Experimental and Toxicologic Pathology 68 (2016) 125–131

Contents lists available at ScienceDirect

Experimental and Toxicologic Pathology

journal homepage: www.elsevier.de/etp

Changes in plasma concentrations of corticosterone and its precursors after ketoconazole administration in rats: An application of simultaneous measurement of multiple steroids using LC–MS/MS.

Tomoaki Tochitani*, Akihito Yamashita, Mami Kouchi, Yuta Fujii, Izumi Matsumoto, Izuru Miyawaki, Toru Yamada, Hitoshi Funabashi

Preclinical Research Laboratories, Sumitomo Dainippon Pharma Co., Ltd., 3-1-98 Kasugade-naka, Konohana-ku, Osaka 554-0022, Japan

ARTICLE INFO

Article history:
Received 9 April 2015
Received in revised form 18 October 2015
Accepted 11 November 2015

Keywords:
Adrenal steroidogenesis
Drug
Simultaneous measurement of multiple steroids
Ketoconazole
Rat
In vivo

ABSTRACT

The adrenal gland is the most common toxicological target in the endocrine system, and inhibition of adrenal steroidogenesis by drugs can be fatal in humans. However, methods to evaluate the drug effect are limited. Recently, simultaneous measurement of multiple steroids, including precursors, has become possible. Here, we evaluated the usefulness of this simultaneous measurement for the evaluation of drug effects on adrenal steroidogenesis in vivo. For this purpose, we measured plasma concentrations of adrenal steroids in rats dosed with ketoconazole, a known inhibitor of adrenal steroidogenesis, and examined its relationship with the changes in histopathology and mRNA expression of steroidogenic enzymes in the adrenal gland. Ketoconazole (150 mg/kg/day) was orally administered to male rats for 7 days. The adrenal weight was high, and the zona fasciculata/reticularis were hypertrophic with an accumulation of lipid droplets. mRNA expression of CYP11A1, a rate-limiting enzyme in adrenal steroidogenesis, was slightly high in the adrenal gland. Plasma concentration of deoxycorticosterone was markedly high, while there were no significant changes in that of corticosterone, progesterone, or pregnenolone. The changes in the adrenal gland and plasma concentration of steroids were thought to reflect inhibited metabolism of deoxycorticosterone to corticosterone through inhibition of CYP11B1, and compensatory reaction for the inhibition. The compensatory reaction was thought to have masked decrease of corticosterone. These results suggest that simultaneous measurement of multiple steroids can enable sensitive evaluation of drug effects on adrenal steroidogenesis in vivo, while providing insight into the underlying mechanism of the effect.

1. Introduction

The adrenal gland is the most common toxicological target organ within the endocrine system (Ribelin, 1984). In the adrenal gland, the cortex is more frequently involved in toxic lesions than the medulla, and drug-induced inhibition of steroidogenesis in the cortex can be fatal in humans (Capen, 2007; Harvey and Everett, 2003). However, the importance of evaluating drug effects on adrenal steroidogenesis is relatively underestimated, and a novel evaluation method is needed (Harvey and Everett 2003; Hinson and Raven, 2006).

ACTH stimulation test is a common method to evaluate effects of drugs on adrenal steroidogenesis, especially glucocorticoid synthesis in vivo (Capen, 2007; Harvey and Sutcliffe, 2010; Yarrington and Reindel, 1996). However, ACTH stimulation test is difficult to incorporate into routine general toxicity studies, and thus additional animal experiments are required. Also, the results usually do not suggest the underlying mechanisms of observed effects.

In humans, it is known that in patients with some congenital adrenal insufficiencies caused by steroidogenic enzyme deficiency, levels of adrenal steroid precursors that would be metabolized by the deficient enzymes are elevated in blood, and this is used in the diagnosis of diseases (Kushnir, 2010; New, 2004). The same steroidogenic enzymes are inhibited by several drugs (Harvey and Sutcliffe, 2010), and similar changes in precursors can be expected also in drug-induced inhibition of the enzymes in vivo. However, precursors of adrenal steroids are rarely measured in experimental animals, probably due to lack of a specific, sensitive method to measure them and to limited sample volumes.

* Corresponding author. Fax: +81 6 6466 5446.
E-mail address: tomoaki-tochitani@ds-pharma.co.jp (T. Tochitani).

http://dx.doi.org/10.1016/j.etp.2015.11.004
0940-2993/

Though steroid hormones have been most commonly measured using ELISA or RIA, mass spectrometry (MS) is now increasingly used to measure steroids (Shackleton, 2010). Also, use of MS has enabled simultaneous measurement of multiple steroids and its precursors, using only small sample amounts (Kushnir, 2010; Maeda et al., 2013).

In this study, designed to evaluate the usefulness of simultaneous measurement of steroid and its precursors for the evaluation of drug effects on adrenal steroidogenesis in vivo, we examined the changes in plasma concentrations of corticosterone and its precursors in rats dosed with ketoconazole, a known inhibitor of adrenal steroidogenesis (Engelhardt et al., 1985; Johansson et al., 2002; Loose et al., 1983), and its relationship with the changes in histopathology and mRNA expression of steroidogenic enzymes in the adrenal gland.

2. Materials and methods

2.1. Chemicals

Ketoconazole and methyl cellulose (MC) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nacalai Tesque, Inc. (Kyoto, Japan), respectively.

2.2. Animals and husbandry

Animal usage was approved by the Committee for the Ethical Usage of Experimental Animals of Sumitomo Dainippon Pharma Co., Ltd. Seven-week old male Sprague-Dawley (Crl:CD) rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and were allowed a one-week acclimation period. In the acclimation period, the animals were acclimated to handling to minimize handling-related stress. The animals were housed individually or 2 animals per cage in a barrier-sustained room with controlled temperature of 24 °C ± 2 °C and relative humidity of 55% ± 10% and a 12-h light (8 a.m.–8 p.m.)/dark cycle. The rats were fed commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd.) and tap water ad libitum.

2.3. Animal experiment

At 8 weeks of age, five rats per group were randomly assigned to control and ketoconazole groups. Ketoconazole was suspended in 0.5% MC solution (final concentration 30 mg/mL). The animals in the ketoconazole group were dosed with 150 mg/kg of ketoconazole by oral gavage once daily around 10 a.m. for 7 days. The dose was selected based on a previous report (Shin et al., 2006). The animals in the control group were dosed with the same volume of 0.5% MC solution in the same manner. During the dosing period, daily observation for clinical signs and mortality was conducted. Also, body weights were measured before and 2 days after the initiation of dosing, and on the day of necropsy.

Six hours after the final dosing, about 1 mL of blood was sampled from the tail vein without anesthesia, using a syringe equipped with a needle. Twenty-four hours after the final dosing, the rats were decapitated and the trunk blood was sampled. The latter time point was selected because this point is commonly set for the blood sampling in routine general toxicity studies, and the former time point was selected expecting higher blood concentration of ketoconazole than that in the latter. At the decapitation, the decapitator was wiped each time and the face of animal was masked to minimize stress. Plasma was prepared by centrifuging the blood sample using EDTA as an anticoagulant, and was stored at −80 °C until the steroid measurement.

2.4. Necropsy and histopathology

After the blood sampling after decapitation (24 h after the final dosing), the bilateral adrenal glands and thymus were rapidly removed, grossly examined, and weighed. The organ weight relative to body weight was calculated using the body weight recorded on the day of necropsy. For histopathology, the right adrenal gland was fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, stained with hematoxylin and eosin (HE), and examined by light microscopy.

For immunohistochemistry, sections of the adrenal gland were subjected to a labeled polymer method using Histofine Simple Stain Rat MAX PO (MULTI) (Nichirei Biosciences Inc., Tokyo, Japan). Primary antibodies used were mouse monoclonal anti-adipophilin antibody (clone AP-125, dilution 1:200, Progen Biotechnik GmbH, Heidelberg, Germany) and mouse monoclonal anti-rat Ki-67 antibody (clone MIB-5, dilution 1:100, Dako, Glostrup, Denmark). For antigen-retrieval, sections were heated in 0.01 M citrate buffer (pH 6.0) using a pressure cooker. Counterstaining was conducted with hematoxylin.

The number of Ki-67-positive cells was counted in 8 randomly chosen 400× magnification fields of the adrenal cortex including the capsule (total area counted was about 0.34 mm^2), and the mean number of positive cells per field was calculated.

RNA extraction and mRNA expression analysis by quantitative real-time reverse transcription PCR (qRT-PCR)

At necropsy, the left adrenal glands of 4 of 5 animals in each group (the animals were selected in an ascending order) were immersed in RNAlater (QIAGEN K.K., Tokyo, Japan) soon after the weighing and were stored at −80 °C until RNA extraction. Total RNA was extracted from the adrenal gland using QIAGEN miRNeasy mini kits in accordance with the manufacturer's protocol. The RNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Epsom, UK) and the quality of all the RNA samples was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Primers used are shown in Table 1. The specificity of each of the primer sequences was verified by presence of a single band after agarose gel electrophoresis, and only one peak in a melting curve analysis that was carried out after each qRT-PCR reaction. cDNA synthesis and the subsequent qRT-PCR reactions were conducted using QuantiFast SYBR® Green RT-PCR Kit (Qiagen GmbH, Hilden, Germany) and Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster city, CA, USA), in accordance with the manufacturers' protocols. The thermal cycler conditions were as follows: 50 °C for 10 min (RT), 95 °C for 5 min (enzyme inactivation), and 40 cycles at 95 °C for 10 s and 60 °C for 34 s. Each measurement was carried out in duplicate.

Table 1
Primers used for quantitative real time-PCR.

Target	GenBank accession no.	Primer sequence (5′-3′)
CYP11A1	NM_017286	F: GGGCAACATGGAGTCAGTTT
		R: TTTCCTCGGCATCTGAAC
CYP21	NM_057101	F: CGACCCCAGATACTAGATGGAAAG
	XM_001076389	R: AATTCCTGGGTCAGCTGCTC
HSD3B1	M38178	F: TGGTGCAGGAGAAAGAACTG
		R: AGACATCAATGACAGCAGCG
HSD3B2	NM_001042619.1	F: GAACCTATTGGAGGCCGGTAT
		R: TCTTCCTCACGGCCATTCAG
CYP11B1	NM_012537	F: ACCATGGAAGCCAGCCATTT
	XM_346808	R: CATGAGCTGTGTGGTGGACT
GAPDH	NM_017008	F: AGCCAAAAGGGTCATCATCT
	XM_216453	R: GGGGCCATCCACAGTCTTCT

F: forward; R: reverse.