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# Effects of ethanol on RhoA/Rho-kinase-mediated calcium sensitization in mouse lung parenchymal tissue

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ABSTRACT

Calcium sensitization by the RhoA/Rho-kinase (ROCK) pathway contributes to the contraction in smooth muscle. Contractile stimuli can sensitize myosin to $Ca^{2+}$ by activating RhoA/Rho-kinase that inhibits myosin light chain phosphatase activity. The present study was aimed at investigating the possible involvement of RhoA/Rho-kinase pathway in contractile responses to agonist (phenylephrine) and depolarizing (KCl) of mouse lung parenchymal tissues. Also, we investigated the effect of ethanol on RhoA/Rho-kinase pathway. Phenylephrine ($10^{-8}$–$10^{-4}$ M) and KCl (10–80 mM) induced sustained contractions in parenchymal strips. Ethanol significantly attenuated the contractions to phenylephrine and KCl. The Rho-kinase inhibitors fasudil ($5 \times 10^{-5}$ M) and Y-27632 ($5 \times 10^{-5}$ M) inhibited contractions to in both control and ethanol-treated parenchymal strips. In addition, the relaxations induced by fasudil ($10^{-4}$ M) and Y-27632 ($5 \times 10^{-4}$ M) on parenchymal strips contracted by phenylephrine but not KCl was decreased in ethanol-treatment group. Also, RhoA, ROCK1 and ROCK2 expressions were detected in mouse lung parenchymal tissue. In ethanol-treated group, expression of RhoA and ROCK1 but not ROCK2 decreased compared to control. Furthermore, ethanol causes apoptotic changes in alveolar type I epithelial cells of parenchymal tissue. These results suggest that RhoA/Rho-kinase signaling pathway plays an important role in phenylephrine- and KCl-induced $Ca^{2+}$ sensitization in mouse lung parenchymal tissue. Also, ethanol may be decrease phenylephrine- and KCl-induced contraction due to lowering the RhoA/Rho-kinase-mediated $Ca^{2+}$-sensitizing by inhibiting RhoA/Rho-kinase pathway in parenchymal tissue. These results may be lead to important insights into the mechanisms of lung diseases due to alcohol consumption.



## 1. Introduction

The pharmacological response of lung parenchymal strip has been attributed to the presence of contractile components including bronchiolar smooth muscle (Lulich et al., 1976; Goldie et al., 1982), parenchymal alveolar interstitial myofibroblasts (Kapanci et al., 1974) and vascular smooth muscle (Goldie et al., 1982). $Ca^{2+}$-dependent activation of myosin light chain kinase (MLCK) is widely accepted the key factor for initiation of smooth muscle contraction, eliciting to phosphorylation of myosin light chain (MLC), leading it to interact with actin (Somlyo and Somlyo, 1994). However, widespread studies reported that the contraction of smooth muscles is also mediated by intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$)-independent pathways (Uehata et al., 1997; Kawano et al., 2002). Since, contractile signaling pathways that directly lead inhibition of MLC phosphatase can enhance contractile force independently from additional increases in $[Ca^{2+}]_i$, this mechanism is called "$Ca^{2+}$ sensitization" (Somlyo and Somlyo, 1994; Chiba and Misawa, 2004). Previous studies have demonstrated that the small G protein RhoA and its downstream effector Rho-kinase/ROK/ROCK play major role in the $Ca^{2+}$ sensitization of smooth muscle contraction due to inhibition of MLC phosphatase activity (Somlyo and Somlyo, 2000; Fukata et al., 2001). RhoA is a member of the family of small GTPases and is a pivotal player in the modulation of various cellular functions such as motility, smooth muscle contraction, proliferation and apoptosis (Jaffe and Hall, 2005; Loirand et al., 2006). Two isoforms of ROCKs, Rho-associated ROCK1/$ROK_\beta$ and Rho-kinase/ROCK2/$ROCK_\alpha$, were identified (Nakagawa et al., 1996). The various contractor agonists, which activate specific heterotrimeric G protein-coupled receptors (GPCR), increase both

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the intracellular calcium (via $G_q/G_i$ proteins) and calcium sensitization (mainly via $G_{12}/G_{13}$ proteins). Previous evidence from variety smooth muscle preparations (Sward et al., 2003; Sakurada et al., 2003; Ghisdal et al., 2003; Tsai and Jiang, 2006) suggest that GTPase RhoA-induced Rho-kinase activation may be a main mediator of $Ca^{2+}$ sensitization in response to GPCR activation. Similarly, receptor-dependent role of Rho-kinase in agonist-induced airway smooth muscle contraction has been reported in guinea-pig trachea smooth muscle (Schaafsma et al., 2004). Also, it has been shown that phenylephrine, α-adrenergic receptor agonist, activates Rho-kinase-mediated $MYTP1^{Thr696\ and\ 853}$ phosphorylation and PKC-mediated $CPI\text{-}17^{Thr38}$ phosphorylation to regulate myosin light chain phosphatase (MLCP) activity and MLC phosphorylation (Tsai and Jiang, 2006). On the other hand, KCl has been used as a contractile agent to bypass GPCRs activation. It is generally accepted that high KCl causes depolarization of membrane potential by activating voltage-operated $Ca^{2+}$ channels and elevating $[Ca^{2+}]_i$ levels (Ratz et al., 2005). Several studies have been reported that KCl can cause $Ca^{2+}$ sensitization and ROCK occurs to be contributing in KCl-induced $Ca^{2+}$ sensitization (Sakurada et al., 2003; Urban et al., 2003; Ratz et al., 2005).

Alcohol (ethanol) is involved in many deleterious effect on the respiratory system, such as respiratory distress syndrome (Moazed and Calfee, 2014), pneumonia (Chalmers et al., 2009; Mehta et al., 2013). The attenuation of smooth muscle contraction by ethanol-treatment have been demonstrated in several studies (Boselli and Govoni, 2000; Kesim et al., 2004; Verde et al., 2014). Furthermore, the effects of ethanol on RhoA activity have been reported and these effects depend on the type of tissue and ethanol exposure conditions. In the airway epithelium, short-term alcohol exposure caused to attenuation of RhoA activity via the NO/cGMP/PKG pathway (Bailey et al., 2011). On the other hand, acute and chronic ethanol treatment did not alter RhoA activity in aorta and liver, respectively (Schaffert et al., 2006; Je et al., 2012). There are contradictory studies about the effect of ethanol on RhoA/Rho-kinase pathway.

To our knowledge, the underlying mechanisms of the increased contractile responsiveness of parenchymal tissue, and the role of RhoA/Rho-kinase pathway in contractions produced with agonist and high KCl has not been studied in lung parenchymal tissue. In the present study, we examined the possible involvement of RhoA/Rho-kinase pathway in contractile responses to phenylephrine and KCl of mouse lung parenchymal tissues. The effect of ethanol on RhoA/Rho-kinase pathway in the lung parenchymal tissue or what mechanism is responsible from the alterations has not been clarified. Also, we investigated the role of RhoA/Rho-kinase pathway in the inhibitory effect of ethanol on the contraction induced by phenylephrine and KCl of lung parenchymal tissues. With this purpose we performed functional, histological and molecular studies in parenchymal lung strips isolated from control and ethanol-treated mice. We firstly determined RhoA, ROCK1 and ROCK2 protein expression of lung tissues isolated from mice. To explore whether RhoA/Rho-kinase-dependent $Ca^{2+}$ sensitization plays a role in the phenylephrine- and high KCl-induced contraction in mouse lung parenchymal tissue, we studied the effects of the structurally unrelated Rho-kinase inhibitors, fasudil and Y-27632, on contractions of parenchymal strips. Furthermore, electron microscopy studies were evaluated in lung tissues isolated from ethanol-treated mice.

## 2. Materials and methods

### 2.1. Experimental animals and ethanol-treatment

Male Swiss albino mice weighing 20–25 g were obtained from Cukurova University Experimental Research and Application Center of Medical Science (DETAUM). Mice were located in Plexiglas cages and kept under environmentally controlled conditions (12 h light/darkness cycles) and allowed free access to food and water. Protocols were approved by local Ethic Committee of the University of Cukurova. Two groups of animals were used as ethanol-treated mice and control mice.

### 2.2. Ethanol treatment

For exposure via the inhalation route, we used a method described by Goldstein and Pal (1971). Mice put in an inhalation chamber and continuously exposed to ethanol vapor by inhalation for 14 days. The inhalation chamber consisted of Plexiglas box (7.5 L) through which ethanol vapor continuously passed. The 95% ethanol delivered to filter paper at a rate of 0.0035 ml/min by an infusion pump. The control group was not exposed to ethanol vapor but only placed in air vapor chambers under conditions similar to those of the ethanol. At the end of the 2 weeks, control and ethanol-treated mice were removed from the chamber. In our previous study, increased blood ethanol level was detected by this model (Aydinoglu et al., 2008). Blood alcohol levels were monitored throughout the experiments and showed elevated levels of alcohol in the ethanol groups of mice. As previously reported, no significant weight loss or nutritional disturbances were observed in mice treated with ethanol.

### 2.3. Tissue preparations

Control and ethanol-treated male mice were killed by cervical dislocation. The lungs from both control and ethanol-treated mice were removed and two slices were cut from the left lobes. Tissues were placed in a Petri dish containing Krebs Henseleit solution (composition in mM; NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, $NaPO_4$ 1.2, glucose 11). Lung strips were mounted under 0.2 g tension in organ bath (10 ml) containing Krebs solution. The bath medium was maintained at 37 °C and gassed with 5% $CO_2$ and 95% $O_2$. The tissue strips were allowed to equilibrate for a period of 60 min during equilibration and the bath solution was replaced every 15 min. The responses were recorded with isotonic transducer (Ugo Basile, 7006) on a recorder (Ugo Basile Gemini, 7070).

### 2.4. Functional studies

To investigate the underlying mechanisms of contractile responsiveness of parenchymal tissue, agonist (phenylephrine, $\alpha_1$-receptor agonist)- and depolarizing (KCl)-induced contractions were obtained in isolated lung tissues from control and ethanol-treated mice as follows. In the first series of experience, phenylephrine-induced contractions were investigated. Following to equilibration period of 60 min, parenchymal strips were exposed to 80 mM KCl in order to calculate percentage of contractile response to phenylephrine. The tissues were then washed out with Krebs solution and the strips were left to re-equilibration for 30 min. After this period, cumulative phenylephrine ($10^{-8}$–$10^{-4}$ M) concentration–response curve was obtained. In the second series of experience, KCl-induced contractions were studied in parenchymal tissue strips isolated from both groups. In this set of experiments, following to equilibration period tissues were precontracted with phenylephrine ($10^{-4}$ M) to calculate to percentage of contractile response to KCl. The tissue was then washed out with Krebs solution and strips were left re-equilibration for 30 min. After this period, cumulative KCl (10–80 mM) concentration–response curve was obtained. Only one agonist or KCl concentration–response curve was studied one each tissue.