Biomedicine & Pharmacotherapy 100 (2018) 132–141

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

# Differential expression of the receptors for thyroid hormone, thyroid stimulating hormone, vitamin D and retinoic acid and extracellular signal-regulated kinase in uterus of rats under influence of sex-steroids

Abu Sadat Md Sayem[a,b], Nelli Giribabu[b], Kamarulzaman Karim[b], Si Lay Khiang[c], Sekaran Muniandy[d], Naguib Salleh[b,*]

[a] Department of Pharmacy, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[b] Department of Physiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[c] Department of Obstetric & Gynaecology, Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia
[d] Department of Biochemistry, MAHSA University College, Bandar Saujana Putra, 42610 Jenjarum, Selangor, Malaysia

ARTICLE INFO

*Keywords:*
TRα-1
TRβ-1
TSHR
VDR
RAR
ERK1/2
Sex-steroids
Uterus

ABSTRACT

Sex-steroids play important role in modulating uterine functions. We hypothesized that these hormones affect expression of proteins in the uterus related to thyroid hormone action. Therefore, changes in expression levels of receptors for thyroid hormone (TRα-1 and TRβ-1), thyroid stimulating hormone (TSHR), vitamin D (VDR) and retinoid acid (RAR) as well as extracellular signal-regulated kinase (ERK1/2) in uterus were investigated under sex-steroid influence.

*Methods:* Two rat models were used: (i) ovariectomised, sex-steroid replaced and (ii) intact, at different phases of oestrous cycle. A day after completion of sex-steroid treatment or following identification of oestrous cycle phases, rats were sacrificed and expression and distribution of these proteins in uterus were identified by Western blotting and immunohistochemistry, respectively.

*Results:* Expression of TRα-1, TRβ-1, TSHR, VDR, RAR and ERK1/2 in uterus was higher following estradiol ($E_2$) treatment and at estrus phase of oestrous cycle when $E_2$ levels were high. A relatively lower expression was observed following progesterone (P) treatment and at diestrus phases of oestrous cycle when P levels were high. Under $E_2$ influence, TRα, TRβ, TSHR, VDR, RAR and ERK1/2 were distributed in luminal and glandular epithelia while under P influence, TSHR, VDR abn RAR were distributed in the stroma.

*Conclusions:* Differential expression and distribution of TRα-1, TRβ-1, TSHR, VDR, RAR and ERK1/2 in different uterine compartments could explain differential action of thyroid hormone, TSH, vitamin D, and retinoic acid in uterus under different sex-steroid conditions.

## 1. Introduction

Sex-steroids play important role in regulating the uterine functions. Under the influence of $E_2$, uterus undergoes a remarkable growth and proliferation, while under the influence of P, extensive secretion and stromal decidualization occurs in preparation for pregnancy [1–3]. $E_2$ and P exert their action via binding to sex-steroid receptor, following which the intracellular signallings are activated, leading to transcription of genes that encode proteins involved in many uterine functions [4,5]. Among the proteins which expression have been reported to be influence by sex-steroids are the receptors for thyroid hormone, TRα and TRβ [6,7], receptor for thyroid stimulating hormone, TSHR [8], receptor for vitamin D, VDR [9], receptor for retinoic acid, RAR [10] and ERK1/2 signaling protein [11]. These proteins participate in thyroid hormone actions.

Besides sex-steroids, thyroid hormone (TH) is also important for many uterine functions [12,13]. This was evidence from deficiency of thyroid hormone which can cause menstrual irregularities and failure of the embryo to implant, that would eventually impair fertility [14]. There are two forms of tyroid hormones i.e. thyroxine (T4) and triiodothyronine (T3) which mediate their action via binding to specific thyroid hormone receptor (TR) [6,15] that isare located intracellularly [16] or in the membrane [17]. Currently, two isoforms of TR i.e. TRα and TRβ have been identified [18,19]. Expression of TR has been detected in the endometrium in humans and rodents [6,7].

Additionally, thyroid stimulating hormone (TSH) also plays

* Corresponding author.
*E-mail addresses:* naguib.salleh@gmail.com, naguib.salleh@yahoo.com.my, naguibsalleh@um.edu.my (N. Salleh).

https://doi.org/10.1016/j.biopha.2018.02.008
Received 1 June 2017; Received in revised form 1 February 2018; Accepted 2 February 2018

important role in the uterus [20]. Among its uterine actions include increases the expression of leukemia inhibitory factor (LIF) and its receptor (LIFR) in the endometrial stroma of humans [6] and primates [21]. TSH is also involved in endometrial glucose transport, in view that expression of *Glut-1* mRNA was up-regulated by TSH in human endometrial stromal cells and Ishikawa (uterine adenocarcinoma) cells [6]. TSH might have direct actions on the uterine functions, since receptors for thyrotropin-releasing hormone (TRH) and TSH were dectected in the monkey uterus following long term treatment with sex-steroids [22]. TSH acts via binding to TSHR which has been found to be expressed in the uterus in humans and rabbits [23,24]. Expression of TSHR and TRs in the uterus was reported to be influence by sex-steroids in which chronic administration of conjugated equine estrogen and medroxyprogesterone acetate to ovariectomized cynomolgus macaques caused up-regulation of TSHR and TR expression in uterine compartments [8]. However, direct effects of individual sex-steroids i.e $E_2$ and P on TSHR and TR expression in the uterus have never been identified.

In the meantime, Vitamin D has been reported to play important role in uterine functions including regulating uterine smooth muscle contraction [25] and uterine cell proliferation [26]. Expression of VDR and enzyme that convert vitamin D to its active form (1α-hydroxylase) has been reported in the uterine tissue [27]. In uterus, VDR is found to be expressed in the follicular and luteal phases of the menstrual cycle [9]. VDR can be localized in both endometrium and myometrium in humans [28]. VDR is a transcription factor located in the nuclei, and this protein mediates the genomic effect of vitamin D (1,25(OH)2D3) [29]. Lack of VDR expression in thyroid disorders has been linked to infertility and pregnancy loss [30]. It was also reported that mice lack VDR had impaired fertility [26].

Retinoic acid (RA) is a low molecular weight acid that is a lipophilic metabolite in terms of Vitamin A. RA is crucially involved in maintenance of the female reproductive system functions, including cell proliferation and differentiation [31]. RA has important role in regulating expression of matrix metalloproteinases (MMP) produced by endometrial stromal cells during decidualization [32,33]. In addition, RA has protective role on the uterus, since growth of cancer cells in the endometrium was reduced following treatment with RA [34]. RA binds to RAR, which is a nuclear receptor [35]. Expression of RAR has been reported in the uterine stroma of mice [36] and uterine epithelium of rats [10] and humans [37]. Expression of RAR in uterus was found to be influence by ovarian steroids, as documented in rats [10] and humans [37]. The functional heterodimer complex of TR and retinoid acid receptor (RAR) interact with specific thyroid hormone responsive element (TRE) on DNA, initiating protein synthesis [38].

ERK1/2, a member of well-known mitogen-activated protein kinase (MAPK), is reported to also be involve in many uterine functions, including proliferation and decidualization [39,40]. ERK1/2 plays critical role in embryo implantation, in mice and humans [41]. ERK1/2 is also involve in mediating the non-genomic effects of thyroid hormone [42–44]. Both $E_2$ and P were reported to regulate ERK1/2 expression in the smooth muscle of uterine artery [11].

In view of the important role of $E_2$ and P for the uterus, it was hypothesized that both hormones could affect uterine expression of TR isoforms, TSHR, VDR, RAR and ERK1/2. As there was currently inadequate information pertaining to the effect of individual sex-steroids on expression of these proteins in the uterus, our study aims to identify changes in expression and distribution of these proteins under different sex-steroid influence.

## 2. Materials and methods

### 2.1. Animals and hormones treatment

All experimental procedures were approved by the Animal Care and Use Committee (IACUC), University of Malaya, Kuala Lumpur. Twelve weeks old adult female Sprague–Dawley (SD) rats were housed in a clean and well-ventilated environment (12 h light and 12 h dark cycle, temperature 24 ± 2 °C). All rats weighing 210 ± 20 g were kept in cages with 5–6 animals in eachcage. Rats were given food pellet (Harlan, Rossdoff, Germany) and tap water *ad libitum*. Ovariectomy was performed under ketamine: xylazine (80 mg/kg: 8 mg/kg; intraperitoneal) anaesthesia in order to eliminate the variation in endogenous sex-steroid levels [45,46]. Twenty-one (21) days after ovariectomy, estradiol benzoate and progesterone (P), dissolved in peanut oil and peanut oil (vehicle) only (Sigma Aldrich Co., St. Louis, USA), were injected behind the neck scruff of these rats daily for three (3) consecutive days. Rats were divided into two (3) groups with six animals per group and the treatments are as follows:

Group 1: peanut oil only (control: C)
Group 2: $E_2$ s.c. at 1 μg/kg/day
Group 3: P s.c. at 4 mg/kg/day

Doses of $E_2$ and P were selected based on previously reported dose [47].

In another cohort of rats, estrus and diestrus phases were identified by vaginal smear. Smears were performed in three consecutive cycles in order to determine the cycle regularity. In this study, all rats used were found to have regular cycle based on the appearance of the cells in the smear during each stages (as described below).

For vaginal smear, secretions were collected immediately following vaginal flushing with 10 μl normal saline (NaCl 0.9%). Secretions were smeared on glass slides which were then observed under a light microscope (Olympus, Japan). Identification of estrous cycle phases was based on the criteria as described by Marcondes et. al., [48]. Briefly, three types of cells can be found in the smear. During proestrus and estrus stages, smears contained predominantly nucleated epithelial and anucleated cornified cells, respectively. Approximately the same proportion of epithelial, leukocytes and cornified cells were observed in smear obtained during metestrus stages, while smear from rats ar diestrus contained predominantly leukocytes. Following estrous cycle phase identification, intact rats were divided into the following groups:

Group 5: Estrus phase (Es)
Group 6: Diestrus phase (Ds)

Though the level of $E_2$ increases at proestrus and decreases at the end of estrus, the latter phase was selected for the study for endogenous $E_2$ effect on protein expression as optimum level for $E_2$ was reported in estrus phase. Additionally, length of the phases was relatively short with the mean duration of each phase was estimated to be only a day. Proestrus phase was not selected in view that the level of $E_2$ is still inclining and have yet to reach the peak. In the meantime, P secretion becomes high during metestrus and diestrus and decreases afterwards [49].

Following completion of sex-steroid treatment and identification of estrous cycle phases, rats were euthanized by anesthetic overdosage using ketamine: xylazine (100 mg/kg: 10 mg/kg) anaesthesia that was administered intraperitoneally. This was then followed by cervical dislocation. The mid-portion of uteri (whole) were removed for protein expression analyses by Western blotting and protein distribution analysis by immunohistochemistry (IHC). Blood was withdrawn via direct heart puncture immediately following sacrifice for determination of plasma sex-steroid levels.

### 2.2. Measurement of plasma sex-steroid levels by enzyme-linked immunosorbent assay (ELISA)

Blood, once collected into the plain tubes was left for 30 min and allowed to clot at room temperature. In order to prepare the plasma, blood was centrifuged at 2500 × *g* for 15 min. Then, plasma (clear fluid) was aliquot and stored at −20 °C for measurement of sex-steroid levels by using enzyme-linked immunoassay (ELISA) kit. ELISA procedures follow the guidelines of the manufacturer(Cayman Chemical-USA, Estradiol ELISA kit-582251 and Progesterone ELISA kit-582601). The kits can measure $E_2$ and P levels between the range from 6.6 to