated with DES for 1 week, and 12.42 ± 0.33 μg/mg of protein in control rats after 2 weeks, 6.31 ± 0.45 μg/mg of protein in rats treated with DES for 2 weeks. The levels of cholesterol ester (white columns) calculated using total and free cholesterol levels were $\sim 44.40 \pm 3.64$ μg/mg of protein in control rats after 1 week, 27.82 ± 1.65 μg/mg of protein in rats treated with DES for 1 week, and 47.88 ± 3.63 μg/mg of protein in control rats after 2 weeks, 21.09 ± 3.22 μg/mg of protein in rats treated with DES for 2 weeks. Significant differences in the cholesterol ester levels at 1 and 2 weeks of treatment were observed between control and DES-treated rats. The data for each group represent the mean $\pm$ s.e.m. of three to five rats. * $P < 0.05$ compared with the control rats.

is transported into the mitochondria by StAR and PBR as the rate-limiting step in steroid hormone biosynthesis. Cytosolic cholesterol is then converted to pregnenolone by P450scc. This enzyme system converts cholesterol to pregnenolone and determines the net steroidogenic capacity. Thus, it serves as the chronic regulator of steroidogenesis. Unfortunately, the expression levels of *Star*, *Pbr* and *P450scc* tended to increase upon DES treatment (Fig. 6), indicating that cholesterol transport and conversion to pregnenolone are not the cause of the observed adrenal insufficiency and the decrease in corticosterone biosynthesis produced by DES. Therefore, we examined the possibility that low cholesterol may cause the DES-promoted changes in the expression of the HDLR and LDLR. The cholesterol used for adrenal steroidogenesis is primarily obtained via SR-B1 (HDLR)-mediated uptake from serum HDL-cholesterol in rodents (Kraemer 2007). The expression of the SR-B1 and LDLR was analysed using western blotting with specific antibodies, and the receptors were found to be increased in the adrenal glands of DES-treated rats, as shown in Fig. 7, indicating that the system by which cholesterol is taken up from the blood may not function efficiently even upon their induction. Therefore, we examined why corticosterone biosynthesis is suppressed despite the expression levels of both receptors being induced. Decreased adrenal corticosterone synthesis was linked to the cholesterol content in the adrenal gland. The mechanism by which adrenal cholesterol is decreased upon DES treatment remains unclear; however, DES was shown to disrupt the cholesterol supply for optimal corticosterone synthesis. The mechanism of DES toxicity in the adrenal glands appears to be distinct from that in the testis, as has been previously reported by us, in which DES influences the proteins associated with steroidogenesis (including P450scc (Maeda *et al.* 2013b)) but does not disrupt cholesterol content of the testis (data not shown). Plasma lipoprotein deficiency in mice as a result of feeding with probucol is associated with decreased adrenal cortex cholesterol levels, a lower basal and stress-induced plasma glucocorticoid level (Hoekstra *et al.* 2010). Recently, in low-HDL-cholesterol subjects, adrenal steroids have been reduced to about 30% of the normal values for controls due to insufficiency of the source for adrenal steroidogenesis (Bochem *et al.* 2013). These previous studies and our results in this study support the hypothesis that reduction of adrenal steroidogenesis is due to adrenal cholesterol insufficiency, which might be caused by the reduction in serum HDL/cholesterol level in DES-treated rats. To explore the mechanism by which adrenal cholesterol is

reduced, further study of serum lipoproteins and cholesterol using short-term DES administration is required.

Adrenal steroids play important roles not only in foetal and neonatal growth and development but also in labour and childbirth (Chida *et al.* 2011, Kaludjerovic & Ward 2012). Therefore, chronic corticosterone suppression induced by DES has the potential to disrupt the regulators of endocrine homeostasis and to facilitate the impairment of stress resistance. These findings constitute a new and important step towards understanding the mechanisms behind DES toxicity, such as reduced fertility, increased vaginal adenocarcinoma, and reduced testicular size and sperm count (Schrager & Potter 2004, Shin *et al.* 2009, Ünüvar & Büyükgebiz 2012). It is difficult to elucidate the mechanism of DES toxicity over the long term from the foetal stage to adulthood due to multiple hormone-related events such as foetal development, delivery and puberty. The adult exposure used in this study was used to reveal some of the direct adverse effects of DES. The adrenal insufficiency and cholesterol imbalance found in this study should be the key targets for investigation of the mechanism of DES toxicity in pregnant animals and fetuses in the future. In addition, DES administration has the potential to disrupt cholesterol homeostasis, which is strictly controlled *in vivo*.

In conclusion, we have shown that DES disrupts corticosteroid biosynthesis via adrenal cholesterol insufficiency. The elucidation of the detailed mechanism behind this new adverse effect may reveal an association between chemical intake and various diseases due to cholesterol imbalance.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-13-0460>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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