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ORIGINAL ARTICLE

# A LARGE-SCALE GENE EXPRESSION SCREENING ON ANTI-INFLAMMATORY EFFECTS OF KETAMINE AND SEVOFLURANE IN RAT C6 GLIOMA CELLS

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**Abstract** In the present study, we have conducted cDNA microarray in C6, rat brain glioma cell line, to assess anti-inflammatory effects of ketamine and sevoflurane in the central nervous system. The cultured C6 cells were treated with ketamine (0-100 μM) and sevoflurane (0 and 0.66 mM). Total RNA was extracted from the cells and labeled with fluorescent dye and then hybridized with microarray slide, containing 1936 genes. Quantitative analysis of each gene expression was confirmed by real-time polymerase chain reaction (PCR). Microarray analyses showed that ketamine downregulated the expression of 4 proinflammatory cytokine genes and upregulated that of 2 anti-inflammatory cytokine genes. On the other hand, sevoflurane downregulated the expression of 2 proinflammatory cytokines but upregulated that of two other proinflammatory cytokines. Furthermore, sevoflurane failed to stimulate the expressions of anti-inflammatory cytokines. Although patterns of cytokine expression in response to ketamine and sevoflurane were different from each other described above, both anesthetics downregulated a key cytokine, interleukin (IL)-1β remarkably in microarray analysis, which was confirmed by real time PCR. These results suggest that both ketamine and sevoflurane show mainly anti-inflammatory properties through the inhibition of IL-1β.

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**Key words:** Ketamine; Sevoflurane; Anti-inflammatory properties; Brain cells.

原 著

# ラット C6 グリオーマ細胞におけるケタミンとセボフルランの抗炎症作用関連遺伝子のスクリーニング

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**抄録** 我々は中枢神経系におけるケタミンとセボフルランの抗炎症作用を評価するため C6，ラットグリオーマ細胞を用いて cDNA マイクロアレイを行った．培養された C6 細胞にケタミン（0-100 μM）及びセボフルラン（0，0.66 mM）処理をおこなった後，RNA を採取，その RNA をフルオレセンで染色，1936 個の遺伝子の乗ったマイクロアレイスライドにハブリダイゼイションさせ，遺伝子発現を観察した．個々の遺伝子発現の定量は更にリアルタイム PCR で確認した．マイクロアレイ解析の結果，ケタミンとセボフルランの抗炎症作用のメカニズムは個々の遺伝子をみると互いに異なっていることが判明した．しかし，両薬剤とも炎症において重要な作用を持つインターロイキン 1β を共通して抑制した．以上より，ケタミンとセボフルランの抗炎症作用のメカニズムは厳密には異なるが，大元のインターロイキン 1β を抑制することで作用することは共通であることが示唆された．

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**キーワード：**ケタミン；セボフルラン；抗炎症作用；脳細胞．

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## Introduction

There are presently 18 cytokines with the name interleukin (IL). Other cytokines have retained their original biological description, such as tumor necrosis factor (TNF). Some cytokines promote inflammation and are called proinflammatory cytokines, such as IL-1, TNF and chemokines, whereas other cytokines suppress the activity of proinflammatory cytokines and are called anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and transforming growth factor (TGF)-β[1].

The intravenous anesthetic ketamine has been recommended for the induction and maintenance for anesthesia and sedation in patients with septicemia and trauma[2]. Various studies have shown that in addition to its anesthetic activity, ketamine has anti-inflammatory effects both in vivo and in vitro [2–15]. In vitro studies demonstrated that ketamine suppressed TNFα, IL-6, IL-8, nuclear factor-κB (NF-κB), and nitric oxide in several types of immuonocompetent cells or cell lines including monocytes, macrophages, and neutrophils[3, 4, 7, 12, 15]. In various animal models, ketamine suppressed the production of TNFα and IL-6 after stimulation of lipopolysaccharide[2, 5, 13, 14]. In clinical studies, ketamine attenuated the systemic release of the proinflammatory cytokine IL-6 in humans during and following cardiopulmonary bypass[9].

On the other hand, volatile anesthetics also have significant nonanesthetic physiologic effects[16]. Volatile anesthetics protect against ischemia-reperfusion injury in the heart[17–25], lung[26, 27], liver[28] and kidney[16, 29]. Furthermore, the mechanism of organ protection by volatile anesthetics has been one of the main topics of anesthetic research. Several studies have suggested that volatile anesthetics protect the heart via activation of adenosine triphosphate-dependent potassium channels[20, 23, 24], adenosine receptors[17] and protein kinase C[17]. However, recent studies have suggested that volatile anesthetics protect the above organs by inhibiting the actions of proinflammatory cytokines[16, 18, 21, 22, 25-27, 29]. Particularly, sevoflurane also has direct anti-inflammatory effects in vivo and in vitro[16, 18, 21, 22, 25-30].

However, there hasn't been a large-scale gene expression survey (cDNA microarray) reported to assess the anti-inflammatory effects of ketamine and sevoflurane in the central nervous system (CNS).

The C6 glioma cell line is a widely used cell line in neurobiological research, and glial cells consisting of astrocytes and microglia are the major components mediating immune responses and inflammation in the CNS. Furthermore, the glial cells play roles in many pathological conditions, including neurodegenerative disease, stroke, traumatic brain injury, infectious diseases and pathological pain[11]. So the glioma model in rats has served as an excellent model of immune response and inflammation behavior with respect to therapeutic drug efficacy in the CNS.

In the present study, we have conducted a large-scale gene expression survey (cDNA microarray) in neural cell line to compare anti-inflammatory effects of ketamine with those of sevoflurane in the CNS.

## Materials and methods

### Cell line

C6, a rat brain glial cell line was obtained from Health Science Resources Bank (Tokyo, Japan). The cells were plated on 100-mm culture dishes and cultured in Hams F10 culture medium with 15% horse serum (ICN Flow 2070033) and 2.5% fetal bovine serum (ICN Flow 101083). The cells were grown in a humidified atmosphere of 95% air and 5% $CO_2$ at 37℃ . The cells were harvested from the dishes with Hams F10 medium with 0.02% EDTA for further passages.

## Ketamine and sevoflurane treatment

C6 cells were incubated for 30 min at 37℃ with Hams F10 medium containing various concentrations of ketamine (0-100 μM, Sigma Chemical Co., St. Louis, MO, USA) or sevoflurane (0 and 0.66 mM, Maruishi Pharmaceutical Co., Osaka, Japan). In the sevoflurane treatment group, sevoflurane was delivered at 5% to the culture medium by bubbling with 95% air and 5% $CO_2$ *via* a standard Sevotec5 variable bypass vaporizer (Datex-Ohmeda, Milwaukee, WI, USA) to reach a final concentration of 0.66 mM. Sevoflurane concentration in the medium was measured by gas chromatography (No. D-5500A, HITACHI Ltd., Tokyo, Japan). In the ketamine treatment group, microarray analyses were performed at 0 and 100 μM of ketamine concentrations. Then, real-time polymerase chain reaction (PCR) analyses were performed at various concentrations of ketamine (0, 3 μM, 10 μM, 30 μM). On the other hand, in the sevoflurane group, both microarray and real-time PCR analyses were performed at the same concentrations (0 and 0.66 mM).

## RNA isolation

Total RNA was extracted from each cell monolayer with an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA) according to the manufacture's protocol. Total RNA was treated with RNase free-DNase (Qiagen, Chatsworth, CA, USA) for eliminating genomic DNA contamination from RNA samples prior to cDNA microarray and real time-PCR.

## cDNA labeling and microarray hybridization

Reverse transcription, labeling and hybridization were performed with Qiagen LabelStar according to the manufacture's protocol. Briefly, total RNA was reverse-transcribed into target cDNA using LabelStar (Qiagen) with Cy3 and Cy5 for control and drug-treated cells, respectively. Labeled cDNAs were purified using MiniElute spin columns (Qiagen). Cy3-labeled and Cy5-labeled target cDNAs were combined, dried and resuspended in hybridization buffer. The target cDNA mixture was hybridized with rat ADME cDNA microarrays (Asahi Technoglass, Tokyo, Japan) which included 1936 cDNA elements. The microarrays were scanned in both the Cy3 and Cy5 channels with an Affymetrix 428 Array Scanner (Santa Clara, CA, USA), and then analyzed using the ImaGene and GeneSight-Lite Software packages (BioDiscovery Inc., El Segundo, CA, USA). After background subtraction and dye bias normalization, poor quality features were excluded from further analysis. Features with low signal intensity in the reference channel were filtered if the signal-to-noise ratio was less than 2.5. The change in expression of each gene was shown as the ratio (Cy5 fluorescence/Cy3 fluorescence) and/or the logarithm of the ratio. The ratio of normalized intensity $\geq 2$ or $\leq 0.5$ (or log ratio $\geq 0.3$ or $\leq -0.3$) was used to define genes with significant changes in expression.

## Real-time polymerase chain reaction (PCR)

Expression of each gene was quantified by SYBR Green-real time PCR with ABI PRISM 7000 (Applied Biosystems, Foster, CA). PCR primer sets used are shown in Table 1. The PCR cycling condition was 94 ℃ for 30 sec, 55 ℃ for 20 sec and 72℃ for 30 sec. Glyceraldehyde-3-phosphodehydrogenase was used as an internal standard. The analyses of the PCR results were conducted using the ΔCt value ($Ct_{target\ gene}$-$Ct_{G3PDH}$). Relative gene expression was obtained by ΔΔCt methods ($\Delta Ct_{sample}$-ΔCtcalibrator) for comparison of every unknown-sample gene expression level. The conversion between ΔΔCt and relative gene expression levels is fold induction = $-2^{-\Delta\Delta Ct}$.

**Table 1** Primers designed for real-time polymerase chain reaction (PCR)

| | |
|---|---|
| IL1-β forward | GATGGCTGCACTATTCCTAATGC |
| IL1-β reverse | AGACTGCCCATTCTCGACAAG |
| G3PDH forward | TGCCAAGTATGATGACATCAAGAAG |
| G3PDH reverse | AGCCCAGGATGCCCTTTAGT |

IL1-β : Interleukin 1-β, G3PDH: Glyceraldehyde-3-phosphodehydrogenase.

**Table 2** Ketamine and sevoflurane-induced change in expression of cytokine genes

| Gene name | Gene ID | cDNA microarray (Log mean ratio) | |
|---|---|---|---|
| | | Ketamine | Sevoflurane |
| Interleukin 1 alpha | 24493 | -1.06 | -0.12 |
| Interleukin 1 beta | 24494 | -0.81 | -1.16 |
| Interleukin 6 signal transducer | 25205 | -0.16 | -0.57 |
| Interleukin 7 | 25647 | -0.52 | 0.97 |
| Tumor necrosis factor superfamily, member 8 | 25069 | -0.79 | 0.52 |
| Latent transforming growth factor beta binding protein 1 | 59107 | 0.32 | 0.05 |
| Transforming growth factor beta stimulated clone 22 | 25564 | 0.54 | -0.09 |

Microarray analyses were performed at 0 and 100 μM of ketamine concentrations or at 0 and 0.66 mM of sevoflurane concentrations. The change in expression of each gene was shown as the ratio (drug-treated/control) and/or the logarithm of the ratio. The ratio of normalized intensity ≥ 2 or ≤ 0.5 (= log ratio ≥ 0.3 or ≤ -0.3) was used to define genes with significant changes in expression. Data are expressed as mean log ratio.
Gene ID = Gene ID in the GeneBank database; mean ratio = mean ratio of the signals of drugs treated samples to those of control samples in C6 cells (n = 2-4).

## Statistical analysis

Data are expressed as mean ± SD. Data obtained from microarray analyses and real-time PCR analyses were compared between groups by unpaired t-test or by Tukey-Kramer test. $P<0.05$ was defined as statistically significant. Statistical analysis was performed using KyPlot 4.0 (KyenceLab, Tokyo, Japan).

## Results

**cDNA microarray**

In the large-scale gene expression survey with cDNA microarray, we found that ketamine downregulated most of the 1936 genes expression in C6 cells represented on Rat ADME microarray (downregulated 829 genes; 43% , not change 650 genes; 33% , upregulated 232 genes; 12% of 1936). On the other hand, sevoflurane did not change most of the genes expression (downregulated 375 genes, 19%; not changed 1280 genes, 66%; upregulated 281 genes, 15% of 1936).

Table 2 summarizes gene expression related to proinflammatory and anti-inflammatory cytokines. Patterns of cytokine expression in response to ketamine and sevoflurane were notably different. Microarray analyses showed that ketamine downregulated the expression of 4 proinflammatory cytokine genes (IL-1α, IL-1β, IL-7, TNF superfamily, member 8), and upregulated that of 2 anti-inflammatory cytokine genes (Latent transforming growth factor beta binding protein 1, Transforming growth factor beta stimulated clone 22). On the other hand, sevoflurane downregulated expressions of 2 proinflammatory cytokines (IL-1β, Interleukin 6 signal transducer) and upregulated that of other 2 proinflammatory cytokines (IL-7, TNF superfamily, member 8). However, 2 anti-inflammatory cytokines (Latent transforming growth factor beta binding protein 1, Transforming growth factor beta stimulated

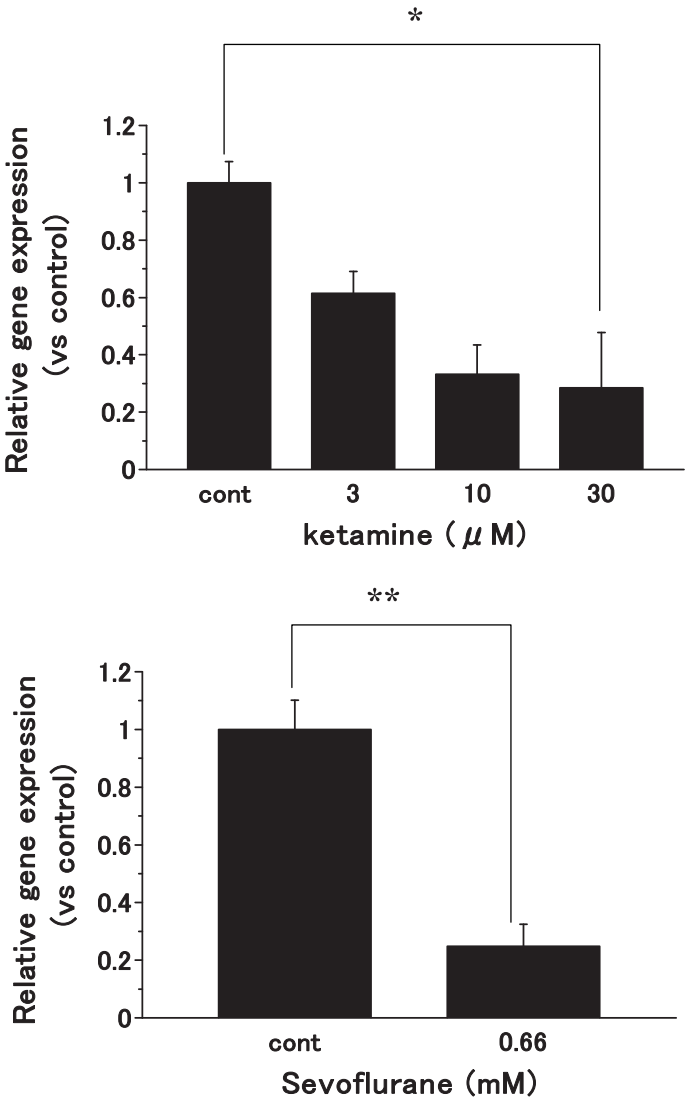


**Fig. 1** Downregulation of IL-1β expression induced by two anesthetics
Relative gene expression levels in C6 cells treated with ketamine (A) and sevoflurane (B) analyzed by real-time PCR. C6 cells were incubated for 30 min with medium containing ketamine (0-30 μM, n = 4) or sevoflurane (0-0.66 mM, n = 4). Each value is expressed as the ratio vs. control value. $^{**}P < 0.01$, $^{*}P < 0.05$ vs. the value of control.

clone 22) and proinflammatory cytokine, IL-1α, were not affected by sevoflurane. In these cytokines, IL-1β is a key cytokine that regulates inflammatory actions. Change of IL-1β expression in response to both ketamine and sevoflurane was the most marked in microarray analyses. Therefore, IL-1β gene was further subjected to real time PCR analysis.

## Interleukin-1β (IL1-β)

As shown in Table 2, microarray analyses revealed that both ketamine and sevoflurane significantly downregulated IL-1β expression in C6 cells (ketamine Log ratio of treated/control, -0.81, $p < 0.01$; sevoflurane Log ratio of treated/control, -1.16, $p < 0.01$). The inhibitory actions of ketamine and sevoflurane on the gene expression of IL-1β were confirmed by real-time PCR. Ketamine up to 30μM significantly and dose-dependently downregulated expression of IL-β in C6 cells (Fig. 1A). Sevoflurane also significantly downregulated it in C6 cells (Fig. 1B).

## Discussion

In the present study, we confirmed anti-inflammatory properties of ketamine and sevoflurane in neural cell line by a large-scale gene expression survey. Ketamine and sevoflurane showed a different detailed pattern of effects on the expression of proinflammatory and anti-inflammatory cytokines; however, both significantly suppressed the key inflammatory cytokine, IL-1β. These findings suggest that ketamine and sevoflurane show mainly anti-inflammatory properties through the inhibition of IL-1β.

The synergism of IL-1 and other cytokines have been reported[1]. Humans who were injected IL-1β experienced fever, headache, myalgias, and arthralgia. IL-1β and TNFα induce adhesion of leukocytes to the endothelial surface prior to emigration into the tissues. Besides, IL-1β and TNFα trigger the inflammatory cascade. Although inflammatory products such as endotoxins trigger it, the cytokine IL-1β and TNFα are particularly effective for stimulating the expression of proinflammatory genes. They also initiate the cascade of inflammatory mediators by targeting the endothelium. That is to say, an inhibition of IL-1β and TNFα results in a marked reduction of inflammatory actions.

In the present study, we showed ketamine and sevoflurane have mainly anti-inflammatory properties through the inhibition of IL-1β, but did not show the effects of these anesthetics on another key cytokine, TNFα. However, many studies have shown that ketamine and sevoflurane inhibit TNFα both in vivo and in vitro. In vitro studies, ketamine reduced lipopolysaccharide (LPS)-induced TNFα production in primary glial cells[11], rat alveolar macrophages[7] and mouse macrophage-like cells[12]. Besides, in cultured human mononuclear cells, ketamine attenuated endotoxin-stimulated TNFα gene expression[6]. Similarly, sevoflurane attenuated TNFα gene expression in human kidney proximal tubule cells (HK-2) following ischemia-reperfusion injury[29]. In various in vivo models, ketamine suppressed the production of TNFα and IL-6 after stimulation of lipopolysaccharide[2, 5, 13, 14]. Similarly, sevoflurane reduced the gene expression of proinflammatory cytokines including TNFα in rats[25, 30], pigs[31], and humans[22]. Based on these studies and the present study, ketamine and sevoflurane appear to have anti-inflammatory properties through the inhibition of both key cytokines, TNFα and IL-1β.

Many studies have shown the anti-inflammatory effects of ketamine both in vivo and in vitro[2–15]. Our study concurs with these findings. On the other hand, results concerning inflammatory response after exposure to inhalation anesthetics are still not consistent. Goto et al. showed that sevoflurane does not affect cytokine concentrations following cataract surgery[32]. On the other hand, Kotani et al. proposed that gene expression of proinflammatory cytokines (IL-1β, IL-1α, TNFα IL-6, and macrophage inflammatory protein-2) increase after inhalation of halothane, enflurane, isoflurane and sevoflurane under mechanical ventilation in rat alveolar macrophages[33]. Besides, sevoflurane increases pulmonary leukotriene C4, nitrite, and nitrate production in pigs, indicating an inflammatory response[31]. In humans, sevoflurane and desflurane increased TNFα, IL-1β and IL-6 in plasma during tympanoplasty surgery[34]. However, many previous studies have proposed that volatile anesthetics have anti-inflammatory effects by inhibiting proinflammatory cytokines in various models[16, 18, 21, 22, 25-30]. Volatile anesthetics protect against ischemia-reperfusion injury in the heart, lung, liver and kidney by inhibiting proinflammatory cytokines. Preconditioning with sevoflurane attenuates the activation of NF-κB and inhibits NF-κB-dependent inflammatory gene expression such as IL-1 and TNFα in rat myocardium[25]. Kidani et al. proposed that sevoflurane pretreatment decreases mortality

rate, severity of hypotension, and acidosis, and inhibits cytokine responses in rats injected with endotoxin[30]. The addition of sevoflurane to cardioplegia is associated with inhibition IL-8 level of neutrophils after cardiopulmonary bypass[21]. Moreover, sevoflurane suppressed the production of IL-6 and IL-8, but not IL-10 and IL-1 receptor antagonist in patients undergoing coronary artery bypass graft surgery[18]. We now show anti-inflammatory properties of sevoflurane in the transcriptional level. These results are congruent with those of the above investigations.

It has been reported that serum concentration of ketamine used in anesthetic doses is less than 80 μM in animals[35, 36] and total plasma level of ketamine is in the range of 33-94 μM immediately after 2.0-2.2 mg kg-1 i.v. administration in humans[11]. So, we considered that the concentrations of ketamine (30-100 μM) used in this study are in a range of clinically relevant concentrations achievable during induction of ketamine anesthesia. On the other hand, the concentrations produced by 1-5% sevoflurane in the physiologic salt solution can be predicted as 0.13-0.67 mM, and recently reported concentrations of sevoflurane in blood samples from rats during steady state anesthesia with 1 minimum alveolar concentration of sevoflurane (2.8%) were 0.66 mM[37]. Therefore, the concentration of sevoflurane used in this study (0.66 mM) is also similar to the concentrations used clinically.

A limitation of our study is that this assessment of the effect of ketamine and sevoflurane on inflammatory cytokines is at a transcriptional level. Therefore, further studies on protein synthesis level are needed to clarify the effect of ketamine and sevoflurane on inflammatory cytokines. Besides, in the present study, real-time PCR have not been performed for other genes except IL-1β. So the difference between ketamine and sevoflurane in the mechanisms of anti-inflammatory properties derived from microarray analysis might be uncertain. It is certain, however, that both agents significantly suppressed the key inflammatory cytokine, IL-1β.

In conclusion, in the present study, we confirmed anti-inflammatory properties of ketamine and sevoflurane in the CNS by a large-scale gene expression survey. Microarray analyses indicate that ketamine and sevoflurane have different detailed mechanisms of anti-inflammatory properties, however, both significantly suppressed key inflammatory cytokine, IL-1β. These findings suggest that ketamine and sevoflurane show mainly anti-inflammatory properties through the inhibition of IL-1β.

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