Up-regulation of the G3PDH 'housekeeping' gene by estrogen

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Abstract. Proteomic and genomic studies commonly involve the assessment of mRNA levels using reverse transcription-polymerase chain reaction (PCR) and real-time quantitative PCR. An internal standard RNA is fundamentally analyzed along with the investigated mRNA to document the specificity of the effect(s) on mRNA and to correct for inter-sample variations. In our studies implementing estrogen treatments on different cell lines, we initially used glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as an internal standard. However, the results of PCR amplification demonstrated that 17β-estradiol enhanced the expression of the G3PDH gene, rendering it impossible to use G3PDH as an unbiased comparative control.

Introduction

For monitoring changes in mRNA expression levels, Northern blot analysis, ribonuclease protection assays, reverse transcription-polymerase chain reaction (PCR) and real-time quantitative PCR (RT-PCR, e.g., Taq Man assay) are among the most common tools in molecular biology studies. In order to assess changes in the expression levels of the investigated mRNA(s), they must be compared to at least one additional cellular RNA, commonly referred to as 'internal standards' (housekeeping genes). To be a valid internal standard, the selected RNA must be expressed at a constant level across all samples (stable) and must also be unaffected by the investigated experimental conditions or treatments (1,2). Hence, internal standards can validate the specificity of effects on mRNA and can correct for inter-sample variation. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is one of the commonly used internal RNA standards.

Our studies involved *in vitro* estrogen treatments (3,4). In such treatments, estrogen (steroid hormone) mainly functions through cellular receptor proteins. The hormone/receptor complexes function as transcription factors which can up-regulate or down-regulate the expression of a particular gene (5,6). However, there is evidence to suggest that steroid hormones have non-conventional mechanisms of action in different types of tissue. One of the characteristics of their so-called 'non-genomic' actions is the binding of steroid hormones to membrane-bound proteins or the induction of rapid changes in plasma membrane properties (7,8). Steroid hormones interact with several enzymes that do not function as direct regulators of gene expression (9). These early studies were considered to present a potential model of the mechanism of steroid action. A stir up effort in this direction re-evolved, demonstrating the ability of estrogen to increase the activity of 17-aminoacyl-t RNA synthetase in rat uterus (10), to auto-phosphorylate tyrosine kinase (11) and to activate adenylate cyclase (12). Moreover, the activity of neuronal nitric oxide synthase was shown to be affected by estradiol in a biphasic manner, whereby nanomolar concentrations activated the enzyme while micromolar concentrations attenuated its activity (13). Subsequent reports indicated beyond reasonable doubt that estrogens use membrane-initiated events to activate cellular mechanisms, in addition to the classical genomic pathway (14-16).

Materials and methods

Cell culture. The *in vitro* effects of estrogen on different gene expression levels were investigated in RAW 264.7, ATDC5 and HFLS cells. The RAW 264.7, HFLS and ATDC5 cells were first cultured in complete medium α-MEM supplemented with 10% fetal calf serum, synoviocyte growth medium (Cell Application Inc., San Diego, CA, USA) and DMEM:Ham F12=1:1 + 5% FBS (Sigma-Aldrich, St. Louis, MO, USA), respectively. Cells were incubated in humidified 5% CO_2 atmosphere at 37˚C.

Exogenous 17β-estradiol treatment. At confluence, 10^{-11} M of 17β-estradiol (Sigma-Aldrich) was added to the cultures for periods of 1, 3, 6, 12 and 24 h, while controls were treated with

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Table I. Sequences of the primers used.

Primer		Sequence
G3PDH	F	GTCTACATGTTCCAGTATGACTCC
	R	AGCCTTCTCCATGGTGGTGAAGAC
L19	F	CTGAAGGTGAAGGGGAATGTG
	R	GGATAAAGTCTTGATGATCTC

an equivalent volume of ethanol (estrogen vehicle). In RAW cells, three different concentrations of 17β-estradiol were applied (10^{-11}, 10^{-8} and 10^{-5} M).

Reverse transcription-polymerase chain reaction. Total RNA was isolated using guanidinium thiocyanate-phenol-chloroform extraction (Trizol; Invitrogen Corp., Carlsbad, CA, USA). The RNA pellets were dissolved in distilled water and quantified using NanoDrop spectrophotometer (ND 1000; Thermo Scientific, Wilmington, DE, USA). Total RNA was reverse transcribed using oligo (dT) primers and reverse transcriptase (Toyobo, Tokyo, Japan) according to the manufacturer's protocol.

Polymerase chain reaction amplification. PCR amplification was performed using KOD-Dash (Toyobo). Gene-specific primers for human G3PDH were used. Amplification was performed as follows: an initial cycle at 94˚C for 10 min; 25 cycles at 94˚C for 30 sec, with an annealing temperature of 60˚C for 2 sec and 72˚C for 30 sec; a final cycle at 72˚C for 10 min.

Gene-specific primers for L19 (ribosomal protein L19) were used (as an internal standard) to test cDNA quality and equalization. PCR was run for 30 cycles using Ampli Taq Gold DNA polymerase (Perkin Elmer, Boston, MA, USA). Amplification was performed as follows: an initial cycle at 95˚C for 11 min; 30 cycles at 95˚C for 1 min, with an annealing temperature of 58˚C and 72˚C for 1 min; a final cycle at 72˚C for 10 min. The sequences of the primers used are listed in Table I.

Following PCR, the reaction products were resolved on 2% agarose gels by electrophoresis. Gel was stained with ethidium bromide to visualize the PCR products.

Results and Discussion

PCR amplification for 25 cycles revealed that 17β-estradiol dynamically up-regulated the expression of G3PDH in three different cell lines with different specificity and function (Fig. 1). A time-dependent increase in expression was observed starting 1 h following treatment. In RAW cells treated with different concentrations of 17β-estradiol (10^{-11}, 10^{-8} and 10^{-5} M), a decrease in the expression of G3PDH was observed with increasing concentrations of 17β-estradiol (Fig. 2). Concordant with our previously reported optimal estrogen concentration (10^{-11} M), beyond which a decrease in the expression of ERβ, RANK and c-fms was documented (3,4), similar effects on G3PDH were demonstrated within the same cells.

In this study, we investigated the G3PDH 'housekeeping' gene for use as an internal control to normalize real-time PCR data in experimental treatments with estrogen. This

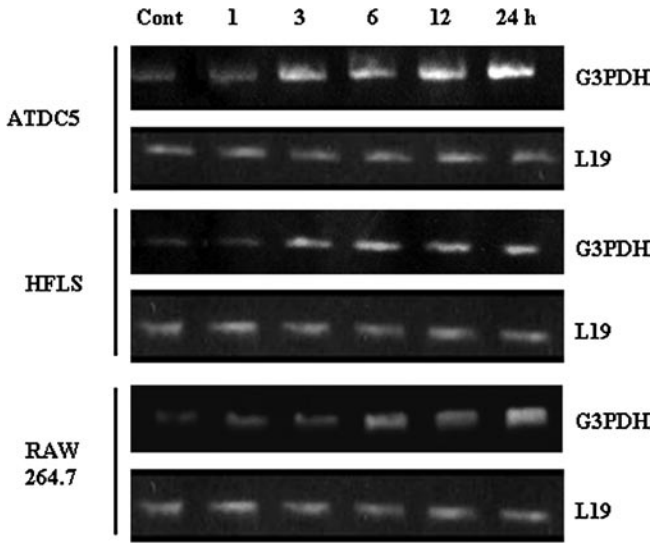

Figure 1. The effect of 17β-estradiol on G3PDH mRNA expression in RAW 264.7, HFLS and ATDC5 cells. Cells were incubated with 10^{-11} M 17β-estradiol for periods of 1, 3, 6, 12 and 24 h. RNA was isolated and RT-PCR was performed. There was a gradual increase in the expression of G3PDH following the addition of estrogen. Cont, control.

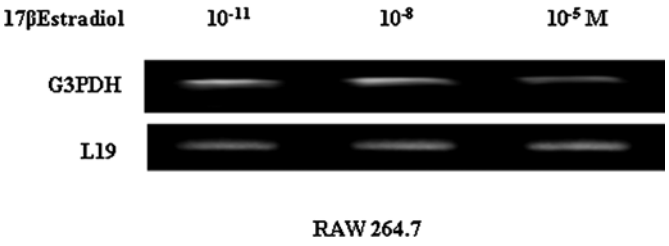

Figure 2. Effect of different concentrations of estrogen on G3PDH. In RAW 264.7 cells treated with different concentrations of 17β-estradiol (10^{-11}, 10^{-8} and 10^{-5} M), a decrease in the expression of G3PDH was observed with increasing concentrations of 17β-estradiol.

'housekeeping' gene has previously been validated for use as an internal control in many experimental systems, but has not been validated for work with estrogen.

A wide range of 'housekeeping' genes have been used as internal controls in expression profiling following exposure to estrogens, including β-actin (17), 16S rRNA (18) and ribosomal proteins (including S2, S3, S8, S15, S27, L4, L5, L8, L13, L21, and L28) (19). However, very few of these studies provided any validation of these genes for use as internal controls prior to their application.

The effect of estrogen on G3PDH expression observed here likely results from its involvement in metabolism, since estrogens, like other sex steroids, are well known to play role in the regulation of the metabolic processes associated with altered energy demands during gonad development and reproduction (20). Moreover, several other metabolic enzymes are known to be controlled by sex steroids, including estrogens (21).

We restricted our analysis to the G3PDH gene due to its widespread use as internal RNA standard. Although the mechanisms by which the estrogen hormone modifies G3PDH expression are as yet unclear, this unexpected finding demonstrated that estradiol within physiological concentrations

regulates the expression of G3PDH and, therefore, controls cell metabolism under specific physiological conditions, for example during proestrus (high level of estrogen) or pregnancy (high level of progesterone).

Our results show that the expression level of one of the most commonly used 'housekeeping' genes is, in fact, regulated by estrogen treatment. We therefore recommend against the use of G3PDH as internal control in real-time PCR studies using estrogen-exposed cells. Given that the data indicate that choosing an inappropriate 'housekeeping' gene as an internal control to normalize expression data for a gene of interest can potentially lead to inaccurate conclusions on estrogen effect pathways, we strongly recommend that the most appropriate 'housekeeping' gene for use as an internal control is first established for the specifics of each estrogen experiment. This selection should take into account the fact that estrogen-regulation of 'housekeeping' genes may vary according to factors such as chemical dose and exposure length, in addition to gender and tissue type.

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