

Evaluation of transcriptional activity of the oestrogen receptor with sodium iodide symporter as an imaging reporter gene

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Background Oestrogen receptors are ligand-dependent transcription factors whose activity is modulated either by oestrogens or by an alternative signalling pathway. Oestrogen receptors interact via a specific DNA-binding domain, the oestrogen responsive element (ERE), in the promoter region of sensitive genes. This binding leads to an initiation of gene expression and hormonal effects.

Objective To determine the transcriptional activity of the oestrogen receptor, we developed a molecular imaging system using sodium iodide symporter (NIS) as a reporter gene.

Methods The NIS reporter gene was placed under the control of an artificial ERE derived from pERE-TA-SEAP and named as pERE-NIS. pERE-NIS was transferred to MCF-7, human breast cancer cells, which highly expressed oestrogen receptor- α with lipofectamine. Stably expressing cells were generated by selection with G418 for 14 days. After treatment of 17 β -oestradiol and tamoxifen with serial doses, the ¹²⁵I uptake was measured for the determination of NIS expression. The inhibition of NIS activity was performed with 50 $\mu\text{mol}\cdot\text{l}^{-1}$ potassium perchlorate.

Results The MCF7/pERE-NIS treated with 17 β -oestradiol accumulated ¹²⁵I up to 70–80% higher than did non-treated cells. NIS expression was increased according to increasing doses of 17 β -oestradiol. MCF7/pERE-NIS

treated with tamoxifen also accumulated ¹²⁵I up to 50% higher than did non-treated cells. Potassium perchlorate completely inhibited ¹²⁵I uptake. When MDA-MB231 cells, the oestrogen receptor-negative breast cancer cells, were transfected with pERE-NIS, ¹²⁵I uptake of MDA-MB-231/pERE-NIS did not increase.

Conclusion This pERE-NIS reporter system is sufficiently sensitive for monitoring transcriptional activity of the oestrogen receptor. Therefore, cis-enhancer reporter systems with ERE will be applicable to the development of a novel selective oestrogen receptor modulator with low toxicity and high efficacy. *Nucl Med Commun* 27:773–777 © 2006 Lippincott Williams & Wilkins.

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Introduction

Oestrogen exerts profound effects on growth, differentiation and function of cardiovascular tissues and the skeletal system as well as reproductive tissues, including the genital tract and mammary gland. The highest amounts of the oestrogen receptor are found in reproductive organs such as mammary gland, ovaries, vagina, and the uterus [1,2]. Oestrogen–oestrogen receptor binding activates the receptor. With activation, the receptor homodimerizes and binds oestrogen receptor elements in the promoter region of specific genes, thereby starting the process of target gene transcription [3,4]. The co-activators and co-repressors are involved in initiation and regulation of gene transcription by the oestrogen receptor [5]. Such co-activators are believed to determine the agonistic and antagonistic properties of

compounds and to be responsible for tissue-specific action of selective oestrogen receptor modulator (SERM)-like tamoxifen or raloxifene [6].

The thyroid gland concentrates iodide by a factor of 20–40 compared to its plasma level [7]. Thyroid follicular cells transport iodide through a specific transporter, the sodium iodide symporter (NIS), the gene of which was identified in 1996 [8]. NIS is an intrinsic membrane protein with 13 transmembrane domains [9]. Because of uptake characteristics of ^{99m}Tc as well as radioiodine by NIS expression, NIS is already proposed to imaging reporter gene [10,11]. There are many advantages of NIS as an imaging reporter gene because of the wide availability of its substrates such as radioiodine and ^{99m}Tc and the well-understood metabolism and clearance

of these substrates in the body. The complicated radiolabelling was not required compared to PET reporter genes, including *HSV1-tk* and the dopamine D₂ receptor [12]. A cis-enhancer reporter system has been used to monitor transcriptional activity of endogenous gene expression, intracellular signal transduction and nuclear receptor [11,13–15].

In this study, we developed an evaluation system for transcriptional activity of the oestrogen receptor using a cis-enhancer reporter method and sodium iodide symporter (NIS) as a reporter gene.

Materials and methods

Construction of the reporter gene, and chemicals

The hNIS gene containing FL*-hNIS/pcDNA3 was provided by Dr S. Jhiang (Ohio State University, Columbus, USA) and pERE-TA-SEAP, including artificial oestrogen responsive element (ERE) was purchased from Clontech (Palo Alto, California, USA). Cytomegalovirus promoter fragment from FL*-hNIS/pcDNA3 was removed by treatment with NruI and HindIII (New England Biolabs, Beverly, Massachusetts, USA). The ERE sequence from pERE-TA-SEAP was amplified by using the polymerase chain reaction (PCR) technique with the PCR primers ERE1-NruI(A) GTGTTTCGCGACGGGAGGTA CTTGGAGCG and ERE1-HindIII(AS) CCGAAGCTTC CATTATATACCCAGATCTAG. The amplified ERE sequence was inserted into NruI and HindIII digested FL*-hNIS/pcDNA3 and this transgene was named as pERE-NIS.

17 β -Oestradiol and tamoxifen were purchased from Sigma Chemical Company (St. Louis, Missouri, USA) and dissolved in DMSO. These were stored at 20°C and protected from light. All chemicals, buffers and solvents were of analytical grade, and unless otherwise stated, were purchased from Sigma. ¹²⁵I in the form of sodium iodide was purchased from NEN (Boston, Massachusetts, USA).

Cell culture and transfections

Human breast cancer cells MCF-7 and MDA-MB-231, which are oestrogen receptor-positive and oestrogen receptor-negative cell, respectively, were obtained from the Korea Cell Line Bank, and maintained as recommended. These cells were grown as a monolayer in RPMI 1640 medium (JBI, Daegu, Korea) supplemented with 292 mg·ml⁻¹ glutamine, 100 000 IU·l⁻¹ penicillin (GIBCO, Grand Island, New York, USA), 100 mg·l⁻¹ streptomycin (GIBCO), and 10% fetal bovine serum (FBS). Plasmid was transfected into cells using lipofectamine plus reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Stable transfectants were selected with 600 μ g·ml⁻¹ of Geneticin (Invitrogen) in RPMI 1640 containing 10% fetal bovine serum for 3 weeks.

Radioiodine uptake assay

Cells were placed in 24-well plates and cultured with RPMI 1640 containing 10% FBS. When the cells reached confluence, they were treated with several doses of 17 β -oestradiol or tamoxifen for 18 h. After the cells had been incubated with the drugs, ¹²⁵I uptake levels were determined. Iodide uptake assays were performed using a modification of the method described by Nakamoto *et al.* [16]. Cells were incubated at 37°C for 30 min in 500 μ l of Hanks balanced salt solution (HBSS) containing 0.5% bovine serum albumin and 10 mmol·l⁻¹ of the sodium salt of 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, pH 7.4, with 18.5 kBq (0.5 μ Ci) carrier-free Na¹²⁵I and 10 μ mol·l⁻¹ of NaI, to yield a specific activity of 740 MBq·mmol⁻¹ (20 mCi·mmol⁻¹). After incubation, the cells were then quickly washed with 3 ml of ice-cold HBSS. The cells were detached with 0.05% SDS and the radioactivity was measured with a gamma counter (Cobra II, Canberra Packard, USA). Unless otherwise stated, the iodide uptake was expressed as pmol per 10⁶ cells. In order to modulate the iodide uptake, the cells were incubated for 30 min in either Na¹²⁵I medium or Na¹²⁵I medium supplemented with 50 μ mol·l⁻¹ potassium perchlorate [17]. All data points were measured in triplicate, and are displayed as means \pm SEM.

Results

Oestrogen receptor activity as a transcription factor is presented by binding with oestrogen or SERMs. To determine the oestrogen receptor activation by oestrogen or SERM binding using a cis-enhancer reporter system, pERE-NIS in breast cancer cells, we used two cell lines, MDA-MB231 and MCF-7. Because MDA-MB231 cells are oestrogen receptor-negative, iodide uptake of pERE-NIS transfected MDA-MB231 treated with 17 β -oestradiol or tamoxifen was not increased (Fig. 1). However, when MDA-MB231 cells were transfected with pCMV-NIS, iodide uptake of the transfected cells was enhanced and normal functionality of NIS was shown. The iodide uptake of pERE-NIS transfected MCF-7 cells (which express the oestrogen receptor) treated with 17 β -oestradiol was increased by up to two-fold than that of non-treated cells (Fig. 2(A)). Iodide uptake was completely inhibited by 50 μ mol·l⁻¹ of potassium perchlorate treatment, a specific inhibitor of NIS. Tamoxifen treatment of pERE-NIS-transfected MCF-7 cells also induced iodide uptake in the same manner as 17 β -oestradiol (Fig. 2(B)). When cells were incubated with increasing concentration of reagents, NIS activity also was increased in a dose dependent manner (Table 1).

After MCF-7 cells had been transfected with pERE-NIS, four independent clones of the stable cell line were selected with treatment of G418 for 14 days. MCF7/pERE-NIS treated with 17 β -oestradiol also accumulated

radioiodine up to 50% higher than did non-treated cells (Fig. 3).

Discussion

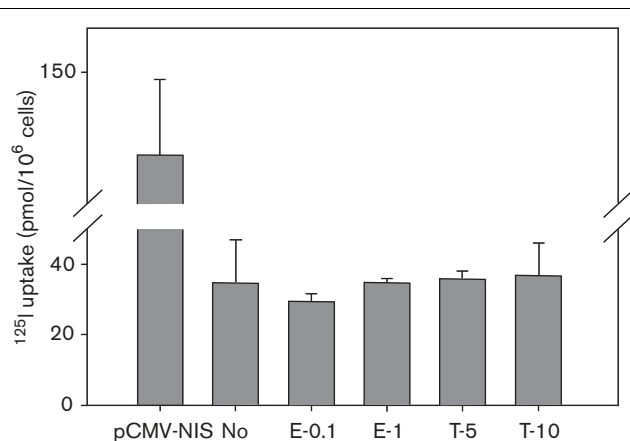
Oestrogen has crucial roles in the proliferation of cancer cells in reproductive organs such as breast and uterus [18,19] and oestrogen-stimulated growth of cells is mediated by the oestrogen receptor. Approximately 70% of human breast tumours express higher amounts of the oestrogen receptor than does normal breast tissue, and oestrogen receptor expression reflects the prognosis of disease. Lower oestrogen receptor expression in breast

tumours often reveals a more aggressive phenotype [20]. Hormone therapy with tamoxifen, a selective oestrogen receptor modulator, is recommended for oestrogen receptor-positive breast cancer in the clinic [21,22]. Therefore, measurement of oestrogen receptor status in breast cancer tissue is important when deciding upon a therapeutic strategy for patients with breast cancer.

There have been several methods for demonstrating the presence and function of the oestrogen receptor in tissues. *In vitro* oestrogen receptor assays such as immunohistochemistry and radioreceptor assays have been used clinically to measure the existence of the oestrogen receptor, but the techniques require tissue that is of limited availability. PET images can be used to assess the *in vivo* functional integrity of the oestrogen receptor using 16α - ^{18}F fluoroestradiol at sites of primary breast carcinoma as well as distant metastases [23]. A molecular imaging method using the cis-enhancer reporter system developed in this study can demonstrate the transcriptional activity of a nuclear oestrogen receptor *in vivo*. This approach to molecular imaging can be used to evaluate the early stages of the oestrogen receptor and to develop new SERMs.

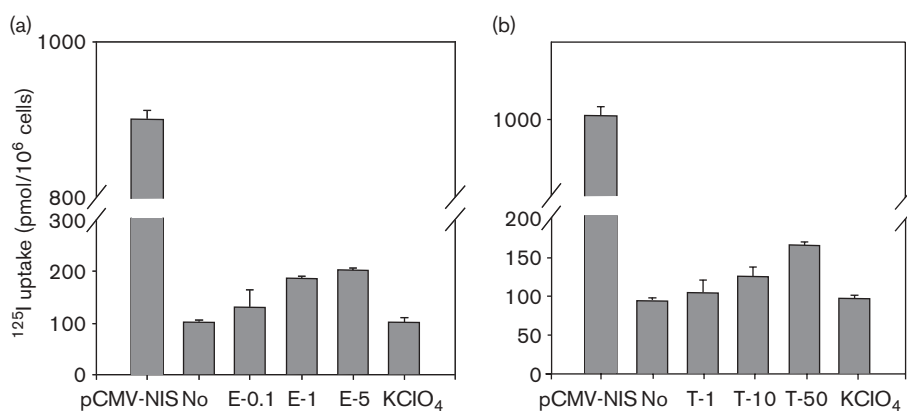
In this study, we developed a cis-enhancer reporter system for monitoring the transcription activity of the oestrogen receptor by determining 17β -oestradiol or tamoxifen binding to the receptor using NIS as a reporter gene. We constructed a reporter gene in which the NIS expression was regulated under the control of enhancer regulatory elements that are responsive to 17β -oestradiol binding. It has been reported that the cis-enhancer reporter system was applied to monitoring of endogenous gene expression and nuclear receptor activity such as retinoic acid [11,13–15].

Fig. 1



^{125}I uptake in pERE-NIS-transfected MDA-MB231 cells treated with either oestradiol or tamoxifen. ^{125}I uptake by these cells was not increased. pCMV-NIS: NIS gene expression controlled by the CMV promoter; No: DMSO only; E-0.1: $0.1 \text{ nmol}\cdot\text{l}^{-1}$ oestradiol; E-1: $1 \text{ nmol}\cdot\text{l}^{-1}$ oestradiol; T-1: $1 \text{ nmol}\cdot\text{l}^{-1}$ tamoxifen; T-10: $10 \text{ nmol}\cdot\text{l}^{-1}$ tamoxifen.

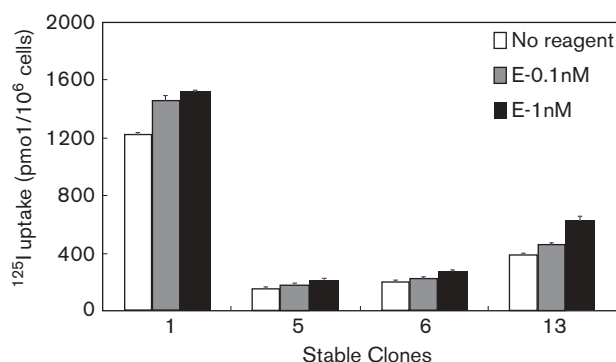
Fig. 2



^{125}I uptake in pERE-NIS transfected MCF-7 cells treated with either oestradiol (a) or tamoxifen (b). ^{125}I uptake into these cells was increased by up to 70–80% compared to that in untreated cells. ^{125}I uptake was completely inhibited by potassium perchlorate treatment. pCMV-NIS: NIS gene expression is controlled by CMV promoter; No: DMSO only; E-0.1: $0.1 \text{ nmol}\cdot\text{l}^{-1}$ oestradiol; E-1: $1 \text{ nmol}\cdot\text{l}^{-1}$ oestradiol; E-5: $5 \text{ nmol}\cdot\text{l}^{-1}$ oestradiol; T-1: $1 \text{ nmol}\cdot\text{l}^{-1}$ tamoxifen; T-10: $10 \text{ nmol}\cdot\text{l}^{-1}$ tamoxifen; T-50: $50 \text{ nmol}\cdot\text{l}^{-1}$ tamoxifen; KClO₄: NIS inhibitor and $5 \text{ nmol}\cdot\text{l}^{-1}$ oestradiol or $50 \text{ nmol}\cdot\text{l}^{-1}$ tamoxifen-treated group.

Table 1 Sodium iodide symporter (NIS) activities in pERE-NIS transfected MCF-7 cells treated with oestradiol or tamoxifen

Conc (nmol·l ⁻¹)	Oestradiol-treated group					Tamoxifen-treated group				
	0	0.1	1	5	5+KClO ₄	0	1	10	50	50+KClO ₄
NIS activity (pmol per 10 ⁶ cells)	101.4±3.8	130.3±33.2	187.0±0.4	202.3±2.2	100.4±8.9	95.3±3.6	104.5±14.4	124.1±13.9	166.1±2.0	96.2±5.7
Activity ratio vs untreated group	1	1.29	1.84	2.00	1.1	1	1.10	1.30	1.74	1.00

Fig. 3

¹²⁵I uptake by MCF-7 cells stably expressing NIS under the control of the oestrogen responsive element (ERE). MCF-7/pERE-NIS cells treated with 17 β -oestradiol also accumulated up to 50% more ¹²⁵I than did untreated cells. Unfilled bars: no reagent; grey bars: 0.1 nmol·l⁻¹ oestradiol; filled bars: 1 nmol·l⁻¹ oestradiol.

When MCF-7 cells, human breast cancer cells that highly express oestrogen receptor- α , were transfected with pERE-NIS and treated with 17 β -oestradiol or tamoxifen, ¹²⁵I uptake was increased to a greater extent than in untreated cells. After the cells had been treated with serial doses of 17 β -oestradiol, the uptake of ¹²⁵I was enhanced according to increasing concentrations. Although NIS activity was not determined in this study, it should be mediate the change of ¹²⁵I uptake in the cells. NIS activity of MCF7/pERE was completely blocked with potassium perchlorate, an NIS specific inhibitor. MDA-MB231 cells are oestrogen receptor negative, and ¹²⁵I uptake of pERE-NIS transfected MDA-MB231 did not increase in spite of treatment of 17 β -oestradiol.

Although the pERE-NIS reporter system is sufficiently sensitive for monitoring oestrogen receptor activity *in vitro*, NIS expression imaging with tumour-bearing mice by a gamma camera was scarcely being acquired in this study (data not shown). We suspected there was little discrepancy in NIS activity between drug-treated and untreated samples *in vivo*. To acquire a nuclear medicine image using a gamma camera, it is necessary to augment the transcriptional activity of the oestrogen responsive element. For example, a two-step transcriptional ampli-

fication (TSTA) system could be applied to enhance the imaging signal. In TSTA, expression of a potent transcriptional activator such as GAL4-VP16 is controlled by weak promoter or enhancer. The expressed activator binds to several GAL4 binding sites which drive the expression of reporter or therapeutic gene [24,25]. The activity of prostate-specific antigen promoter and carcinoembryonic antigen promoter could be enhanced by the TSTA system [24,26].

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