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Cytoskeleton interruption in human hepatoma HepG2 cells induced by ketamine occurs possibly through suppression of calcium mobilization and mitochondrial function

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Suppressive effects of ketamine on hepatocyte cytoskeletons

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ABBREVIATIONS:

ATP, adenosine triphosphate; CYP, cytochrome P450-dependent monooxygenase; PBS, phosphate-buffered saline; RT-PCR, reverse-transcriptase polymerase chain reaction

ABSTRACT

Ketamine is an intravenous anesthetic agent often used for inducing and maintaining anesthesia. Cytoskeletons contribute to the regulation of hepatocyte activity of drug biotransformation. In this study, we attempted to evaluate the effects of ketamine on F-actin and microtubular cytoskeletons in human hepatoma HepG2 cells and its possible molecular mechanisms. Exposure of HepG2 cells to ketamine at ≤ 100 μM, which corresponds to clinically relevant concentrations, for 1, 6, and 24 h did not affect cell viability. Meanwhile, administration of therapeutic concentrations of ketamine obviously interrupted F-actin and microtubular cytoskeletons. In parallel, levels of intracellular calcium concentration- and time-dependently decreased following ketamine administration. Analysis by confocal microscopy further revealed that ketamine suppressed calcium mobilization from an extracellular buffer into HepG2 cells. Exposure to ketamine decreased cellular adenosine triphosphate (ATP) levels. The mitochondrial membrane potential and complex I NADH dehydrogenase activity were both reduced following ketamine administration. Ketamine did not change the production of actin or microtubulin messenger (m)RNA in HepG2 cells. Consequently, ketamine-caused cytoskeletal interruption led to suppression of CYP3A4 expression and its metabolizing activity. Therefore, this study shows that therapeutic concentrations of ketamine can disrupt F-actin and microtubular cytoskeletons possibly through suppression of intracellular calcium mobilization and cellular ATP synthesis due to downregulation of the mitochondrial membrane potential and complex I enzyme activity. Such disruption of the cytoskeleton may lead to reductions in CYP3A4 activity in HepG2 cells.

Introduction

Ketamine, an intravenous anesthetic agent, is often applied to induce and maintain anesthesia during surgical procedures (White et al., 1982). Clinically, ketamine has more stable hemodynamics than barbiturates or inhaled anesthetic agents, so it is usually used in critically ill patients to induce anesthesia (Bourgoin et al., 2003). Application of ketamine can be associated with increases in cardiac output, arterial blood pressure, and heart rate through modulation of central and sympathetic nervous system outputs or a reduction of endothelial nitric oxide production (Cook et al., 1991; Chen et al., 2005). In the cellular host defense system, ketamine has been reported to attenuate the activities of neutrophils, leukocytes, and macrophages (Weigand et al., 2000; Chang et al., 2005; Wu et al., 2008). Previous studies also showed that ketamine can interact with and affect the functions of other tissues, including the liver and neurons (Chan et al., 2005; Takadera et al., 2006).

Cytoskeletons are key organelles for maintaining cell morphologies, polarity, movement, and other activities (Canton and Litchfield, 2006). Disturbances in cytoskeletal remodeling result in mitochondrial dysfunction, release of reactive oxygen species, and cell dysfunction (Gourlay and Ayscough, 2005; Adrain et al., 2006; Cherng et al., 2008). Actin and microtubulin are two major monomers respectively contributing to the polymerization of F-actin and microtubular cytoskeletons (Hill, 1981). Cytoskeletal remodeling requires calcium and energy (Molitoris et al., 1996; Fache et al., 2005). Previous studies showed that calcium mobilization can trigger cytoskeletal remodeling and regulate cell activities (L'ubica et al., 2004; Fache et al., 2005). The ischemia-reperfusion process leads to mitochondrial dysfunction and adenosine triphosphate (ATP) depletion, and such decreases in cellular ATP levels induce cytoskeleton disruption, which can damage endothelial cells (Nishimura et al., 1998). Mitochondria are important energy-producing organelles and participate in ATP biosynthesis through the respiratory chain reaction (Papucci et al., 2003). Maintenance of the mitochondrial membrane potential is critical for mitochondrial functions and ATP synthesis. Our previous study further showed that ketamine decreases the mitochondrial membrane potential and cellular ATP synthesis, which then suppress phagocytosis and the oxidative ability of macrophages (Chang et al., 2005).

Hepatocytes play critical roles in the metabolism of endogenous and exogenous substances. Previous studies revealed that interruption of cytoskeleton polymerization may lead to hepatocyte dysfunction or even death. In rat hepatocytes, change in F-actin cytoskeleton was shown to affect storage-operated calcium channels and organization of the endoplasmic reticulum (Wang et al., 1997). Microtubular cytoskeleton is reported to participate in regulation of cytochrome P450-dependent monooxygenase (CYP) 1A, 2B, 2C, or 3A expressions possibly through activation of glucocorticoid receptor or aryl hydrocarbon receptor (Dvorak et al., 2005). Meanwhile, little is known about the effects of ketamine on cytoskeletal remodeling. Thus, this study was designed to evaluate the effects of ketamine on F-actin and microtubular cytoskeletons of hepatoma HepG2 cells and its possible mechanisms. CYP3A4, the major isoform of CYPs in human liver, contributes to ketamine metabolism (Hijazi and Boulieu, 2002). Although CYP3A4 is constitutively expressed, it can also be regulated by xenochemicals (Martinez-Jimenez et al., 2007). Therefore, the other aim of this study was further to evaluate the effects of ketamine-caused cytoskeleton disruption on CYP3A4 expression.

Materials and Methods

Cell Culture, Drug Treatment, and Cytotoxicity assay. Human hepatoma HepG2 cells purchased from American Type Culture Collection (Rockville, MD, USA) were cultured according to a previous method (Chen et al., 2000). Ketamine was dissolved in phosphate-buffered saline (PBS) (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). Concentrations of ketamine of $\leq$ 100 μM, which correspond to clinical plasma concentrations (Domino et al., 1982; Grant et al., 1983), were chosen as the treated dosages in this study. To avoid drug interaction, when HepG2 cells were exposed to ketamine, the culture medium did not contain serum and antibiotics. Levels of γ-glutamyltranspeptidase and lactate dehydrogenase in the culture medium were measured to evaluate the cytotoxicity of ketamine to HepG2 cells as described previously (Wu et al., 2007).

Confocal Microscopic Analyses of F-Actin and Microtubular Cytoskeletons. The F-actin and microtubular cytoskeletons in HepG2 cells were visualized as described previously (Cherng et al., 2008). For imaging analysis of F-actin filaments and the microtubule cytoskeleton, cells were stained with phalloidin-FITC (Molecular Probes, Eugene, OR, USA) or immunodetected using a mouse monoclonal antibody labeled with FITC against mouse α-tubulin (Molecular Probes). A confocal laser scanning microscope (Model FV500, Olympus, Tokyo, Japan) was utilized to analyze the samples. Images were acquired and quantified using FLUOVIEW software (Olympus).

Analyses of Intracellular Calcium Levels and Calcium Mobilization. Intracellular calcium levels and calcium mobilization were analyzed to determine if ketamine can modulate the bradykinin-induced calcium influx as described previously (Chen et al., 2005). After drug treatment, HepG2 cells were treated with Fluo-3/AM and bradykinin. Levels of intracellular calcium were immediately detected using a multi-label counter (Wallac, Turku, Finland). Calicium mobilization was observed and analyzed using a confocal laser scanning microscope (Olympus). Images were acquired and quantified using FLUOVIEW software (Olympus).

Detection of Cellular ATP Levels. Levels of cellular ATP in HepG2 cells were

determined by a bioluminescence assay according to the protocol of Molecular Probes' ATP Determination kit (Molecular Probes) as described previously (Wu et al., 2005). The luminent light (560 nm) emitted by the luciferase-mediated reaction of ATP and lucifenin was detected by a multilabel counter (Wallac).

Quantification of the Mitochondrial Membrane Potential. The mitochondrial membrane potential was determined as described previously (Chang et al., 2006). After drug treatment, HepG2 cells were harvested and incubated with 3,3'-dihexyloxacarbocyanine ($DiOC_6$), a positively charged dye, at 37 °C in a humidified atmosphere of 5% CO_2. The fluorescent intensities in cells were analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA).

Assay of Mitochondrial NADH Dehydrogenase Activity. To determine the effects of ketamine on mitochondrial function, NADH dehydrogenase activity was detected using a colorimetric assay following the method of Wu et al. (2005). After drug treatment, HepG2 cells were cultured with new medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for another 3 h. The formazan products were dissolved in dimethyl sulfoxide and spectrophotometrically measured.

Assay of Erythromycin *N*-Demethylase Activity. After drug treatment, microsomal fractions of HepG2 cells were prepared as described previously (Chen et al., 2000). Erythromycin *N*-demethylation activity was determined by following the formation of formaldehyde using the Nash reagent (Nash, 1953).

Immunodetection of CYP3A4 and β_2M. Levels of CYP3A4 and β_2M in HepG2 cells were immunodetected following a previously described method (Chen et al., 2003). CYP3A4 protein was immunodetected using a goat polyclonal antibody against human CYP3A4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). β_2M protein was immunodetected using a mouse monoclonal antibody against human β_2M (Santa Cruz Biotechnology) as an internal control. Intensities of the immunoreactive protein bands were determined using a digital imaging system (UVtec, Cambridge, UK).

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Assay. Messenger (m)RNA from HepG2 cells exposed to ketamine was prepared for RT-PCR analyses of α-

actin, β-actin, microglobulin, CYP3A4, and β_2M mRNA following the instructions of the ready-to-go RT-PCR beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK) as described previously (Wu et al., 2008). Oligonucleotides for the PCR analyses of α-actin, β-actin, α-tubuline, microglobulin, CYP3A4, and β_2M mRNA were designed and synthesized by Mission Biotech (Taipei, Taiwan). The oligonucleotide sequences of these primers were 5'-GCTCACGGAGGCACCCCTGAA-3' and 3'-TACGATTGTTACAGGATAGTC-5' for α-actin; 5'-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3' and 3'-CGTCTACACCTAGTCGTTCGTCCTCATACTGC-5' for β-actin; 5'-CTCGCGCTACTCTCTCTTTCTGG-3' and 3'-AATTCACCCTAGCTCTGTACATTCG-5' for microglobulin; 5'-TGCTGTCTCCAACCTTCACC-3' and 3'-CCACCACTACTAAGGTTCGAT-5' for CYP3A4; and 5'-GTCTACATGTCTCGATCCCACTTA A -3' and 3'- CTCGCGCTACTCTCTCTTTCTGG -5' for β_2M. According to our preliminary results, the 35 cycles of PCR reactions were chosen as our analyzing condition. The PCR reaction was carried out using 35 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 2 min. The PCR products were electrophoretically separated. These DNA bands were photographed and quantified with the aid of a digital imaging system (UVtec).

Statistical Analysis. Statistically significant differences between the control and ketamine-treated groups were determined using Student's t-test, and differences were considered statistically significant at p values of < 0.05. Statistical analysis between groups over time was carried out using two-way analysis of variance (ANOVA).

Results

Toxicity of Ketamine to HepG2 cells. Cell viability was assayed to evaluate the toxicity of ketamine to HepG2 cells (data not shown). Exposure to 25, 50, 75, and 100 μM ketamine for 24 h did not affect the release of γ-glutamyltranspeptidase and lactate dehydrogenase. When the concentration reached 1000 μM, ketamine caused significant 2.5- and 3.2-fold increases in release of γ-glutamyltranspeptidase and lactate dehydrogenase, respectively.

Disruption of F-Actin and Microtubule Cytoskeletons by Ketamine. Confocal microscopy was used to determine the effects of ketamine on F-actin and microtubular cytoskeletons of HepG2 cells (Fig. 1). In untreated HepG2 cells, long-form and regular F-actin filaments were observed (Fig. 1A). Administration of ketamine at a clinically relevant concentration of 100 μM for 1 h did not affect F-actin cytoskeletons. After exposure for 6 h, ketamine obviously disrupted polymerization of F-actin filaments in HepG2 cells. Remodeling of the F-actin cytoskeleton was time-dependently suppressed following ketamine administration for 24 h (Fig. 1A). The fluorescent intensities of HepG2 cells were quantified and analyzed (Fig. 1B). Exposure of HepG2 cells to 100 μM ketamine for 6 and 24 h significantly decreased F-actin cytoskeletal formation by 54% and 72%, respectively.

In control hepatocytes, the microtubular cytoskeleton was uniformly distributed (Fig. 1C). Treatment of HepG2 cells with a therapeutic concentration of ketamine (100 μM) for 1 h did not affect the microtubular cytoskeleton. After exposure for 6 h, ketamine at 100 μM obviously interrupted polymerization of the microtubular cytoskeleton in HepG2 cells. Remodeling of the microtubular cytoskeleton in HepG2 cells exhibited much greater disturbance after exposure to ketamine for 24 h (Fig. 1C). The fluorescent intensities were quantified and analyzed (Fig. 1D). Exposure of HepG2 cells to a clinically relevant concentration of 100 μM ketamine for 6 and 24 h caused significant decreases of 48% and 77% in the microtubular cytoskeleton, respective.

Reduction of Bradykinin-induced Calcium Mobilization by Ketamine. Levels of intracellular calcium in HepG2 cells were quantified to determine the mechanism of ketamine-caused suppression of F-actin and microtubular cytoskeletons (Fig. 2). Exposure

of HepG2 cells to 25 μM ketamine did not influence bradykinin-enhanced intracellular calcium levels (Fig. 2A). After administration of 50 μM ketamine for 24 h, bradykinin-caused increases in the levels of intracellular calcium were significantly decreased by 23%. Treatment of HepG2 cells with 75 and 100 μM ketamine for 24 h caused significant 42% and 61% decreases in intracellular calcium levels (Fig. 2A). Exposure to a therapeutic concentration of ketamine (100 μM) for 1 h significantly decreased the bradykinin-augmented intracellular calcium amount by 19% (Fig. 2B). After administration of 100 μM ketamine for 6 and 24 h, levels of intracellular calcium were respectively reduced by 40% and 63% (Fig. 2B).

A confocal analysis was used to confirm the effects of ketamine on bradykinin-induced calcium mobilization (Fig. 3). Before bradykinin stimulation, low levels of intracellular calcium were detected in control and ketamine-treated hepatocytes (Fig. 3A, D, G, J). When untreated HepG2 cells were exposed to bradykinin for 3 min, the amounts of intracellular calcium were obviously enhanced (Fig. 3B), and the intracellular calcium levels reached a maximum after 5 min (Fig. 3C). In 1-h-treated HepG2 cells, pretreatment with ketamine obviously suppressed bradykinin-induced calcium influx (Fig. 3E, F). The suppressive effects of ketamine on bradykinin-induced calcium mobilization became much more apparent in 6- and 12-h-treated HepG2 cells (Fig. 3H, I, K, L).

Suppression of Cellular ATP Biosynthesis by Ketamine. Cellular ATP levels were quantified to evaluate the roles of mitochondria in ketamine-involved suppression of F-actin and microtubular cytoskeletons (Fig. 4). Exposure of HepG2 cells to 25 μM ketamine for 24 h did not change the levels of cellular ATP (Fig. 4A). After administration of 50 μM ketamine, the amounts of cellular ATP had significantly decreased by 24%. When the concentrations reached 75 and 100 μM, ketamine caused significant 41% and 44% reductions in cellular ATP synthesis (Fig. 4A). Treatment of HepG2 cells with ketamine for 1 h did not influence cellular ATP levels (Fig. 4B). Levels of intracellular ATP were significantly reduced by 31% and 48% following ketamine administration for 6 and 24 h, respectively.

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Downregulation of the Mitochondrial Membrane Potential and Complex I NADH Dehydrogenase Activity by Ketamine. To determine the mechanism of the ketamine-caused reduction in cellular ATP synthesis, the mitochondrial membrane potential and mitochondrial complex I NADH dehydrogenase activity were assayed (Fig. 5). Exposure of HepG2 cells to a therapeutic concentration of ketamine (100 μM) for 1 h did not affect the mitochondrial membrane potential (Fig. 5A). When administered for 6 and 24 h, ketamine significantly decreased the membrane potential of hepatocyte mitochondria by 25% and 46%, respectively. Treatment of HepG2 cells with a clinically relevant concentration of ketamine for 1 h did not influence the activity of mitochondrial complex I NADH dehydrogenase (Fig. 5B). Meanwhile, after exposure for 6 and 24 h, ketamine significantly decreased mitochondrial complex I activity by 29% and 40%, respectively.

Effects of Ketamine on α- and β-Actin and Microtubulin mRNA Productions. Biosyntheses of α- and β-actin and microglobulin mRNA in HepG2 cells were analyzed to evaluate the effects of ketamine on the expressions of these cytoskeletal monomers (Fig. 6). In untreated HepG2 cells, α- and β-actin mRNAs were detected (Fig. 6A, *top and middle panels*, lane 1). After exposure to a therapeutic concentration of ketamine for 1, 6, and 24 h, the syntheses of α- and β-actin mRNAs were not influenced (lanes 2~4). Levels of β_2M mRNA were determined as the internal standards (Fig. 6A, *bottom panel*). These DNA bands were quantified and analyzed (Fig. 6B). Administration of a clinically relevant concentration of ketamine did not change the production of α- or β-actin mRNA in HepG2 cells. Microglobulin mRNA was detected in control hepatocytes (Fig. 6C, *top panel*, lane 1). Treatment of HepG2 cells with a clinically relevant concentration of ketamine for 1, 6, and 24 h did not affect synthesis of microglobulin mRNA (lanes 2~4). The amounts of β_2M mRNA were determined as the internal standards (Fig. 6C, *bottom panel*). These DNA bands were analyzed and are shown in Fig. 6D. Exposure of HepG2 cells to ketamine did not influence microglobulin mRNA synthesis.

Inhibition of Erythromycin *N*-Demethylation and CYP3A4 protein and mRNA Productions by Ketamine. To evaluate the effects of ketamine-induced suppression of F-actin and microtubular cytoskeletons on the metabolic capacity of HepG2 cells, CYP3A4

expression and activity were analyzed (Fig. 7). In untreated HepG2 cells, the activity of CYP3A4 involved in erythromycin *N*-demethylation was detected (Fig. 7A). However, after exposure to ketamine for 6 and 24 h, the CYP3A4-involved *N*-demethylation of erythromycin was significantly decreased by 31% and 54%, respectively. CYP3A4 protein and mRNA were detected in untreated HepG2 cells (Fig. 7B, D, *top panels*, lane 1). Administration of a clinically relevant concentration of ketamine for 1 h did not change CYP3A4 protein and mRNA productions (lane 2). Meanwhile, after exposure for 6 and 24 h, ketamine obviously inhibited the syntheses of CYP3A4 protein and mRNA (lanes 3 and 4). Levels of β_2M protein and mRNA were determined as the internal standards (Fig. 7B, D, *bottom panels*). These protein and DNA bands were quantified and analyzed (Fig. 7C, E). Treatment of HepG2 cells with ketamine for 6 and 24 h caused significant 37% and 49% decreases in CYP3A4 protein synthesis (Fig. 7C). Simultaneously, the levels of CYP3A4 mRNA inhibited by 28% and 49% following ketamine administration for 6 and 24 h, respectively (Fig. 7E).

Discussion

This study shows that therapeutic concentrations of ketamine (≤ 100 μM) can induce dysfunction of HepG2 cells. Administration of neither 25, 50, nor 100 μM ketamine was cytotoxic to HepG2 cells. Concentrations of ketamine used in this study are within the range of clinical relevance (Domino et al., 1982; Grant et al., 1983). Under such clinically relevant concentrations, ketamine suppressed polymerization of F-actin and microtubular cytoskeletons. Levels of intracellular calcium and cellular ATP significantly decreased following ketamine administration. Cytoskeletons and mitochondria are two key organelles which are involved in maintaining cell activities (Canton et al., 2006). In parallel with the suppression of cytoskeletal remodeling, exposure of HepG2 cells to a therapeutic concentration of ketamine (100 μM) inhibited CYP3A4 expression and its enzyme activity. In hepatocytes, CYP3A4 is a major enzyme which participates in the metabolism of xenobiotics (Martinez-Jimenez et al., 2007). Our previous studies showed that a therapeutic concentration of ketamine can suppress macrophage activities and reduce endothelial nitric oxide synthesis (Chang et al., 2005; Chen et al., 2005). In this study, we further demonstrated that clinically relevant concentrations of ketamine can lead to dysfunction of HepG2 cells due to suppression of cytoskeletal remodeling, calcium mobilization, ATP synthesis, and enzyme activity rather than the death mechanism.

Ketamine can suppress polymerization of F-actin and microtubular cytoskeletons. Treatment of HepG2 cells with a therapeutic concentration of ketamine (100 μM) significantly decreased polymerization of F-actin and microtubular cytoskeletons. Actin and microtubulin are two monomers which respectively comprise F-actin and microtubular cytoskeletons (Ready et al., 1990; Thai et al., 2006). Treatment of HepG2 cells with ketamine did not affect the biosyntheses of α- and β-actins or microtubulin mRNA. Thus, it is possible that ketamine suppresses F-actin and microtubular cytoskeletons by means other than through inhibition of α- and β-actins or microtubulin expression. A previous study showed that levels of calcium can trigger cytoskeletal remodeling and regulate cell motility (L'ubica et al., 2004; Fache et al., 2005). The present results revealed that ketamine alleviated the bradykinin-induced enhancement of intracellular calcium. In addition,

cytoskeletal remodeling is energy-dependent (Molitoris et al., 1996). Our results showed that ketamine caused significant decreases in cellular ATP levels in HepG2 cells. Therefore, possible reasons explaining the ketamine-caused suppression of remodeling of F-actin and microtubular cytoskeletons are decreases in cellular calcium and ATP levels rather than inhibition of monomer syntheses.

Ketamine can decrease bradykinin-induced augmentation of cellular calcium levels in HepG2 cells. Exposure of HepG2 cells to ketamine for 6 and 24 h significantly decreased the bradykinin-enhanced calcium mobilization. Bradykinin increases calcium influx through stimulation of the bradykinin-B2 receptor (Pallone et al., 1998). This endogenous bradykinin-B2 receptor is found in hepatocytes (Yayama et al., 2007). Thus, we suppose that ketamine might interact with bradykinin-B2 receptor during 6-h- and 24-h-treated periods. To prove our suggestion, our other study is cloning B2 receptor cDNA from HepG2 cells. After that, we will evaluate the effects of ketamine on calcium mobilization and cytoskeleton remodeling in B2 recepter-overexpressed HepG2 cells. The suppressive effects of ketamine on intracellular calcium have been reported in a variety of cells and are known to sequentially affect cell activities. In resistant mesenteric arteries, ketamine can decrease voltage-gated calcium influx and norepinephrine-induced calcium release and causes a contractile response in smooth muscle cells (Akata et al., 2001). A decrease in intracellular levels of calcium can be associated with reduced nitric oxide synthesis in endothelial cells (Fleming and Busse, 1999). Therefore, ketamine can suppress intracellular calcium concentrations in HepG2 cells, which sequentially causes dysfunction of cytoskeletal remodeling and even hepatocyte activities.

Ketamine at a therapeutic concentration decreased cellular ATP synthesis via suppressing the mitochondrial membrane potential and complex I NADH dehydrogenase activity. Treatment of HepG2 cells with ketamine reduced the mitochondrial membrane potential. Maintenance of the mitochondrial membrane potential is critical for ATP synthesis (Papucci et al., 2003). Additionally, complex I NADH dehydrogenase activity participates in the respiratory chain reaction (Wu et al., 2005). In parallel with reductions in the membrane potential and complex I activity in hepatocyte mitochondria, ketamine

decreased cellular ATP levels. Thus, the ketamine-caused suppression of cellular ATP synthesis is due to a reduction in the mitochondrial membrane potential and NADH dehydrogenase activation. Our previous study also showed that ketamine can inhibit mitochondrial ATP synthesis, which thus suppresses macrophage activities (Chang et al., 2005). ATP is also needed for polymerization of F-actin and microtubular cytoskeletons (Nishimura et al., 1998). In mouse endothelial cells, an ATP defect induces alterations of the F-actin cytoskeleton and leads to cell damage and microvascular dysfunction (Suurna et al., 2006). Therefore, a therapeutic concentration of ketamine can decrease cellular ATP levels, and such inhibition may induce interruption of F-actin and microtubular cytoskeletons and decrease hepatocyte activities.

Ketamine inhibits CYP3A4 expression and its enzyme activity. Ketamine at a therapeutic concentration decreased microsomal erythromycin *N*-demethylase activity. Although midazolam and testosterone can also be used as the substrates for CYP3A4 assay, the present study have further determined that ketamine could inhibit CYP3A4 protein and mRNA productions in HepG2 cells. CYP3A4 has been reported to be a major enzyme catalyzing erythromycin *N*-demethylation (Wang et al., 1997). In the rat liver, ketamine can decrease CYP3A activity (Meneguz et al., 1999). Thus, the present results further demonstrate that ketamine can reduce CYP3A4 enzyme activity, and the suppressive mechanism occurs at least at a pretranslational level. Because CYP3A4 is the principal enzyme responsible for ketamine metabolism in human liver (Hijazi and Boulieu, 2002), we suggest that the ketamine-induced reduction of CYP3A4 enzyme activity may lead to suppression of hepatocyte clearance of this intravenous anesthetic agent. Previous studies showed that ketamine can modulate CYP gene expression (Meneguz et al., 1999; Chan et al., 2005, 2006). Cytoskeletal disruption has been shown to severely impair basal and inducible expressions of human CYP genes (Dvorak et al., 2005). Our present results show that ketamine can suppress F-actin and microtubular cytoskeletons. Therefore, one of the possible mechanisms explaining the ketamine-caused inhibition of CYP3A4 mRNA production is via suppression of F-actin and microtubular cytoskeletons.

In conclusion, the present study shows that therapeutic concentrations of ketamine (≤

100 μM) can suppress polymerization of F-actin and microtubular cytoskeletons in HepG2 cells. Meanwhile, ketamine did not affect actin or microtubulin mRNA production. Instead, ketamine alleviated the bradykinin-induced increase in intracellular calcium levels and mobilization. In addition, ketamine decreased the mitochondrial membrane potential, complex I NADH dehydrogenase activity, and cellular ATP levels in HepG2 cells. Simultaneously, ketamine inhibited CYP3A4 mRNA synthesis, and this enzyme is involved in the *N*-demethylation of erythromycin. Ketamine at clinical concentrations is not cytotoxic to HepG2 cells. Therefore, according to the present data, we suggest that ketamine at clinically relevant concentrations can disturb polymerization of F-actin and microtubular cytoskeletons possibly via suppression of calcium mobilization and mitochondrial ATP synthesis rather than inhibition of their monomer expressions. The ketamine-caused suppression of cytoskeletal remodeling may lead to inhibition of CYP3A4 expression. There are certain limitations to this study. Because this *in vitro* study used a human cancer cell line instead of human primary hepatocytes, we cannot draw definite, clinically relevant conclusions about the possible effects of ketamine on hepatocyte functions.

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Footnotes

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Figure legends

FIG. 1. Effects of ketamine on polymerization of the F-actin and microtubule cytoskeletons. HepG2 cells were exposed to 100 μM ketamine for 1, 6, and 24 h. The F-actin filaments in human hepatocytes were stained with phalloidin-FITC and visualized using confocal microscopy (A). The microtubule cytoskeleton in HepG2 cells was immunodetected using a mouse monoclonal antibody labeled with FITC against mouse α-tubulin and observed using confocal microscopy (C). The fluorescent intensities in human hepatocytes were analyzed and quantified (B, D). Each value represents the mean ± SD for 6 individual experiments. * Values significantly differ from the respective control, $p < 0.05$.

FIG. 2. Effects of ketamine on levels of intracellular calcium in human hepatocytes. HepG2 cells were exposed to 25, 50, 75, and 100 μM ketamine for 24 h or to 100 μM ketamine for 1, 6, and 24 h. Intracellular calcium was stained with Fluo-3 dye. After bradykinin administration, the fluorescence intensities were detected using a fluorescence spectrophotometer. Each value represents the mean ± SD for 6 individual experiments. * Values significantly differ from the respective control, $p < 0.05$.

FIG. 3. Effects of ketamine on bradykinin-induced calcium mobilization. HepG2 cells were exposed to 100 μM ketamine for 1, 6, and 24 h. Intracellular calcium was stained with Fluo-3 dye. After bradykinin administration, calcium mobilization from the extracellular buffer to the cytoplasm and the nuclei were laser-scanned using a confocal microscope.

FIG. 4. Effects of ketamine on biosynthesis of cellular adenosine triphosphate (ATP). HepG2 cells were exposed to 25, 50, 75, and 100 μM ketamine for 24 h (A), or to 100 μM ketamine for 1, 6, and 24 h (B). Levels of cellular ATP in HepG2 cells were quantified by a bioluminescence assay. Each value represents the mean ± SD for 6 individual experiments. * Values significantly differ from the respective control, $p < 0.05$.

FIG. 5. Effects of ketamine on the mitochondrial membrane potential and complex I NADH dehydrogenase activity. HepG2 cells were exposed to 100 μM ketamine for 1, 6, and 24 h. The mitochondrial membrane potential was quantified using flow cytometry (A). Activity of mitochondrial complex I NADH dehydrogenase was assayed by a colorimetric method (B). Each value represents the mean ± SD for 6 individual experiments. * Values significantly differ from the respective control, $p < 0.05$.

FIG. 6. Effects of ketamine on α- and β-actin and microtubulin mRNA productions. Messenger RNA from HepG2 cells exposed to 100 μM ketamine for 1, 6, and 24 h were prepared for RT-PCR analyses of α- and β-actin mRNA (A, *top and middle panels*) and microtubulin mRNA (C, *top panel*). Amounts of β_2M mRNA were determined as an internal standard (A and C, *bottom panels*). These DNA bands were quantified and analyzed (B and D). Each value represents the mean ± SD for 6 individual experiments. * Values significantly differ from the respective control, $p < 0.05$. M, DNA 100-bp marker.

FIG. 7. Effects of ketamine on erythromycin *N*-demethylase activity and CYP3A4 protein and mRNA productions. HepG2 cells were exposed to 100 μM ketamine for 1, 6, and 24 h. Microsomal erythromycin *N*-demethylase activity was determined following formation of formaldehyde using the Nash reagent (A). CYP3A4 protein and mRNA were detected using immunoblotting and RT-PCR analyses (B and D, *top panels*). Amounts of β_2M protein and mRNA were determined as internal standards (B and D, *bottom panels*). These protein and DNA bands were quantified and analyzed (C and E). Each value represents the mean ± SD for 6 individual experiments. * Values significantly differ from the respective control, $p < 0.05$. M, DNA 100-bp marker.

Fig. 1

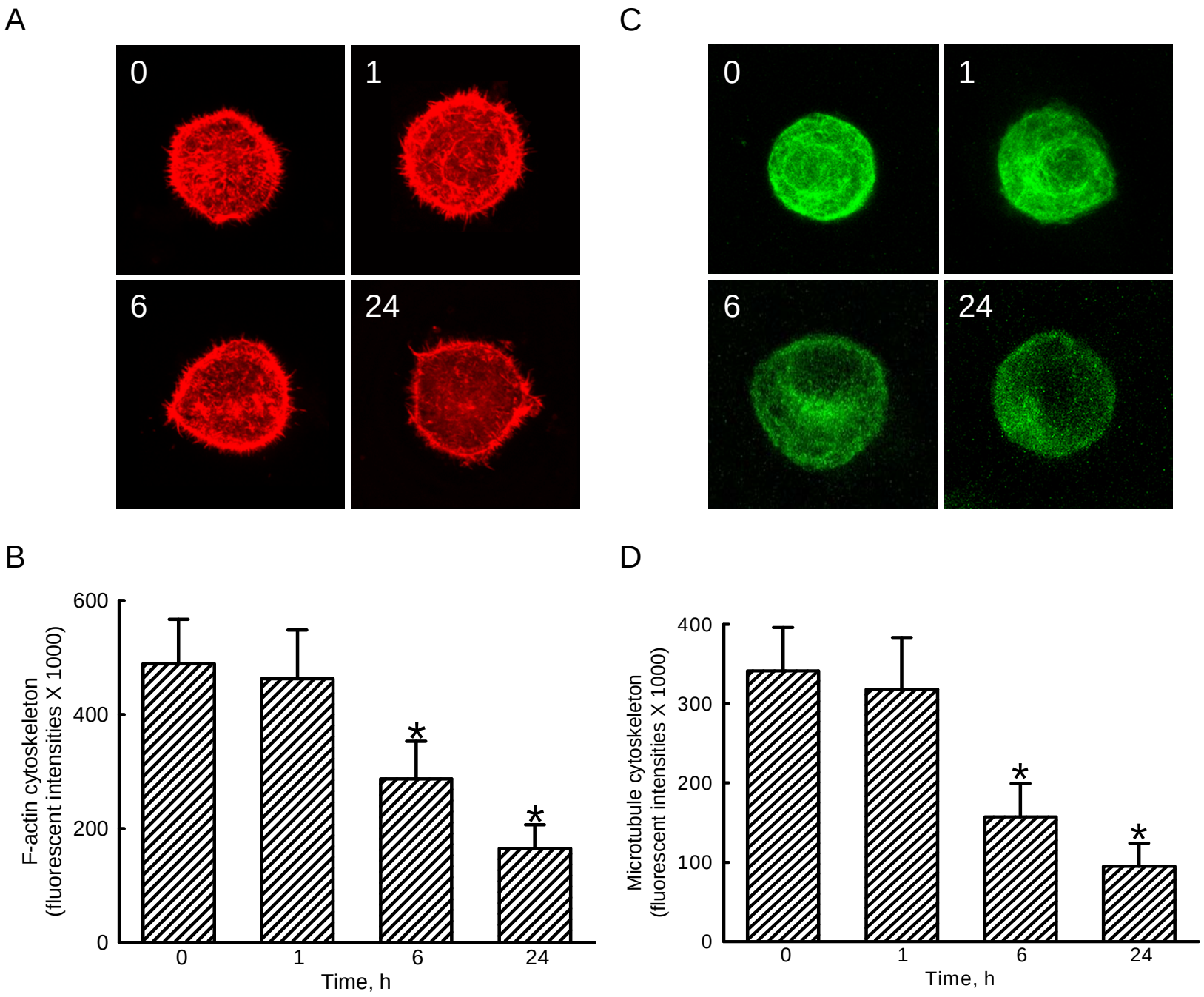

Fig. 2

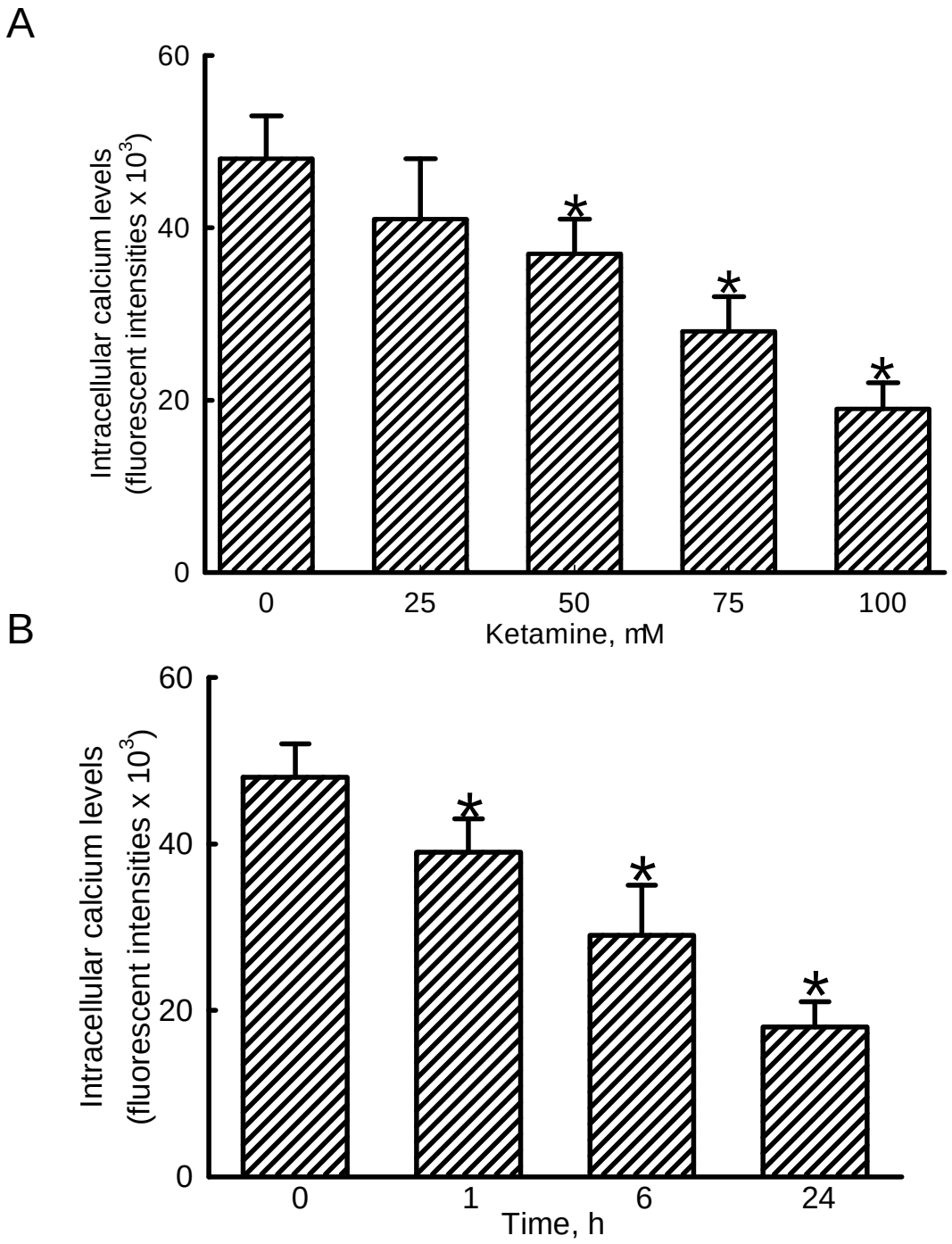

Fig. 3

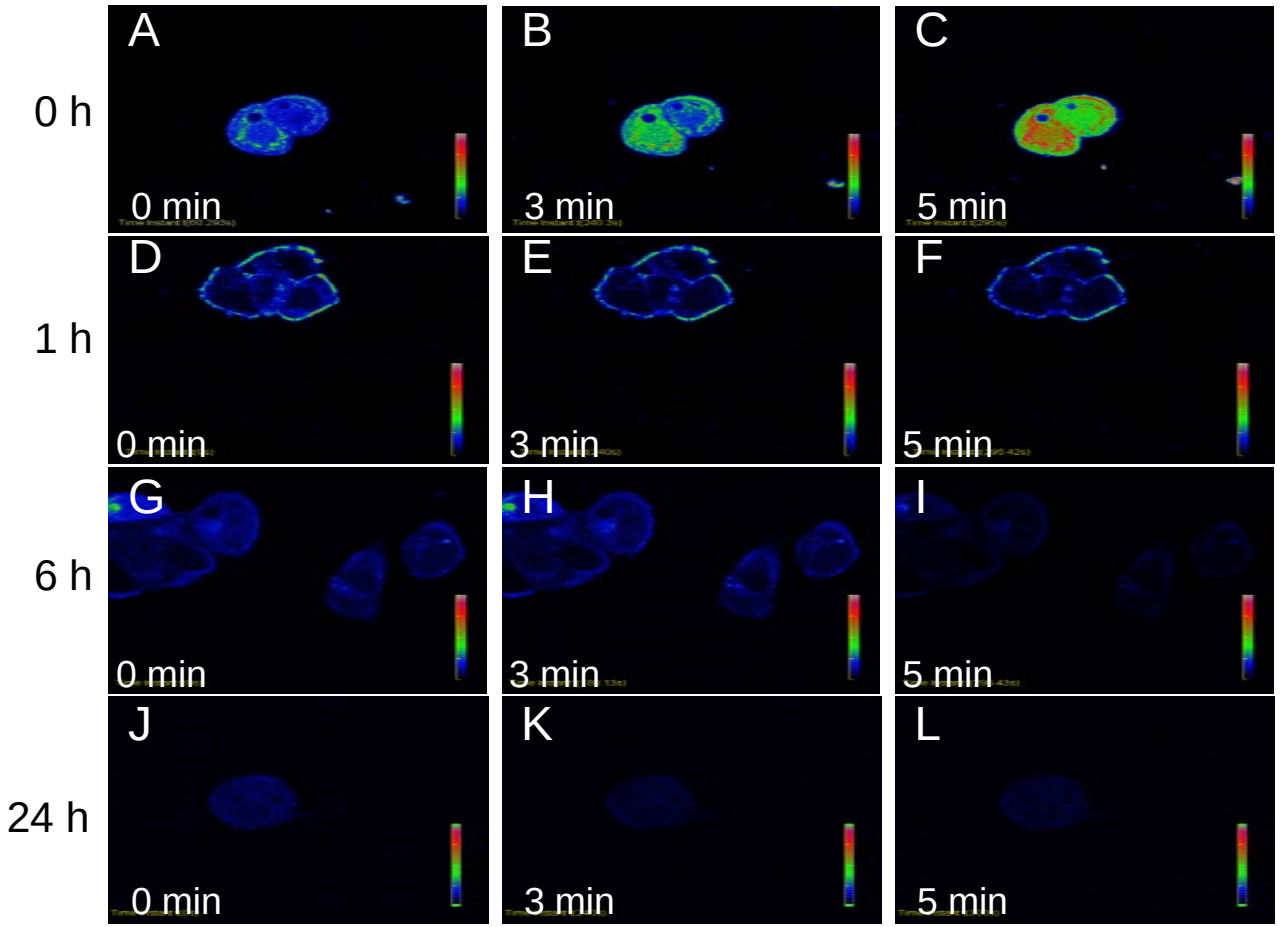

Fig. 4

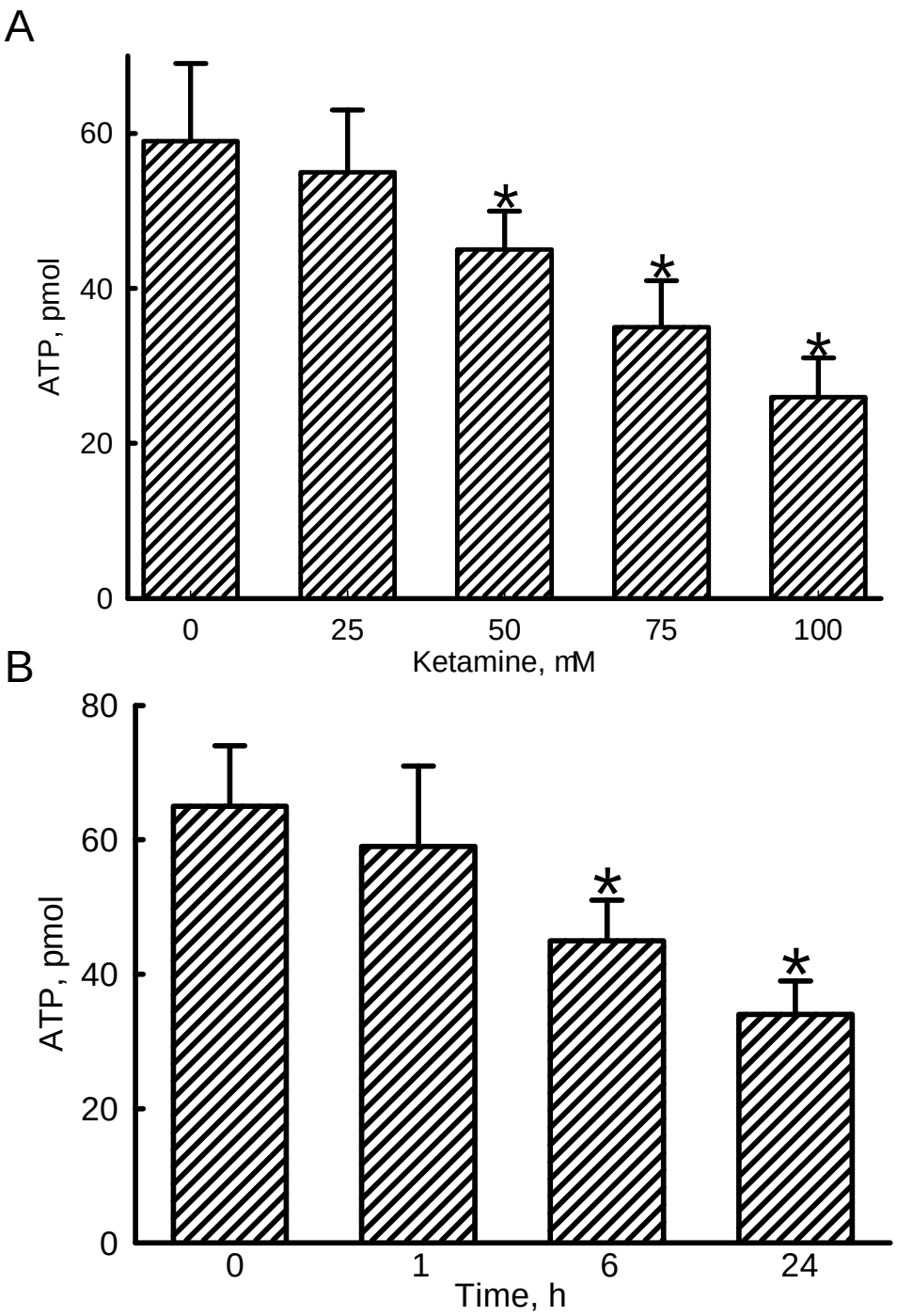

Fig. 5

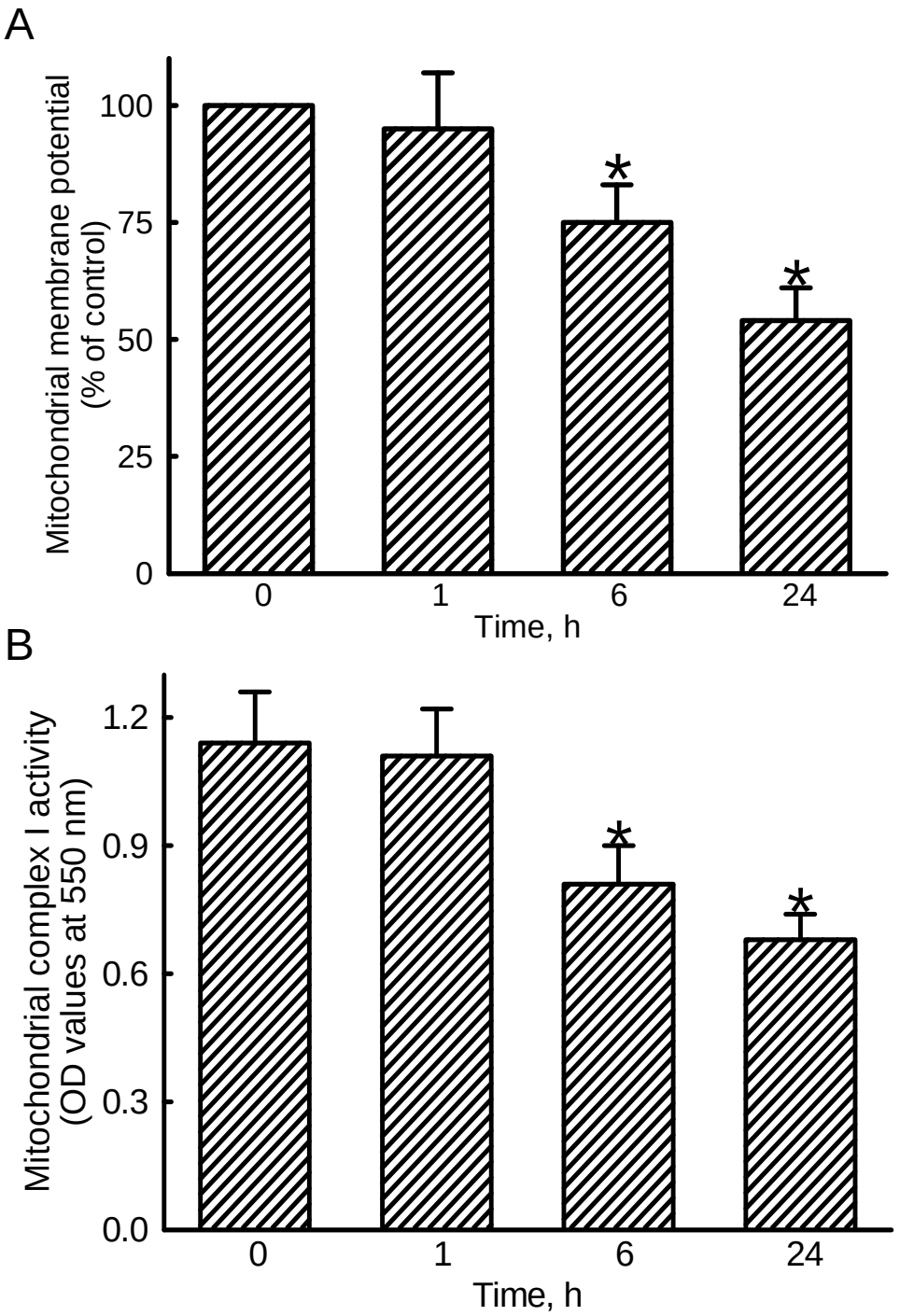

Fig. 6

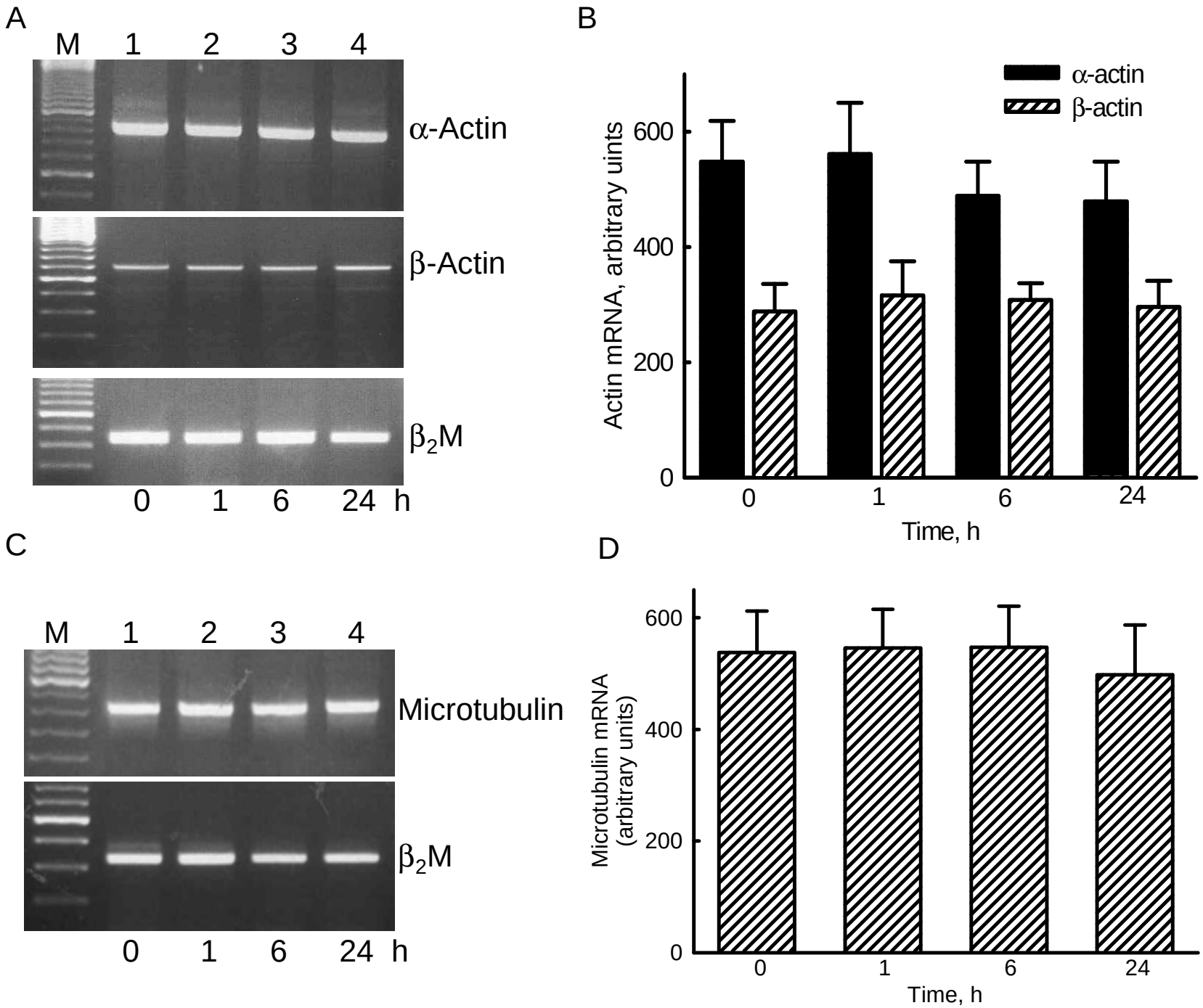

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Fig. 7