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ABSTRACT

Gender Differences in Pharmacokinetics of Anesthetics

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The gender aspect in pharmacokinetics and pharmacodynamics of anesthetics has attracted little attention. Knowledge of previous work is required to decide if gender-based differences in clinical is justified. Females have 20-30% greater sensitivity to the muscle relaxant effects of vecuronium and rocuronium. When rapid onset of or short duration of action is very important, gender-modified dosing may be considered. Males are more sensitive than females to propofol. It may therefore be necessary to decrease the propofol dose by 30-40% in males compared with females in order to achieve similar recovery times. Females are more sensitive than males to opioid receptor agonists, as shown for morphine as well as for pentazocin. On the other hand, females may experience respiratory depression and other adverse effects more easily if they are given the same doses as males. These examples illustrate that gender should be taken into account as a factor that may be predictive for the dosage of several anesthetic drugs. Moreover, there is an obvious need for more research in this area in order to further optimize drug treatment in anesthesia.

key words：pharmacokinetics, drug metabolism, gender differences, anesthetics

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Letter

Functional analysis of transcriptional activity of cytosine and adenine (CA) repeats polymorphism in the estrogen receptor β gene

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ABSTRACT — Cytosine and adenine (CA) repeats polymorphism (D14S1026) iterating "cytosine and adenine" nucleotide motifs is one of the genomic microsatellites in intron 5 of the estrogen receptor β (ER β) gene (14q22-24). Relations between CA repeats polymorphism and several diseases have been shown. Although the relation between number of CA repeats and gene transcription has been actively studied using several genes, results have remained contradictory until this time. In this study, we examined the functional effects of CA repeats polymorphism on transcriptional activity based on our knowledge of the ER β gene. After preparing four types of reporter gene constructs containing 15, 18, 24 or 27 CA repeats, luciferase reporter gene assays were performed. Relative luciferase activities of these constructs were not significantly different from that of the no inserted vector and variation of CA repeats did not affect these activities. Our results indicate that CA repeats polymorphism might not only affect transcriptional activity but also other processes of gene expressions. Further studies are needed to clarify the specific functions of CA repeats polymorphism in the ER β gene.

Key words: Estrogen receptor β, Polymorphism, Microsatellite, Transcription

INTRODUCTION

Estrogen plays important roles in many biological actions mediated by estrogen receptors α (ER α) and β (ER β), both members of the nuclear receptor superfamily (Gustafsson, 2003; Heldring *et al.*, 2007). In ER genes (ER α gene, 6q25.1; ER β gene, 14q22-24), various polymorphisms exist and have been studied regarding their relation with several diseases such as osteoporosis, osteoarthritis, breast cancer and Alzheimer's disease (Anghel *et al.*, 2006; Forsell *et al.*, 2001; Fytili *et al.*, 2005; Gennari *et al.*, 2005; Riancho *et al.*, 2006).

Cytosine and adenine (CA) repeats polymorphism with iteration of "cytosine and adenine" nucleotide motifs is one of the genomic microsatellites, and so far, it has been detected in the genomes of every organism as well as in intron 5 of the human ER β gene (D14S1026) (Tautz and Renz, 1984; Li *et al.*, 2002; Gennari *et al.*, 2005). We previously identified a correlation between this polymorphism, the symptoms and medications of climacteric disorder patients (Takeo *et al.*, 2005; Negishi *et al.*, 2006). Recently, the functional significance of such polymorphic microsatellites has been proven in various biological phenomena as well as the traditional consideration of evolutionary neutral DNA markers (Comings, 1998; Li *et al.*, 2002). Especially, the relation between number of CA repeats and gene transcription has been actively studied using several genes; however, results have remained contradictory up to this time (Agarwal *et al.*, 2000; Buerger *et al.*, 2004; Gebhardt *et al.*, 1999; Saha *et al.*, 2005; Tadokoro *et al.*, 2004; Gonzalez *et al.*, 2007). Additionally, there is no work on this polymorphism in the ER β gene until this time. In this study, we examined the functional effects of CA repeats polymorphism on transcriptional activity in the ER β gene except for promoter regions.

MATERIALS AND METHODS

Subjects

Genomic DNA samples were obtained from blood of healthy volunteers (11 males and 40 females). Precautions

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were taken to ensure that all genetic information used in this study remained confidential. This study was conducted with the approval of the local Ethics Committee of the Graduate School of Pharmaceutical Sciences, Chiba University and in accordance with the ethical and scientific standards embodied in the World Medical Association Declaration of Helsinki.

Analysis of CA repeats polymorphism

Genomic DNA was extracted from human peripheral blood leukocytes with the QIAamp DNA Mini Kit (QIAGEN, Inc., Hilden, Germany) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was performed in a total volume of 75 μl reaction mixture with the following components: 150 ng of human genomic DNA, oligonucleotide primers designed to amplify polymorphic CA repeats in intron 5 of the human ER β gene (forward: 5'-CAA TTC CCA ATT CTA AGC CT-3' and reverse: 5'-TGC CTG GCC TAA AGA AGA AT-3') at 0.4 μM, dNTP Mixture (TaKaRa Bio, Inc., Otsu, Japan) at 200 μM, 7.5 μl of 10× Reaction Buffer (containing 15 mM $MgSO_4$) (Transgenomic, Inc., Omaha, USA) and 2.5 U of Optimase Polymerase (Transgenomic, Inc.), to a total volume of 75 μl by adding MilliQ water. The amplification profiles were as follows: 35 cycles denaturing at 94 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 72 °C for 30 sec. These products, purified with the QIAquick PCR Purification Kit (QIAGEN), were used in the following analysis.

Analysis of CA repeats polymorphisms was conducted by dye-terminator cycle sequencing analysis using the Dye Terminator Cycle Sequencing-Quick Start Kit (Beckman Coulter, Inc. Fullerton, USA) and CEQ2000 DNA Analysis System (Beckman Coulter, Inc.) according to the manufacturers' protocols.

Reporter gene constructs

The CA repeats region in intron 5 of the ER β gene was amplified by PCR from human genomic DNA using the primers with the *Kpn* I site (forward: 5'-ACT GGG TAC-CCA ATT CCC AAT TCT AAG CCT-3' and reverse: 5'-TCA GGG TAC CTG CCT GGC CTA AAG AAG AAT-3'). Following PCR analysis under the same condition of the CA repeats analysis, each PCR product was digested with *Kpn* I (TaKaRa Bio, Inc.) and inserted into the corresponding site of the pGL-3 promoter vector encoding firefly luciferase (Promega Corp., Madison, USA). All constructs were purified using the PureYield Plasmid Midiprep System (Promega, Corp.) according to manufacturer's protocol. Nucleotide sequences were confirmed according to the CEQ2000 Dye terminator sequencing protocol (Beckman Coulter, Inc.).

Transient transfection and luciferase assay

HeLa cells (obtained from Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) were cultured at 37°C in DMEM with 5% fetal bovine serum as a normal growth medium under humidified atmosphere containing 5% CO_2. HeLa cells were cultured in 12-well plates (1.6×10^5/well) for 24 hr without antibiotics so that they were 90-95% confluent before transfection.

Transfection mixtures containing Lipofectamine 2000 (Invitrogen Corp., Carlsbad, USA), 1.57 μg of each reporter gene construct and 0.03 μg of the pRL-SV40 vector encoding *Renilla* luciferase (Promega, Corp.) were incubated for 20 min at room temperature. Following medium change to OPTI-MEM (Invitrogen Corp.), the mixtures were added directly to each well and the cells were incubated for 4 hr at 37°C under humidified atmosphere containing 5% CO_2. After transfection, the cells were cultured for 24 hr in normal growth medium.

Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Corp.), with a TD-20/20 luminometer (Turner Designs Inc., Sunnyvale, USA), and firefly luciferase activity of the reporter gene was normalized by *Renilla* luciferase activity. These experiments were performed independently four times, each in triplicate or quadruplicate. In the statistical analysis, each vector construct was compared with the no inserted vector using Dunnett's test.

RESULTS

Fig. 1A shows the schemes of the various reporter gene constructs containing CA repeats. Recent studies indicate that the frequency distribution of CA repeats polymorphism in the ER β gene ranges from about 15 to 27 repeats (Anghel *et al.*, 2006; Forsell *et al.*, 2001; Fytili *et al.*, 2005; Gennari *et al.*, 2005; Riancho *et al.*, 2006). Based on this knowledge, four types of constructs were prepared containing 15, 18, 24, or 27 repeats.

Relative luciferase activities of these constructs were not significantly different from that of the no inserted vector. Furthermore, variation of CA repeats did not affect these activities (Fig. 1B).

DISCUSSION

In this study, we analyzed the functional effects of CA repeats polymorphism in the ER β gene on transcriptional activity using luciferase reporter gene assay. However,

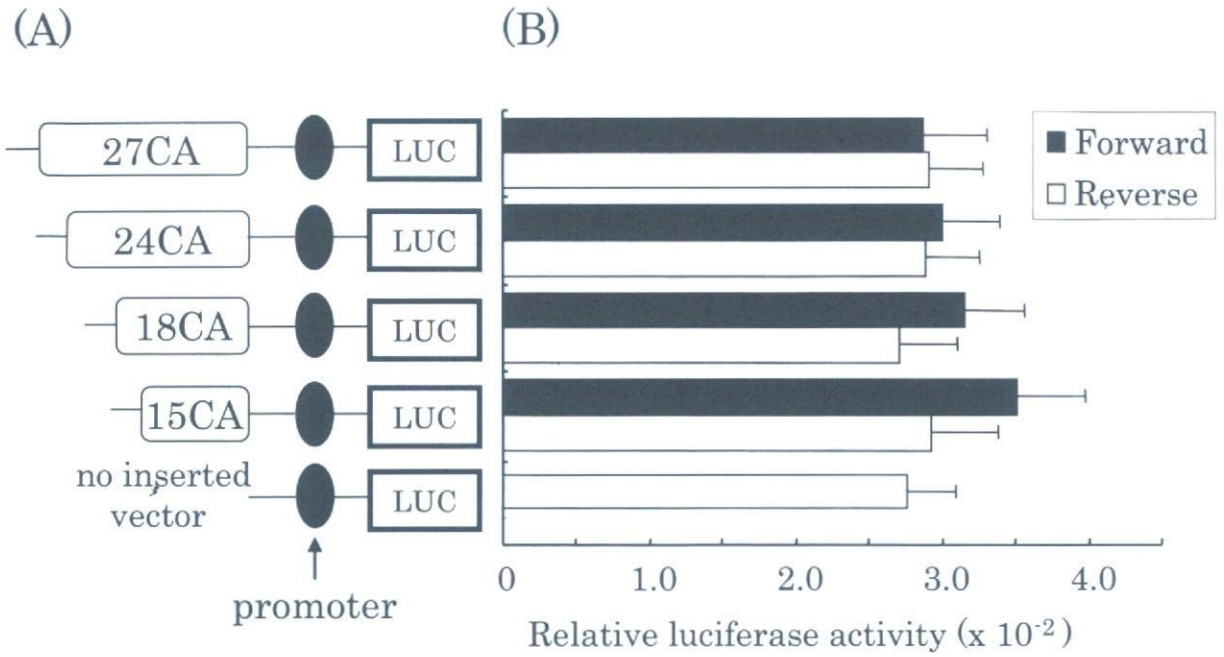

Fig. 1. Relative luciferase activities of each construct in transient transfectants.
(A) Scheme of various constructs containing CA repeats.
(B) Relative luciferase activity of each construct is shown as mean±S.E. of four independent experiments.

relative luciferase activities of these constructs were not significantly different from that of the no inserted vector. Furthermore, variation of CA repeats did not affect these activities.

We examined transcriptional activity using the pGL-3 promoter vector carrying the SV40 promoter in preference to the investigation of the promoter region of the ER β gene, considering that, to the best of our knowledge, the present study is the first approach to investigating CA repeats polymorphism in the ER β gene, and that there are many conflicting results between research studies of CA repeats polymorphism in several genes (Agarwal *et al.*, 2000; Buerger *et al.*, 2004; Gebhardt *et al.*, 1999; Saha *et al.*, 2005; Tadokoro *et al.*, 2004). In this respect, we examined the frequency distribution of CA repeats polymorphism in the ER β gene, which ranges from 15 to 27 repeats. After obtaining no significant results, the data indicate that there is less necessity for using the promoter region of the ER β gene.

Our results also indicate the different findings of other previous studies. In the study of CA repeats polymorphism in the neurotrophin-3 (NTF-3) gene by Tadokoro *et al.*, they used the same luciferase vector as ours and demonstrated that the insertion of the CA repeats region increased the relative luciferase activity in HeLa cells, while there was no difference in the activity between 21 and 23 repeats (Tadokoro *et al.*, 2004). We were not able to inspect the activity of these repeats because of the quantitative restriction in the sample. Therefore, the possibility remained that specific repeat number could affect transcriptional activity, not repeat length. However, it was mentioned that repeat length could be plausibly associated with gene expression rather than specific repeat number (Comings, 1998), and many functional studies of repeat length have actually been reported (Hui *et al.*, 2003; Li *et al.*, 2002). Therefore, the possibility mentioned above is not very much expected. Moreover, these contradictions regarding the relationship between repeat length and transcriptional activity were conceivably caused by differences in the details of the methods employed.

Meanwhile, it was reported that the abundance of $(TG/CA)_{n\geq 12}$ repeats and gene transcriptional levels show an inverse relationship, that is, highly transcribed genes were poorly populated with $(TG/CA)_{n\geq 12}$ repeats (Sharma *et al.*, 2007). These findings indicate that the whole quantity of CA repeats located in many areas of each gene is possibly more associated with gene expression. Taking this possibility into consideration, CA repeats polymorphism might affect not only transcriptional activity but also many other processes, such as regulation of gene activity (RNA splicing, translation, *etc.*), chromatin organization (chromosomal organization, DNA struc-

ture, *etc.*) and regulation of DNA metabolic processes (DNA replication, recombination, cell cycle, *etc.*) (Hui *et al.*, 2003; Hui *et al.*, 2005; Li *et al.*, 2002; Lorenz *et al.*, 2007). However, these mechanisms are very complicated (Comings, 1998; Li *et al.*, 2002).

In conclusion, our present results indicate that CA repeats polymorphism might affect not only transcriptional activity but other processes. Further studies are needed to elucidate the detailed functions of CA repeats polymorphism in the ER β gene.

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