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Original Article

Sugammadex reverses vecuronium-mediated inhibition of reactive oxygen species production in endothelial cells and vascular smooth muscle cells

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Abstract: Neuromuscular blockers, including vecuronium and rocuronium, have been widely used during various operations. Sugammadex, a γ-cyclodextrin with a high binding capacity for neuromuscular blockers, has been widely used for the rapid and complete reversal of induced neuromuscular blockade via encapsulation. However, the effects of sugammadex on neuromuscular blocker-induced changes in reactive oxygen species (ROS) production remain largely unknown. The present *in vitro* study was conducted, aiming to bridge this gap in knowledge. The effects of neuromuscular blockers vecuronium or rocuronium (alone) and neuromuscular blockers combined with sugammadex, and sugammadex (alone) on ROS production, induced by various stimuli, were examined using 2',7'-dichlorodihydrofluorescein diacetate staining. The aim was to measure intracellular ROS. Sugammadex attenuated vecuronium-mediated inhibition of ROS production induced by angiotensin II. It also oxidized low-density lipoprotein in vascular smooth muscle cells and endothelial cells, respectively. However, these effects were not observed with rocuronium. Sugammadex did not significantly change vecuronium- and rocuronium-mediated inhibition of ROS production induced by lipopolysaccharides in macrophage cell RAW264.7. Sugammadex, alone, did not significantly alter ROS production. Present results suggest that sugammadex reverses vecuronium-mediated inhibition of ROS production induced by oxidized low-density lipoprotein and angiotensin II in endothelial and vascular smooth muscle cells. This may be due to the encapsulation of vecuronium by sugammadex.

Keywords: Sugammadex, vecuronium, rocuronium, reactive oxygen species, oxidized low-density lipoprotein, angiotensin II

Introduction

Oxidative stress, which occurs when oxidant levels exceed antioxidant levels, is caused by oxidative damage in cells, tissues, and organs [1]. Endogenous reactive oxygen species (ROS) are produced in the mitochondria, endoplasmic reticulum, plasma membrane, and peroxisomes [1]. Oxidized low-density lipoprotein (oxLDL) and angiotensin II (Ang II) produce ROS, contributing to endothelial dysfunction and vascular remodeling, as observed in hypertension and atherosclerosis [2, 3]. *In vivo*, sepsis also induces the production of ROS, including superoxide anion and hydrogen peroxide. This leads to oxidative damage, cytochrome c release, and apoptosis [4]. It has been reported that desflurane, a widely-used inhaled anesthetic for operations, causes oxidative stress or damage [5, 6]. Additionally, cardiovascular surgery involving cardiopulmonary bypass produces ROS, due to ischemia and reperfusion [7, 8]. Neuromuscular blockers, including vecuronium and rocuronium, have been widely used to pro-

vide strong neuromuscular blockade for various operations. Sugammadex, also known as γ-cyclodextrin, has been commonly used to rapidly reverse neuromuscular blockade produced by vecuronium and rocuronium via encapsulation of the steroidal neuromuscular blocker [9]. Levels of malonyl dialdehyde production and decreases in superoxide dismutase and catalase activity in patients treated with different anesthetics have been reported as follows: Spinal anesthesia > halothane > vecuronium [10]. Vecuronium has shown partial antioxidant activity [11]. Furthermore, vecuronium and rocuronium restore impaired acetylcholine-induced nitric oxide-mediated relaxation due to ROS production [12]. However, the effects of sugammadex on changes in ROS production induced by vecuronium and rocuronium remain largely unknown. The goal of the present study was to examine the effects of sugammadex on vecuronium- and rocuronium-mediated changes in ROS production induced by various stimuli in vascular smooth muscle cells (VSMCs), endothelial cells (ECs), and macrophage cells. The hypothesis was that sugammadex inhibits steroidal neuromuscular blocker-induced antioxidant activity.

Materials and methods

Materials

Human low-density lipoprotein (LDL) was purchased from Merck Millipore (Billerica, MA, USA). Ang II and 2',7'-dichlorohydrofluorescein diacetate (DCFH-DA) were purchased from Calbiochem (La Jolla, CA, USA). Dulbecco's High Glucose Modified Eagle's Medium (DMEM) and antibiotics (penicillin/streptomycin) were provided by Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). Vecuronium and rocuronium were obtained from Reyon Pharmaceutical Company (Seoul, Korea) and Hanlim Pharmaceutical Company (Gyeonggi-do, Korea), respectively. Sugammadex was obtained from Merck Sharp & Dohme (Patheon Manufacturing Services LLC, Greenville, NC, USA). Lipopolysaccharide (LPS; *Escherichia coli* 0111:B4) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culturing

Human EC line EA. hy926 (ATCC® CRL-2922) and RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). They were cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, and 10 μg/mL streptomycin. The cells were incubated in a humidified 5% CO_2 incubator. VSMCs were isolated from rat thoracic aorta by enzymatic dissociation. They were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were sub-cultured every 2 or 3 days with trypsin/EDTA. Cells between passage numbers 2 and 10 were used at 80% confluence for experimentation.

Preparation of oxLDL

Human LDL was oxidized, as described in a previous study [13]. Briefly, LDL was oxidized with 5 mM $CuSO_4$ for 16 hours at 37°C after dialysis against phosphate-buffered saline, aiming to remove EDTA. The extent of LDL oxidation was then assessed by the formation of thiobarbituric acid-reactive substances.

Determination of ROS production

Non-fluorescent DCFH-DA is converted to highly fluorescent dichlorofluorescein upon intracellular oxidation by ROS, as described previously [14]. Accordingly, the DCFH-DA staining method was used to examine intracellular ROS levels. The cells were serum-starved overnight and pretreated with vecuronium (3×10^{-4} M) or rocuronium (3×10^{-4} M) for 10 minutes. Next, sugammadex (10^{-4} M) was added. This was followed by incubation for an additional 50 minutes (**Figure 1A**). ECs, VSMCs, and RAW264.7 cells were then stimulated with oxLDL (100 μg/mL), Ang II (100 ng/mL), and LPS (1 μg/mL), respectively, for 3 hours (**Figure 1A**). Cells treated with the indicated reagents were incubated with 10 μM DCFH-DA for 30 minutes. Afterward, they were harvested and washed twice with phosphate-buffered saline. Fluorescence intensity levels were measured at emission, with excitation wavelengths of 535 and 485 nm, respectively, using a microplate fluorescence reader (Tecan Austria GmbH, Grödig, Austria).

Statistical analysis

Present results were confirmed by three independent experiments performed in triplicate. Data were analyzed using one-way analysis of

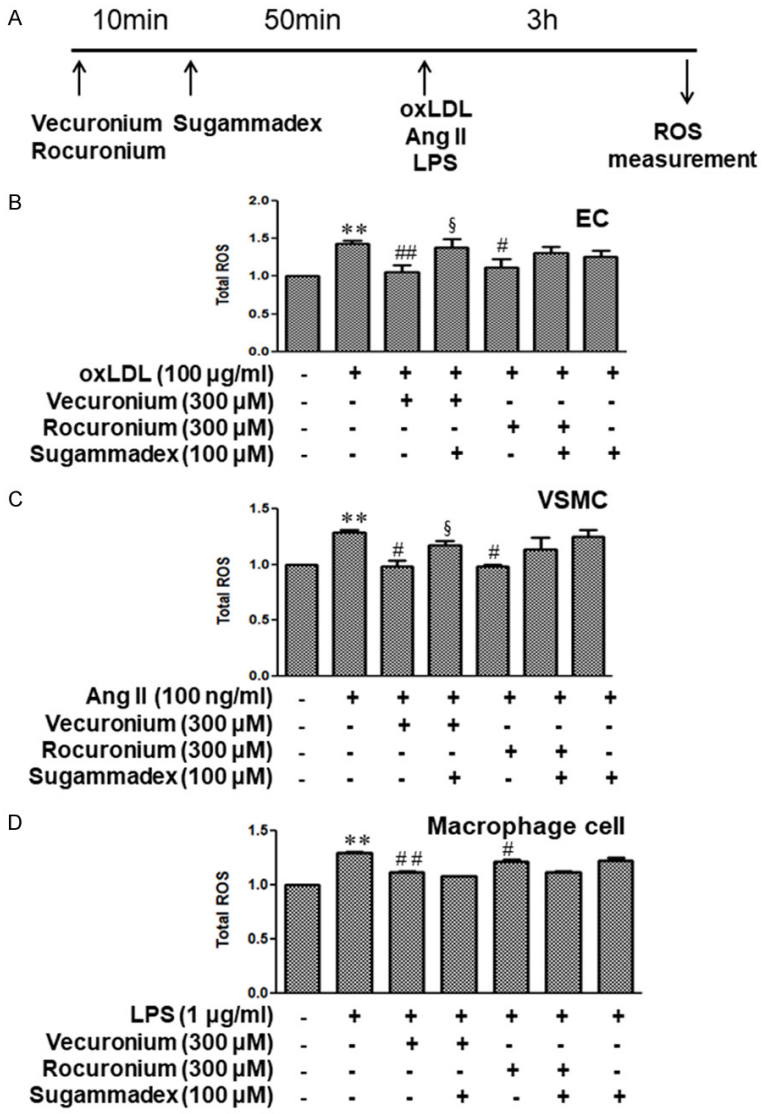

Figure 1. Effects of sugammadex on vecuronium- or rocuronium-mediated inhibition of reactive oxygen species (ROS) production induced by oxidized low-density lipoprotein (oxLDL; B), angiotensin II (Ang II; C), and lipopolysaccharide (LPS; D) in endothelial cells (ECs; B), vascular smooth muscle cells (VSMCs; C), and macrophage cell line RAW264.7 cells (D), respectively. ECs, VSMCs, and macrophage cells were pretreated with vecuronium (3×10^{-4} M) or rocuronium (3×10^{-4} M) for 10 minutes, then sugammadex (10^{-4} M) was added (A). After 50 minutes, ECs, VSMCs, and macrophage cells were stimulated with oxLDL (100 μg/mL), Ang II (100 ng/mL), and LPS (1 μg/mL) for 3 hours, respectively. Intracellular ROS levels were determined as described in the Methods (A). Present data are expressed as the relative changes (fold of control) at 3 hours after stimulation with oxLDL, Ang II, and LPS alone. Data are presented as the mean ± SEM of three independent experiments. (B) $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus oxLDL alone. $^{\S}P < 0.05$ versus vecuronium plus oxLDL. (C) $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ versus Ang II alone. $^{\S}P < 0.05$ versus vecuronium plus Ang II. (D) $^{**}P < 0.01$ versus control. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus LPS alone.

variance with Bonferroni's post-hoc analysis using GraphPad Prism 5.0 software (GraphPad, Inc., La Jolla, CA, USA). All data are presented as the mean ± standard error of the mean. *P* values < 0.05 are considered statistically significant.

Results

OxLDL (100 μg/mL) increased ROS production in human endothelial EA. hy926 cells (*P* < 0.01 versus control; **Figure 1B**). Vecuronium and rocuronium reduced oxLDL-induced ROS production (*P* < 0.01 and *P* < 0.05, respectively, versus oxLDL alone; **Figure 1B**). Sugammadex reversed vecuronium-induced inhibition of ROS production evoked by oxLDL (*P* < 0.05 versus vecuronium plus oxLDL; **Figure 1B**), while rocuronium-induced inhibition of ROS production was not significantly altered (**Figure 1B**). Ang II (100 ng/mL) increased ROS in VSMCs (*P* < 0.01 versus control; **Figure 1C**). Vecuronium and rocuronium decreased Ang II-induced ROS levels (*P* < 0.05 versus Ang II alone; **Figure 1C**). Sugammadex also reversed vecuronium-induced inhibition of ROS production evoked by Ang II (*P* < 0.05 versus vecuronium plus Ang II; **Figure 1C**). However, it did not reverse rocuronium-induced inhibition of ROS production by Ang II (**Figure 1C**). LPS (1 μg/mL) increased ROS production in macrophage RAW264.7 cells (*P* < 0.01 versus control; **Figure 1D**). Vecuronium and rocuronium decreased LPS-induced ROS production (*P* < 0.01 and *P* < 0.05, respectively, versus LPS alone; **Figure 1D**). However, sugammadex did not significantly alter vecuronium- or rocuronium-mediated inhibition of ROS production induced by LPS (**Figure 1D**) in RAW264.7 cells. Additionally, sugammadex alone did not significantly alter ROS produced

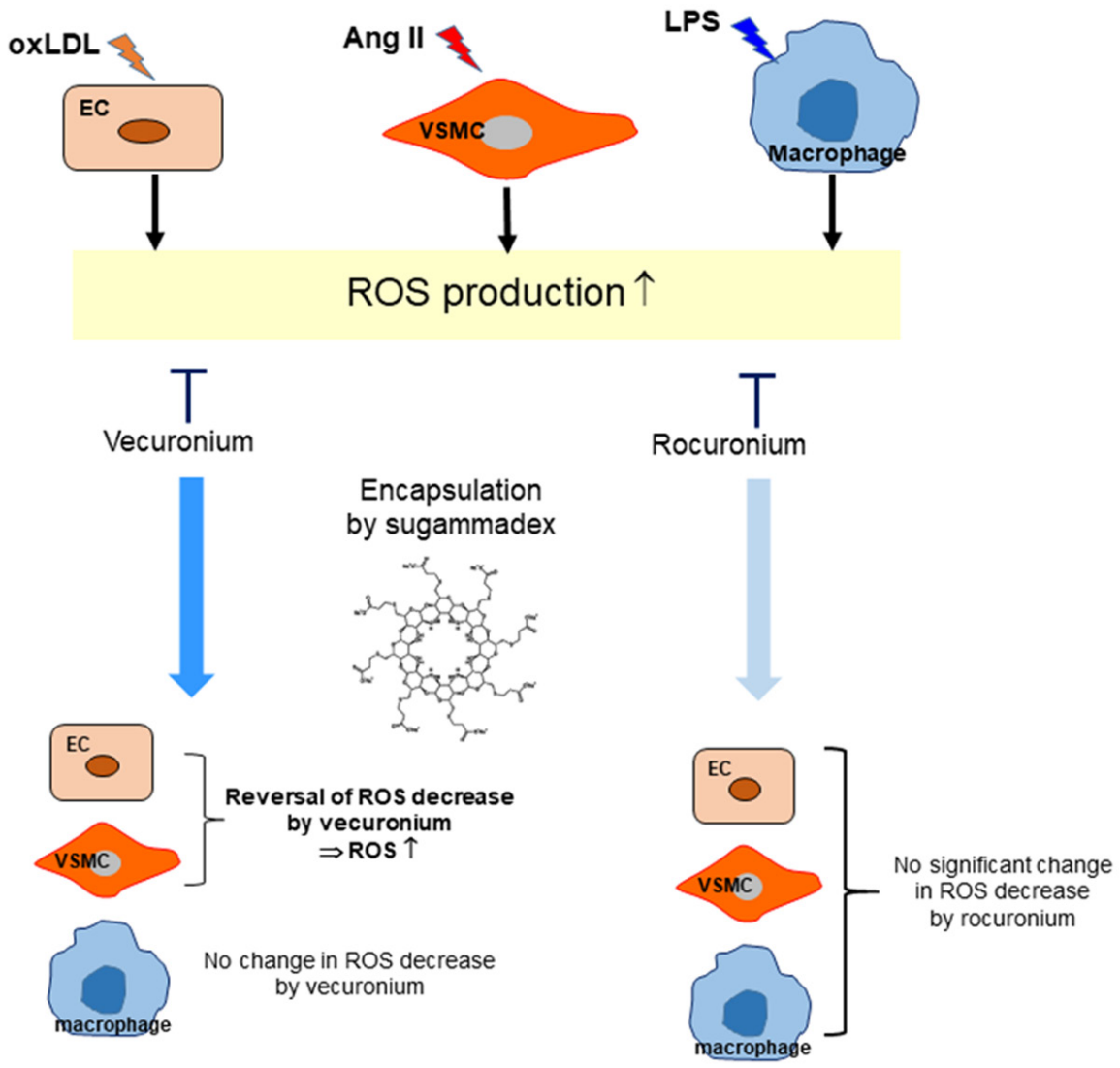

Figure 2. Schematic presentation of the effects of sugammadex on vecuronium- or rocuronium-mediated inhibition of reactive oxygen species (ROS) production induced by oxidized low-density lipoprotein (oxLDL) in endothelial cells (EC), angiotensin II (Ang II) in vascular smooth muscle cells (VSMC), and lipopolysaccharide (LPS) in macrophages.

by oxLDL, Ang II, and LPS in ECs, VSMCs, and RAW264.7 cells, respectively, (**Figure 1B-D**).

Discussion

The present study is the first to suggest that sugammadex inhibits vecuronium-mediated inhibition of ROS produced by oxLDL and Ang II in the endothelium and vascular smooth muscles, respectively (**Figure 2**). OxLDL, shear stress, and cytokines in atherosclerosis activate LDL receptor-1, leading to adhesion molecules and ROS production [2]. Subsequently, upregulation of LDL receptor-1 causes impaired endothelium-dependent nitric oxide-mediated relaxation [2]. In addition, Ang II, produced from angiotensin I by angiotensin-converting enzymes showing increased activity in hypertension, induces mitochondrial ROS production involved in hypertension-related vascular pathophysiology [3, 15]. Furthermore, in a sepsis model induced by administration of LPS, LPS binds to toll-like receptor 4. This produces ROS from damaged mitochondria and induces inflammatory cytokines [16]. Thus, statin, Ang II receptor inhibitor, and angiotensin-converting enzyme inhibitor are used to treat atherosclerosis and hypertension, contributing to the alleviation of ROS production [2, 15].

Present results suggest that vecuronium and rocuronium reduced ROS produced by oxLDL and Ang II, indicating that vecuronium and rocuronium have antioxidant activities [10-12]. Sugammadex attenuated vecuronium-mediated inhibition of ROS produced by oxLDL and Ang II in ECs and VSMCs, respectively (**Figure 2**). However, sugammadex did not significantly attenuate rocuronium-mediated inhibition of ROS produced by oxLDL and Ang II (**Figure 2**). Sugammadex showed a tendency to slightly further decrease rocuronium-mediated reduced levels of ROS production induced by LPS in RAW264.7 cells (**Figure 1D**). Furthermore, sugammadex alone did not change ROS production (**Figure 1B-D**).

The magnitude of the association rate constant, which indicates the binding affinity of sugammadex to the steroidal neuromuscular blocker, was reported as follows: Rocuronium > vecuronium > pancuronium [17]. Despite the lower affinity of sugammadex for vecuronium, compared to rocuronium, sugammadex-induced reversal of neuromuscular blocker inhibition of ROS production in the current study was significant in the vecuronium group. However, it was not significant in the rocuronium group. This discrepancy may be related to the following factors. First, ROS measurement in ECs and VSMCs was performed in the current experiment. Isothermal titration calorimetry, to measure the association rate constant as an indicator of binding affinity between sugammadex and neuromuscular blockers, was used in a previous experiment [17]. Second, sugammadex interacts with neuromuscular blockers at a 1:1 ratio. The current study used three-fold higher molar concentrations of neuromuscular blockers than those of sugammadex [9]. Future studies examining the detailed cellular signal pathways responsible for sugammadex-mediated reversal of ROS inhibition induced by vecuronium are necessary.

Present results have clinical relevance. When a patient with atherosclerosis or hypertension requires the rapid and complete reversal of vecuronium-induced neuromuscular blockade, the use of sugammadex should be avoided. This is because sugammadex-mediated increases in ROS levels may contribute to endothelial dysfunction and vascular remodeling [9, 18]. However, administration of sugammadex to produce complete reversal from neuromuscular blockade induced by vecuronium or rocuronium in patients with sepsis can be conducted, as sugammadex did not significantly affect the inhibition of ROS production by these blockers. In clinical extrapolation of these results, it should be considered that the concentration (3×10^{-4} M) of vecuronium and rocuronium used in the current experiment is higher than the clinically relevant concentrations of vecuronium (1.6×10^{-6} M) and rocuronium (1.1×10^{-5} M) [19, 20].

In conclusion, current results suggest that sugammadex inhibits the vecuronium-induced attenuation of ROS produced by oxLDL and Ang II in ECs and VSMCs, respectively. This may be associated with the encapsulation of vecuronium by sugammadex (**Figure 2**).

Disclosure of conflict of interest

None.

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