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Effects of fluorotelomer alcohol 8:2 FTOH on steroidogenesis in H295R cells: Targeting the cAMP signalling cascade

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ABSTRACT

Previous studies have demonstrated that perfluorinated chemicals (PFCs) can affect reproduction by disruption of steroidogenesis in experimental animals. However, the underlying mechanism(s) of this disruption remain unknown. Here we investigated the effects and mechanisms of action of 1H, 1H, 2H, 2H-perfluoro-decan-1-ol (8:2 FTOH) on steroidogenesis using a human adrenocortical carcinoma cell line (H295R) as a model. H295R cells were exposed to 0, 7.4, 22.2 or 66.6 μM 8:2 FTOH for 24 h and productions of progesterone, 17α-OH-progesterone, androstenedione, testosterone, deoxycorticosterone, corticosterone and cortisol were quantified by HPLC-MS/MS. With the exception of progesterone, 8:2 FTOH treatment significantly decreased production of all hormones in the high dose group. Exposure to 8:2 FTOH significantly down-regulated cAMP-dependent mRNA expression and protein abundance of several key steroidogenic enzymes, including StAR, CYP11A, CYP11B1, CYP11B2, CYP17 and CYP21. Furthermore, a dose-dependent decrease of cellular cAMP levels was observed in H295R cells exposed to 8:2 FTOH. The observed responses are consistent with reduced cellular cAMP levels. Exposure to 8:2 FTOH resulted in significantly less basal (+GTP) and isoproterenol-stimulated adenylate cyclase activities, but affected neither total cellular ATP level nor basal (−GTP) or NaF-stimulated adenylate cyclase activities, suggesting that inhibition of steroidogenesis may be due to an alteration in membrane properties. Metabolites of 8:2 FTOH were not detected by HPLC-MS/MS, suggesting that 8:2 FTOH was not metabolized by H295R cells. Overall, the results show that 8:2 FTOH may inhibit steroidogenesis by disrupting the cAMP signalling cascade.
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Introduction

Perfluorinated chemicals (PFCs) are emerging pollutants and are widely present in the environment, and in the blood of wildlife and humans (Lau et al., 2007), which has generated scientific and regulatory concern in terms of their potential health risk for humans and wildlife. Recently, some PFCs have been shown to have potential endocrine-disruptive activities in vertebrates. For example, perfluorooctanoic acid (PFOA) lowered concentrations of testosterone and cholesterol in serum in rats (Biegel et al., 2001; Martin et al., 2007), and modified the mRNA expression of genes associated with cholesterol biosynthesis and steroid metabolism in fetal mouse liver (Rosen et al., 2007). Exposure to perfluorodecanoic acid (PFDA) decreased circulating concentrations of testosterone and dihydrotestosterone in male rats (Bookstaff et al., 1990). Furthermore, decreases in mRNA expression and the protein abundance of steroidogenic enzymes were observed after acute and chronic perfluorododecanoic (PFDoA) exposure in male rats. This resulted in significantly lower concentrations of plasma testosterone, compared to unexposed animals (Shi et al., 2007, 2009). These studies suggest that PFCs can affect steroidogenic activities, however, the cellular mechanisms remain unknown.

Fluorotelomer alcohols (FTOHs) belong to a class of telomerised fluorinated chemicals and have been used for a variety of commercial and industrial applications in paints, polishes, coatings, adhesives, waxes, polymers, and electronics (Dinglasan-Panlilio and Mabury, 2006). Recently, these chemicals were found to have a wide distribution in the ambient atmosphere (Jahnke et al., 2007; Stock et al., 2004), including in indoor air and dust (Shoeib et al., 2006; Strynar and Lindstrom, 2008). Previous studies have found that FTOHs

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can be biotransformed to perfluorinated acids (Wang et al., 2005, 2009). Specifically, in both rat and mouse hepatocytes, 1H, 1H, 2H, 2H-perfluoro-decan-1-ol (8:2 FTOH) is metabolized to PFOA and PFNA (Henderson and Smith, 2007; Martin et al., 2005), both of which have been reported to occur in human and wildlife. Hence, FTOH-based substances have been proposed as possible indirect sources for some PFC exposure. Furthermore, exposure to 1H, 1H, 2H, 2H-perfluorooctan-1-ol (6:2 FTOH) or 8:2 FTOH significantly altered plasma concentrations of testosterone and estradiol in zebrafish through effects on the hypothalamic–pituitary–gonadal (HPG) axis (Liu et al., 2009a,b). However, the cellular mechanisms underlying such steroidogenic disruption has remained unclear.

The present study investigated the mechanism(s) of alteration of steroidogenic functions by 8:2 FTOH in H295R human adrenocortical carcinoma cells. H295R cells retain physiological characteristics of zonally undifferentiated fetal adrenal cells and express all the key enzymes of steroidogenesis (Fig. 1). Upon cAMP stimulation, these cells produce all the adrenal steroid hormones found in the adult adrenal cortex (Gazdar et al., 1990; Sanderson, 2006). H295R cells have been employed for studying the biological effects and mechanisms of action of a variety of chemicals (e.g., Ding et al., 2007; Hecker et al., 2006, 2007; Hilscherova et al., 2004; Li and Wang, 2005; Sanderson, 2006; Zhang et al., 2005). In the present study, HPLC-MS/MS was used to examine the effects of 8:2 FTOH on the production of seven steroid hormones: progesterone, 17α-OH-progesterone, androstenedione, testosterone, deoxycorticosterone, corticosterone and cortisol. Real-time quantitative polymerase chain reaction (qRT-PCR) and Western blotting analyses were employed to characterize alterations in mRNA expression and protein abundance, respectively, of steroidogenic enzymes. In addition, cellular cAMP and ATP levels, and basal adenylate cyclase activity were measured. Basal activity, independent of G-proteins, was also measured to assess expression and constitutive adenylate cyclase activity. In order to evaluate the roles of interactions between 8:2 FTOH and G-protein or membrane receptors, the basal activities in the presence of NaF or isoproterenol, which elicit maximal G-protein and β-adrenergic stimulation, respectively, were assessed.

Materials and methods

Chemicals. 1H, 1H, 2H, 2H-perfluoro-decan-1-ol (8:2 FTOH) (purity >97%) was obtained from Strem Chemicals (Newburyport, MA, USA). Surrogate deuterated standards, progesterone-d9, 17α-OH-progesterone-d8, androstenedione-d7, testosterone-d5, deoxycorticosterone-d8, were provided by C/D/N Isotope (Pointe-Claire, Quebec, Canada). Cortisol-d4 was provided by Cambridge (Andover, MA, USA). 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Duchefa (Haarlem, the Netherlands). All other reagents used were obtained from Sigma (St. Louis, MO, USA).

Cell culture and chemicals exposure. The H295R human adrenocortical carcinoma cell line was obtained from the American Type Culture Collection (ATCC, Beltsville, VA, USA) and was cultured in DMEM/F12 medium (Sigma), containing 1% insulin-transferring sodium selenite plus Premix (ITS) (BD Bioscience, Bedford, USA) and 2.5% Nu-Serum (BD Bioscience) at 37 °C in a 5% CO_2 atmosphere. For chemical exposure, H295R cells were seeded in different size of plates depending on the type of assays. The medium volume of every well for 96-well, 24-well and 6-well plate was 0.2, 1.0 and 3.0 mL, respectively. After culture for 24 h, cells were exposed to 0, 7.4, 22.2 or 66.6 μM 8:2 FTOH dissolved in dimethyl sulfoxide (DMSO; Sigma) for 24 h. Control treatments received 0.1% DMSO (v/v). The exposure concentrations were chose based on the information from previous studies (Lau et al., 2007; Martin et al., 2007).

Cell viability assay. Cell viability was determined by measuring MTT activity using previous method (Hansen et al., 1989). Briefly, H295R cells were seeded into 24-well plates (Corning Life Sciences, Corning, NY, USA) at a density of 3×10^5 cells/ml. After culture for 24 h, the cells were exposed to 0, 7.4, 22.2 and 66.6 μM 8:2 FTOH for 24 h, then the culture medium was removed and MTT activity was determined. Four replicates were used in each experiment.

Quantification of steroid hormones. For the quantification of steroid hormone concentrations in culture medium samples were extracted by ethyl acetate/hexane (v/v, 50/50) followed by LC-MS/MS analysis. Surrogate deuterated standards, progesterone-d9, 17α-OH-progesterone-d8, androstenedione-d7, testosterone-d5, deoxycorticosterone-d8, and cortisol-d4, were spiked into samples before the extraction. For all steroid hormones, chromatography was performed using nano-pure water and methanol. Sample extracts were separated on a Betasil C18 (100×2.1 mm, 5 μm particle size) column from Thermo (Waltham, MA, USA) and then analyzed using an ABI SCIEX 3000 triple quadrapole tandem mass spectrometer (ABI SCIEX, Milford, MA, USA). All data were acquired and processed with AB Sciex Analyst 1.4.1 software

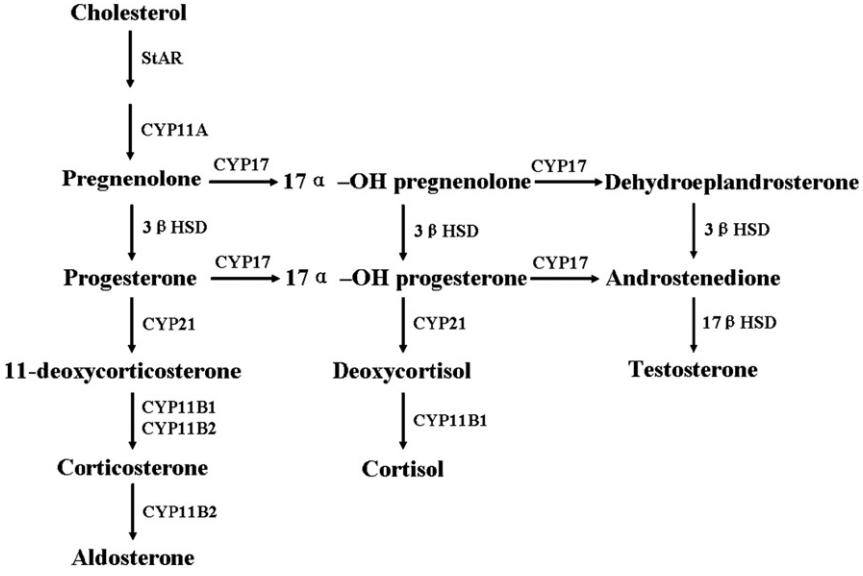

Fig. 1. Steroidogenic pathway in the H295R cells.

(Applied Bioscience, Foster City, CA, USA). Three replicates were used in each experiment.

Western blotting analysis. Western blotting analysis was performed as previously described (Yu et al., 2008; Zhang et al., 2009) with some modifications. Briefly, cells were seeded in 6-well plates (Corning Life Sciences). After exposure, cells were lysed and the protein content was determined. 50 μg cytoplasmic protein were denatured, electrophoresed and transferred onto a polyvinylidene difluoride (PVDF) membrane. The transferring efficiency was evaluated for equal protein in each lane using a reversible dye (PIERCE, IL, USA). The membrane was blocked and blots were probed with an anti-human steroidogenic acute regulatory protein (StAR), side-chain cleavage enzyme (CYP11A), steroid 11β hydroxylase (CYP11B1), aldosterone synthetase (CYP11B2), steroid 17α-hydroxylase/17, 20-lyase (CYP17) and steroid 21-hydroxylase (CYP21) antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Following primary antibody incubation the membrane was washed and incubated with either a horseradish peroxidase-conjugated anti-mouse or anti-goat antibody depending on the origin of the primary antibodies (Santa Cruz Biotechnology Inc.). Each secondary antibody was diluted 1:2000 in skim milk blocking solution. The immunoblot analysis was performed using the Amershanm™ ECL Plus Western Blotting Detection System (GE Healthcare, Baie-d'Urfe, QC, Canada). The quantification of the relative expression of steroidogenic enzymes was performed by using BandScan 5.0 software. Three replicates were used in each experiment.

Quantitative real-time PCR assay. Briefly, cells were seeded in 24-well plates (Corning Life Sciences). After exposure, the culture medium was removed for further hormone analysis and cells were used for total RNA isolation (Agilent Technologies, Santa Clara, CA, USA). The RNA purity was determined by measuring the 260/280 nm ratios and 1% agarose-formaldehyde gel electrophoresis with ethidium bromide staining. The purified RNA was used immediately for reverse transcription or stored at −80 °C until analysis. First-strand cDNA synthesis was performed using an Iscript™ cDNA synthesis kit (BioRad, Mississauga, ON, Canada) with a 1 μg aliquot of cellular total RNA. Quantitative real-time PCR (qRT-PCR) was analyzed on the ABI 7300 real time PCR system (Applied Biosystems) using SYBR® Green PCR master mix (Applied Biosystems). The primer design and concentrations, thermal cycle profiles and quantification of target genes were performed as described previously (Zhang et al., 2005). The housekeeping gene prophobilinogen deaminase (PBGD) did not vary under experimental conditions in the present study (data not shown) and was used as an internal control. Gene expression was measured in triplicate and this experiment was repeated three times.

Quantification of ATP and cAMP. Cellular ATP and cAMP were determined using commercial kits following the manufacturer's specifications. Briefly, for the ATP assay, H295R cells were grown in 96-well plates (Corning Life Sciences) for 8:2 FTOH exposure. After exposure, the medium was removed and the cells were used for ATP content detection using a kit (ViaLight® Plus Proliferation and Cytotoxicity BioAssay Kit, Lonza, Rockland, ME, USA). For the cAMP assay, cells were seeded in 6-well plates (Corning Life Sciences). After exposure, the medium was removed and cells were used to determine the cAMP content using a cAMP enzyme immunoassay kit (Sigma). Three replicate wells were used in each experiment.

Adenylate cyclase activity. Adenylate cyclase activity was determined using the protocol described in earlier studies (Liu et al., 2005; Song et al., 1997). Briefly, cells were plated in 100×20 mm Petri dishes. After 8:2 FTOH exposure, cells were collected and homogenized. Membranes were obtained by two different centrifugation steps (1000*g* for 5 min and 40,000*g* for 20 min at 4 °C) and the protein content was determined. 50 μg of membrane protein was used for each assay. Adenylate cyclase activity was determined in four different ways. First, basal activity was evaluated by incubating the membrane protein for 15 min at 37 °C in reaction buffer (50 mM Tris–HCl (PH 7.4), 5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM ATP, 0.1 μM GTP, 0.2 IU pyruvate kinase, 0.1 U myokinase and 2.5 mM phosphoenol-pyruvate). Second, basal activity independent of G-protein was determined to assess expression and constitutive adenylate cyclase activity by measuring the basal activity in the absence of GTP. Third, the net G-protein-linked response was assessed by measuring the basal activity in the presence of 10 mM NaF, which elicits maximal G-protein stimulation. Finally, receptor-targeted effects were evaluated by measuring the basal activity in the presence of 100 μM isoproterenol, which elicits maximal β-adrenergic stimulation. Three replicate wells were used in each experiment.

Determination of metabolites of 8:2 FTOH. H295R cells were seeded into 24-well plates (Corning Life Sciences) at a concentration of 3×10^5 cells/ml. After exposure for 24 h, the cells and medium were collected for metabolites extraction using previous method with some modifications (Yeung et al., 2006), followed by HPLC-MS/MS analysis. Briefly, the sample and 2 mL of methyl-*tert*-butyl ether (MTBE) were added to a polypropylene tube, and the mixture was shaken for 20 min and centrifuged for 15 min at 3000*g*. The organic layer was collected. Sample extracts were separated on a Betasil C18 column from Thermo (Waltham, MA, USA) and then analyzed using an ABI SCIEX 3000 triple quadrapole tandem mass spectrometer (ABI SCIEX, Milford, MA, USA). All data were acquired and processed with AB Sciex Analyst 1.4.1 software (Applied Bioscience, Foster City, CA, USA). A total of 12 PFCs were identified and quantified by comparison to authentic standards. These metabolites included perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHS), perfluorooctanesulfonate (PFOS), perfluoro-n-butyric acid (PFBA), perfluoropentanoic acid (PFPnA), perfluoroheptanoic acid (PFHA), perfluoroheptanoic acid (PFHpA), perfluorooctanoate (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFuDA), perfluorododecanoic acid (PFDoDA). The detection limit for all of these compounds was 200 ng/L. Three replicate wells were used in each experiment.

Statistical analysis. Normality and homogeneity of variances of each parameter were determined by the Kolmogorov–Smirnov and Levene's test, respectively. One-way analysis of variance (ANOVA) and Tukey's multiple range tests were used to determine significant differences between control and exposure treatments. The criterion for statistical significance was $p<0.05$.

Results

H295R cell viability

No significant change in cell viability was observed in any of the 8:2 FTOH treatments after 24 h as indicated by MTT activity (data not shown).

Effects on production of the steroid hormones

No statistically significant effects on progesterone production were observed after 22.2 or 66.6 μM 8:2 FTOH exposure compared with the control (Table 1). No significant change in production of any of the steroid hormones was observed in cells exposed to 7.4 μM 8:2 FTOH. Only testosterone production, which was decreased by 18.6%, was affected by exposure to 22.2 μM 8:2 FTOH. However, exposure to 66.6 μM of 8:2 FTOH significantly inhibited production of 17α-OH-progesterone, androstenedione, testosterone, deoxycorticosterone,

Table 1
Steroid hormone concentrations (mean ± S.E.M.) in H295R cells exposed to various concentrations of 8:2 FTOH for 24 h.

Steroid hormones	8:2 FTOH concentrations			
	0 mg/L	7.4 μM	22.2 μM	66.6 μM
Progesterone (ng/mL)	0.74 ± 0.10	0.71 ± 0.04	0.66 ± 0.03	0.69 ± 0.12
17α-OH-Progesterone (ng/mL)	6.12 ± 0.09	5.94 ± 0.32	5.52 ± 0.23	4.56 ± 0.27*
Androstenedione (ng/mL)	35.20 ± 2.60	31.73 ± 1.12	31.07 ± 1.00	18.87 ± 3.65*
Testosterone (ng/mL)	0.43 ± 0.01	0.40 ± 0.04	0.35 ± 0.01*	0.16 ± 0.05*
Deoxycorticosterone (ng/mL)	38.90 ± 0.10	36.73 ± 0.33	30.05 ± 6.17	24.10 ± 0.40*
Corticosterone (ng/mL)	21.33 ± 0.53	19.86 ± 0.97	16.67 ± 4.15	11.73 ± 2.37*
Cortisol (ng/mL)	4.93 ± 0.20	4.77 ± 0.17	4.69 ± 0.05	2.54 ± 0.31*

*$P<0.05$ indicates significant difference between exposure groups and the corresponding control group.

corticosterone and cortisol by 25.5%, 46.4%, 62.8%, 38.1%, 45.0%, and 48.5%, respectively (Table 1).

Effects on mRNA expression and protein abundance of the steroidogenic enzymes

Exposure to 22.2 or 66.6 μM 8:2 FTOH significantly down-regulated StAR, CYP11A, CYP11B1, CYP11B2 and CYP21 mRNA expression by 15.0% and 35.1%, 17.1% and 18.8%, 17.2% and 53.5%, 24.7% and 35.7%, and 26.2% and 34.8%, respectively. In contrast, CYP17 mRNA expression was down-regulated by 22.3% only in the 66.6 μM exposure groups compared with the control (Fig. 2). No significant effects on mRNA expression of 3βHSD (3β-hydroxysteroid dehydrogenase isomerase) or 17βHSD (17β-hydroxysteroid dehydrogenase) were observed after 8:2 FTOH exposure at all the tested concentrations compared with the control (Fig. 2). Exposure to 22.2 or 66.6 μM 8:2 FTOH significantly down-regulated CYP11A and CYP21 protein expression by 40.2% and 65.2%, 54.9% and 54.3%, respectively. StAR, CYP11B1, CYP11B2 and CYP17 protein expressions were down-regulated by 42.4%, 51.0%, 43.3% and 44.7% only in the 66.6 μM exposure groups, respectively, compared with the control (Fig. 3).

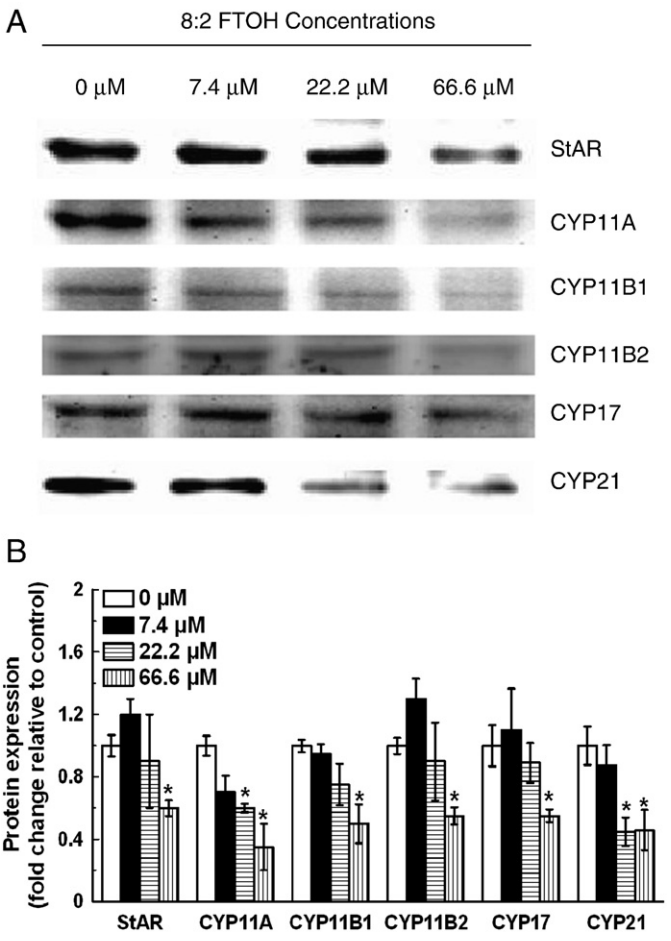

Fig. 3. Abundance of steroidogenic enzymes in H295R cells exposed to 0, 7.4, 22.2 or 66.6 μM 8:2 FTOH for 24 h. (A) Representative Western blot of steroidogenic enzymes from control and 8:2 FOH-exposed cells; (B) Quantification of the relative expression of steroidogenic enzymes in control and treatment groups. Values represent mean ± S.E.M. of three replicate samples. Significant differences ($p<0.05$) between control and exposure groups are indicated by an asterisk (*).

Effects on cellular cAMP and ATP contents and adenylate cyclase activity

Exposure to 22.2 or 66.6 μM 8:2 FTOH significantly decreased cellular cAMP levels by 28.4% and 46.0%, respectively, compared with the control (Fig. 4). No statistically significant effect on cellular ATP content after 8:2 FTOH exposure was detected at any of the tested concentrations (data not shown). Exposure to 66.6 μM 8:2 FTOH significantly depressed basal adenylate cyclase activity by 27.6%, whereas the activity remained unchanged at the lesser concentrations (7.4 or 22.2 μM) (Table 2). Similarly, statistically significant decreases (19.4%) were observed in isoproterenol-stimulated adenylate cyclase activity after 66.6 μM 8:2 FTOH exposure, whereas no significant change in this enzyme activity was observed in cells exposed to 7.4

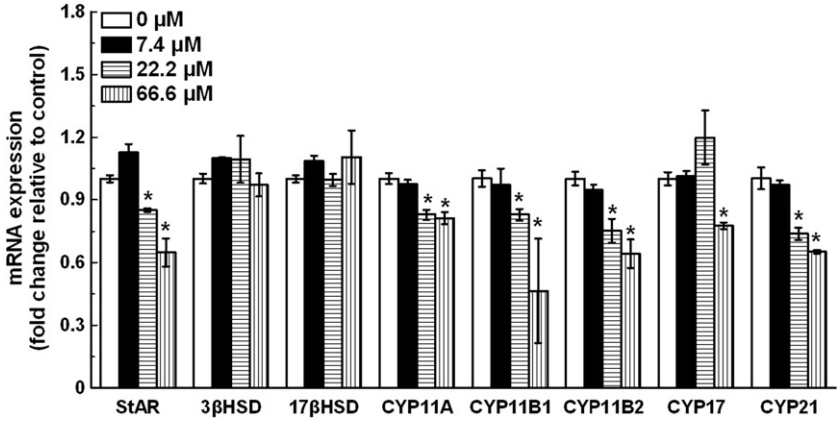

Fig. 2. mRNA expression of steroidogenic genes in H295R cells exposed to 0, 7.4, 22.2 or 66.6 μM 8:2 FTOH for 24 h. Values represent mean ± S.E.M. of three replicate samples. Significant ($p<0.05$) differences between control and exposure groups are indicated by an asterisk (*).

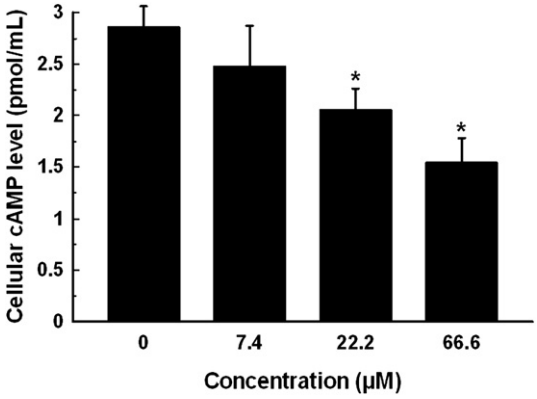

Fig. 4. Cellular cAMP levels in the H295R cells exposed to 0, 7.4, 22.2 or 66.6 μM 8:2 FTOH for 24 h. Values represent mean ± S.E.M. of three replicate samples. Significant differences ($p<0.05$) between control and exposure groups are indicated by an asterisk (*).

or 22.2 μM 8:2 FTOH (Table 2). Exposure to 8:2 FTOH did not significantly affect basal activity independent G-protein and NaF-stimulated activity compared with the control (Table 2).

Metabolite measurement

In the current study, 12 metabolites have not been observed in 8:2 FTOH treated H295R cells.

Discussion

In the present study, a significant inhibition of steroidogenesis was observed in H295R cells exposed to 8:2 FTOH, as demonstrated by the decreased production of the steroid hormones, 17α-OH-progesterone, androstenedione, testosterone, deoxycorticosterone, corticosterone and cortisol. The mechanisms of the inhibitory effects involved down-regulation of StAR, CYP11A, CYP11B1, CYP11B2, CYP17 and CYP21 mRNA expression and protein abundance. In addition, the down-regulation of mRNA expression and protein abundance of key steroidogenic enzymes was accompanied by decreased cellular cAMP. Furthermore, our results suggest that the decreased cAMP content was due to reduced membrane receptor-mediated adenylyl cyclase activities and not due to depressed cellular ATP concentrations.

Exposure to fluorotelomer alcohols (FTOHs) has been implicated as a possible pathway of exposure that results in PFCs accumulation in humans and wildlife. Previous studies reported that certain PFCs, such as PFOA, PFDA, and PFDoA decreased serum testosterone level in rats under toxicological dosages (Bookstaff et al., 1990; Martin et al., 2007; Shi et al., 2007, 2009). The serum PFCs concentrations (5 μg/mL to 260 μg/mL) measured in those dosed animals were in the same range as the concentrations of FTOHs applied to H295R cells in this study (Lau et al., 2007; Martin et al., 2007). It has been shown that exposure to 6:2 FTOH or 8:2 FTOH significantly disrupted plasma concentrations of sex steroids in zebrafish with a LOEC of 30 μg/L for both chemicals (Liu et al., 2009a,b). The present study further expanded on investigating the underlying mechanisms of this inhibitory effect: exposure to 8:2 FTOH significantly decreased 17α-OH-progesterone, androstenedione and testosterone production by H295R cells, whereas no effect on progesterone production was observed. These results suggested that the reduction in testosterone level reported previously might partially result from decreased production of upstream hormones (e.g., 17α-OH-progesterone, androstenedione). In addition, exposure to 8:2 FTOH in H295R cells also caused significant decreases in deoxycorticosterone, corticosterone and cortisol production. These hormones are associated with a variety of physiological processes including metabolism, stress responses, immune responses, vasoconstriction, growth, development, salt and water homeostasis and vascular tone (Mommsen et al., 1999; Osborn, 1991). Studies have shown that decreased production of these hormones may impair normal physiological processes and cause pathologic effects *in vivo* (Peter et al., 1999; Ueshiba et al., 2002).

This study further examined whether the inhibitory effects of 8:2 FTOH on steroidogenesis resulted from the down-regulation of mRNA expression and the consequent protein abundance of steroidogenic enzymes. Exposure to 8:2 FTOH significantly down-regulated StAR, CYP11A, CYP11B1, CYP11B2, CYP17 and CYP21 mRNA expression, and protein abundance in a dose-dependent manner. These enzymes play key roles in steroid hormone biosynthesis (Fig. 1). Therefore, the down-regulation of mRNA expression and protein abundance could lead to the decreased steroid hormone production. It has been previously demonstrated that exposure to PFDoA significantly decreases StAR and P450scc (CYP11A) mRNA expression and protein abundance resulting in decreased serum testosterone concentrations in male rat testes (Shi et al., 2007, 2009). The authors speculated that decreased testosterone level, at least in part, was due to the reduced expression of StAR and CYP11A. On the other hand, we did not observe a significantly inhibitory effect on progesterone production. Progesterone biosynthesis is catalyzed by 3βHSD using pregnenolone as substance, and then is converted to 17α-OH progesterone and 11-deoxycorticosterone by CYP17 and CYP21, respectively (Fig. 1). In the present study, 8:2 FTOH did not affect 3βHSD mRNA expression, but significantly down-regulated CYP17 and CYP21 mRNA expression and protein abundance. Therefore, the down-regulation of CYP17 and CYP21 mRNA expression and protein abundance could result in accumulation of progesterone. That is a possible reason for unchanged progesterone production after 8:2 FTOH treatment despite the down-regulation of StAR and CYP11A mRNA expression and protein abundance, which could result in less pregnenolone, the precursor of progesterone. Taken together, the observation that decreased mRNA expression was associated with a decrease in protein abundance suggests that 8:2 FTOH acts at the level of gene transcription.

cAMP is an important secondary messenger that stimulates steroid hormone biosynthesis in the human adrenal cortex (Sewer and Watermen, 2001; Stocco et al., 2005). The transcriptional profiles of most steroidogenic genes (e.g., StAR, CYP11A, CYP11B, CYP17 and CYP21) are cAMP-dependent (Sewer and Watermen, 2001). Many chemicals have been hypothesized to disrupt steroidogenesis via the cAMP pathway, including triazines, forskolin, vinclozolin, isobutyl, and methylxanthine (Hilscherova et al., 2004; Sanderson et al., 2000, 2002). To test the hypothesis that 8:2 FTOH exposure inhibits steroidogenesis (including decreased mRNA expression, protein abundance and hormones levels) by reducing cellular cAMP levels, cellular cAMP content was examined. In cells exposed to 22.2 or

Table 2
Adenylate cyclase activity (mean ± S.E.M.) in H295R cells exposed to various concentrations of 8:2 FTOH for 24 h.

Adenylate cyclase activities[a]	8:2 FTOH concentrations			
	0 μM	7.4 μM	22.2 μM	66.6 μM
Basal activity (+GTP)	135.18 ± 13.21	143.48 ± 11.59	125.42 ± 14.03	97.81 ± 2.02*
Basal activity (−GTP)	104.54 ± 3.85	114.75 ± 8.94	118.03 ± 7.87	97.74 ± 5.30
NaF-stimulated activity	818.65 ± 66.14	1007.59 ± 63.30	779.61 ± 104.03	777.54 ± 66.11
Isoproterenol-stimulated activity	179.60 ± 8.91	190.22 ± 12.12	184.86 ± 8.11	144.74 ± 1.25*

*$P<0.05$ indicates significant difference between exposure groups and the corresponding control group.
[a] Adenylate cyclase activities were expressed as pmol cAMP/min/mg protein.

66.6 μM 8:2 FTOH cAMP content was significantly decreased by 28.4% and 45.9%, respectively, which indicates that the decrease in cellular cAMP may lead to inhibition of steroidogenesis. This result is consistent with those of the previous study reporting that a 50% decrease in cellular cAMP level significantly reduced aldosterone production by approximately 75% in bovine adrenal glomerulosa cells treated with atrial natriuretic peptide (ANP) (MacFarland et al., 1991).

Adenylate cyclase is the enzyme responsible for catalyzing conversion of cellular ATP to cAMP via a G-protein-coupled receptor pathway (Stocco et al., 2005). In the present study, the decreased basal adenylate cyclase activity and absence of effect on ATP levels suggest that 8:2 FTOH could decrease cellular cAMP by depressing basal adenylate cyclase activity. To further investigate the mechanism (s) of 8:2 FTOH effects on adenylate cyclase, we assessed the constitutive, NaF-stimulated, and isoproterenol-stimulated activity of adenylate cyclase in 8:2 FTOH exposed cells. 8:2 FTOH exposure significantly depressed the isoproterenol-stimulated adenylate cyclase activity, but no effects on constitutive activity and NaF-stimulated activity were observed. These results suggested that 8:2 FTOH could affect a membrane G-protein-coupled receptor pathway by changing membrane properties and thus depress membrane receptor-stimulating adenylate cyclase activity. As stated above, 8:2 FTOH can be metabolized to PFOA and PFNA in rat and mouse hepatocytes. To further verify whether 8:2 FTOH could be metabolized and possible effects from these metabolites on H295R cells, we also measured 12 metabolites. However, in the present study, none of these metabolites were detected in H295R cells and culture medium, indicating that 8:2 FTOH was not metabolized by the cells. These results suggested that the inhibitory effects on steroidogenesis were affected by 8:2 FTOH, not its metabolites.

In summary, we hypothesize that 8:2 FTOH has the potential to depress steroidogenesis (including decreased mRNA expression, protein abundance, and hormone levels) through reduced cellular cAMP levels. Inhibition of cAMP biosynthesis by adenylate cyclase as a result of interaction with membrane receptors appears to be the most likely mechanism for decreased cAMP levels. This study clearly underlines the need for *in vivo* studies to examine the accumulation and disposition of 8:2 FTOH in the body as well as determining effects on steroidogenesis. Furthermore, it would be useful to determine whether metabolites of 8:2 FTOH or the compound itself are responsible for the observed effects on steroidogenesis.

Conflict of interest statement
The authors declare that there are no conflicts of interest.

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