Available online at www.sciencedirect.com
ScienceDirect
Toxicology 243 (2008) 23–30
TOXICOLOGY
www.elsevier.com/locate/toxicol

Effect of tacrolimus on the cauda epididymis in rats: Analysis of epididymal biochemical markers or antioxidant defense enzymes

Akihiko Hisatomi *, Shozo Sakuma, Michio Fujiwara, Jiro Seki

Drug Safety Research Laboratories, Astellas Pharma Inc., 1-6 Kashima 2-chome, Yodogawa-ku, Osaka 532-8514, Japan

Received 15 June 2007; received in revised form 7 September 2007; accepted 10 September 2007
Available online 22 September 2007

Abstract

The effect of tacrolimus on epididymal biochemical markers was investigated following single daily subcutaneous doses of 1, 2 and 3 mg kg^{-1} day^{-1} for 2 weeks to male adult rats. The tacrolimus 2 and 3 mg kg^{-1} day^{-1} groups showed a significant and dose-dependent decrease in sperm count in the cauda epididymis. Among tissue levels of L-carnitine, α-glucosidase and acid phosphatase, only L-carnitine level in the cauda epididymis was significantly reduced in the tacrolimus 3 mg kg^{-1} day^{-1} group. However, no significant difference was seen in the plasma L-carnitine. It was suggested that lowering of L-carnitine in the cauda epididymis was attributable to the adverse effect on epididymal function to transport and/or concentrate L-carnitine. Since L-carnitine has been reported to have antioxidant potential, antioxidant defense enzymes in the cauda epididymis such as superoxide dismutase (SOD), catalase, glutathion peroxidase and glutathione reductase were evaluated. The results showed no significant differences in activities, confirming that the treatment with tacrolimus did not affect the activities of these antioxidant enzymes. In conclusion, this study indicates that tacrolimus induces a decrease in L-carnitine level in the cauda epididymis, which is probably caused by impairment of epididymal function to transport and/or concentrate L-carnitine from bloodstream, and a decrease in sperm count.

Keywords: Tacrolimus; FK506; Rat; Epididymis; Sperm count; L-Carnitine; Antioxidant defense enzyme

1. Introduction

Tacrolimus (Prograf, FK506) is a macrolide immunosuppressant produced by *Streptomyces tsukubaensis*. Although tacrolimus is in worldwide use as an immunosuppressive drug for organ transplantation and atopic dermatitis, it causes some toxicological effects in laboratory animals (Hirano et al., 1994; Mitamura et al., 1994). It has also been reported that tacrolimus affects sperm count and sperm motility in rat epididymis (Hisatomi et al., 1996; Kuwata and Takenaka, 2001). Our previous study showed that tacrolimus decreases sperm count and motility in rats through a direct effect on sperms in the epididymis, but has no effect on sperm production in the testes (Hisatomi et al., 1996). In addition, since it is shown that FKBP (FK506-binding protein; a major pharmacological target molecule of tacrolimus) plays a role in sperm motility (Walensky et al., 1998), it can be inferred that FKBP is involved in the tacrolimus-induced decrease of sperm motility, although there may be other factors not attributable to the direct pharmacological properties of tacrolimus.

* Corresponding author.
E-mail address: akihiko.hisatomi@jp.astellas.com (A. Hisatomi).

0300-483X/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2007.09.017

The epididymis has unique physiological features such as absorption, metabolization, secretion, sperm maturation and sperm storage. Since the chemical composition of the epididymal tissue fluid plays an important role in both sperm maturation and sperm storage, it is possible that chemical substances could perturb these processes and produce toxic effects (Thomas and Thomas, 2001). Indeed, several chemical substances that induce epididymal toxicity have also been shown to produce changes in biochemical markers or antioxidant defense enzymes in the epididymis (Fourie et al., 2001; D'cruz and Mathur, 2005). Here, we hypothesize that tacrolimus also may affect chemical composition in the cauda epididymis, since we confirmed that tacrolimus induces decrease of sperm count and motility, and histopathological change in the cauda epididymis in rats. Therefore, we investigated the effect of tacrolimus on epididymal biochemical markers or antioxidant defense enzymes which are relevant to epididymal function.

2. Materials and methods

2.1. Materials

Male Crl:CD(SD) rats were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan) and used at 9 weeks old 319–388 g after quarantine and acclimation to the lab environment for at least 7 days. During the acclimation and study periods, the animals were kept in a room set at a temperature of $23 \pm 1\,^{\circ}C$, relative humidity of $55 \pm 5\%$ and 16.2 air changes/hour, and allowed free access to a standard laboratory diet and tap water. All of the animal experimental procedures were approved by the Committee for Animal Experiments of Astellas Pharma Inc. (Tokyo, Japan).

The test substance (10 mg tacrolimus (FK506) injectable formulation; Astellas Pharma Inc.) and vehicle control substance (tacrolimus (FK506) placebo injectable formulation; Astellas Pharma Inc.) used in the present study were diluted to appropriate concentrations with physiological saline (Otsuka Pharmaceutical Factory Inc. (Tokushima, Japan)) to prepare dosing solutions.

5,5′-Dithiobis-2-nitrobenzoic acid (DTNB), acetyl coenzyme A (AcCOA), *p*-nitrophenyl-α-D-glucopyranoside (PNPG), L-carnitine, carnitine acetyltransferase (CAT) and 4-nitrophenyl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan) or Nacalai Tesque Inc. (Kyoto, Japan).

2.2. Methods

2.2.1. Treatment

Male rats were divided into four dose groups (nine animals/group), namely tacrolimus 1, 2 and 3 mg kg^{-1} day^{-1} groups and one control (tacrolimus placebo (equivalent to 3 mg kg^{-1} day^{-1} tacrolimus)) group. The rats were dosed subcutaneously once a day for 2 weeks. Body weight was recorded before dosing on day 1 and on days 4, 8, 11 and 15. The day after the final administration, blood was collected from the abdominal aorta under ether anesthesia, left standing at room temperature, and centrifuged at 3000 rpm for 10 min to separate serum, and it was stored at $-80\,^{\circ}C$ until analysis. The epididymis was removed and cleaned of external fat and blood, then sectioned into two parts (the cauda and the caput-corpus), and they were also weighed separately.

2.2.2. Epididymal sperm count

The left cauda epididymis was dissected minutely in 1 mL of phosphate buffered saline (PBS, pH 7.4), then the volume was brought up to 10 mL with the same solution. After gentle shaking for 5 min, the suspension was strained through gauze. Epididymal sperms were counted with a hemocytometer and the sperm count per 100 mg (wet weight) of cauda epididymis was calculated.

2.2.3. Biochemical studies in the cauda epididymis

The right cauda epididymis was minced with scissors then homogenized in four volumes of PBS (pH 7.4) containing 1 mM ethylenediamine tetraacetic acid (EDTA). The homogenate was then centrifuged at 15,000 rpm for 20 min at $4\,^{\circ}C$. The supernatant was used for biochemical analysis, and protein content was measured by the method of Bradford (1976) using an assay kit (Protein Quantification Kit Rapid; Dojindo Laboratories, Kumamoto, Japan).

2.2.3.1. Epididymal biochemical markers.

L-Carnitine, α-glucosidase and acid phosphatase were examined as epididymal biochemical markers.

L-Carnitine was determined by a modification of the DTNB method (Pearson et al., 1974) using an Ultrafree-MC centrifugal filter (30 kDa cut-off, Millipore Corporation (Bedford, MA, USA)) for deproteinization of the sample solution (Fourie et al., 2001). A 0.1 mL portion of the deprotenized solution was added to 1.2 mL of reaction medium containing 0.1 mol/L Tris–HCl buffer (pH 7.5), AcCOA 0.12 mL (1.2 mg/mL), DTNB 30 μL (2 mg/mL) and CAT 6 μL (1.6 mg/mL). The mixture was incubated at $37\,^{\circ}C$ for 30 min, then 2 mL of Tris–HCl buffer was added and absorbance was measured at 412 nm. L-Carnitine concentration was assayed using the standard curve method and expressed as μmol/mg protein (protein weight before deproteinization).

α-Glucosidase activity was determined on the basis of the method of Wang et al. (1993). The incubation medium contained 1.2 mL of 100 mM phosphate buffer (pH 6.8), 0.4 mL of 10 mg/mL PNPG and 0.1 mL of sample solution (same volume buffer as for the blank control). The reaction mixture was incubated at $37\,^{\circ}C$ for 4 h, and 1 M Na_2CO_3 was added to stop the reaction. Absorbance was measured at 400 nm, and *p*-nitrophenol content was estimated according to the *p*-nitrophenol standard curve. α-Glucosidase activity was expressed as nmol *p*-nitrophenol liberated/mg protein.