



## Effect of Kisdolone on Isolated Rat's Tracheal Smooth Muscle

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**Purpose:** Prednisolone (Kisdolone) is an anti-inflammatory drug that is mainly used for patients with chronic obstructive airway diseases (COAD), such as asthma and chronic obstructive pulmonary diseases (COPD). The objective of this study was to determine the effects of Kisdolone on the trachea of rats *in vitro*. **Materials and Methods:** We tested the effectiveness of Kisdolone on isolated rat trachea submersed in Krebs solution in a muscle bath. Changes in tracheal contractility in response to the application of parasympathetic mimetic agents were measured. The following assessments of Kisdolone were performed: (1) Effect on tracheal smooth muscle resting tension; (2) effect on contraction caused by  $10^{-6}$  M methacholine; (3) effect of the drug on electrically-induced tracheal smooth muscle contractions. **Result:** Our results demonstrated that no significant effects were induced by the addition of  $10^{-8}$ – $10^{-5}$  M Kisdolone on tracheal tension after methacholine treatment, indicating that Kisdolone had no anti-cholinergic effect. It alone had a minimal effect on the basal tension of the trachea as the concentration increased. Furthermore, Kisdolone did not affect electrical field stimulation-induced spike contraction, which represent the Kisdolone could not antagonize the parasympathetic innervation responsible for trachea smooth muscle contraction. **Conclusion:** The immediate effect of Kisdolone on COAD may be indirectly. Therefore, solely use of inhalation Kisdolone without  $\beta_2$ -agonist in treating acute asthma or COPD attack may be more suboptimal than co-administration of them.

Key words: Prednisolone, *in vitro* study, trachea, smooth muscle, cholinergic effect, electrical field stimulation

### INTRODUCTION

Chronic obstructive airway disease (COAD) is a type of obstructive lung disease characterized by chronically poor airflow. It typically worsens over time. The main symptoms include shortness of breath, cough, and sputum production.<sup>1</sup> There is no known cure for COAD, but the symptoms are treatable and its progression can be delayed.  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) agonists and the glucocorticoids, both typically being administered by inhalation, are the common two drug classes used in COAD.<sup>2</sup>  $\beta_2$ AR agonists worked primarily on airway smooth muscle cells, causing relaxation within seconds or minutes, whereas glucocorticoids primarily improved airway function via their anti-inflammatory action,

the genomic pathway, within hours or days.<sup>3,4</sup> It is well known that the glucocorticoid can synergize with the spasmolytic action of  $\beta_2$ AR. Therefore, co-administration of them is an optimal treatment for many patients since their combination can be more effective than either monotherapy. In recent studies, researchers showed that glucocorticoids can also exert much more rapid response, probably through the nongenomic pathway,<sup>5</sup> corresponding to the stimulation within seconds or minutes. However, most of those studies are mainly emphasize and explain how glucocorticoids help  $\beta_2$ AR in relaxing the constricting smooth muscle. Seldom studies discuss the direct effect of single use of glucocorticoids on airway tone during an acute COAD attack.

During an acute COAD attack, the trachea plays an important role in reducing pulmonary function as it contracts. Smooth muscle, the main structure of the airway walls, plays a major role in the contraction of the trachea. Its excessive contraction may be one of the crucial factors that directly cause the COAD syndrome.<sup>6</sup> Therefore, the effect of glucocorticoids on the airway smooth muscle merits further exploration. Our previous report developed a simple and rapid test for screening parasympathetic mimetic agents, and potential tracheal contraction agents.<sup>7</sup> We try to use this technique to identify how glucocorticoids affect the isolated trachea smooth muscle

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directly *in vitro*. Understanding of these information will further future pharmacotherapeutics strategies.

## MATERIALS AND METHODS

### Rat trachea tissue preparation

Twenty rats were anesthetized by intraperitoneal administration of pentobarbital (60 mg/kg), and two pieces of trachea about 5 mm in length were removed from each rat. The equipment and process were designed based on our previous study.<sup>7,8</sup> The tracheal specimen was mounted using two steel plates and submersed in a 30-mL muscle bath at 37°C. The bath was filled with 30-mL of Krebs solution consisting of (mmol/L): NaCl (118), KCl (4.7), CaCl<sub>2</sub> (2.5), MgSO<sub>4</sub> 7H<sub>2</sub>O (1.2), KH<sub>2</sub>PO<sub>4</sub> (1.2), NaHCO<sub>3</sub> (25.0), and glucose (10.0). The upper side of the tracheal strip was attached to a Grass FT-03 force displacement transducer (AstroMed, West Warwick, RI, USA) using a steel plate and a 3-0 silk ligature. The other side of the strip was fixed to a steel plate attached to a bath. A passive tension of 0.5 g was applied to the strips and subsequent changes in tension were recorded continuously using Chart V4.2 software (PowerLab; AD Instruments, CO Springs, CO). The chemicals used were of the highest purity available. All chemical reagents were obtained from Sigma (St. Louis, MO, USA).

### Methacholine test

We tested methacholine as a tracheal contraction drug. This contracting agent is a synthetic choline ester that acts as a nonselective cholinergic agonist. It is worthy of note that the drug-induced relaxation of tissue was dependent on prior partial contraction of the smooth muscle in response to methacholine. Preliminary tests showed the tracheal strip immersed in the bath solution used for subsequent experiments did not contract when basal tension was applied. Before drug assays were conducted, isolated tracheas were equilibrated in the bath solution for 30-45 min, during which continuous aeration with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> was applied. Stepwise increases in the amount of drugs used were used to study the contraction or relaxation responses of tracheal strips. When the concentration of Kidsolone is 10<sup>-5</sup> M, we add xylocaine, which is classified as an anti-cholinergic drug, to thoroughly assay the effect of Kidsolone on 10<sup>-6</sup> M methacholine-induced contraction. All drugs were administered by adding a defined volume of stock solution to the tissue bath solution. In each experiment, one untreated strip served as a control.

### Electrical field stimulation test

Electrical field stimulation (EFS; 5 Hz, 5-ms pulse duration, at a voltage range of 30-90 V, trains of stimulation for 5 s)

was applied to the trachea strip with two wire electrodes placed parallel to the trachea strip and connected to a direct-current stimulator (Grass S44; Grass Instrument Co., Quincy, MA, USA). An interval of 2 min was imposed between each stimulation period to allow recovery from the response. Stimulation was applied contiguously to the trachea at 37°C.

### Kidsolone assessments

The following assessments for Kidsolone were performed:

- (1) Effect on tracheal resting tension – this test examined the effect of the drug on the simulating condition of the resting trachea condition;
- (2) Effect on contraction caused by 10<sup>-6</sup> M of methacholine – this procedure was concerned with examining postsynaptic events such as muscle receptor blockade, enhancement, and second messengers; and
- (3) Effect of Kidsolone on electrically induced contractions – electrical stimulation of this tissue causes parasympathetic nerve remnants in the trachea to release the transmitter acetylcholine.

If there is interference with transmitter release, electrical stimulation does not cause contraction. Thus, presynaptic events were seen more easily with this procedure. Concentrations of drugs are expressed as concentrations present in the 30-mL bath solution. In each experiment, repetitions were performed at least 6 times.

### Statistical analysis

Data were presented as mean values and standard deviation. The differences between mean values were compared using Student's *t*-test, and were assumed to be significant at *P* < 0.05.

### Ethical considerations

The research protocol has been reviewed and approved by an animal experiment review board (IACUC-08-188).

## RESULTS

The degree of contraction or relaxation of tracheal strips was estimated from the tension applied to the transducer. Tracheal contraction induced by a small dose of methacholine was easily detected (not shown), and the tissue remained in a contracted state until the drug was rinsed from the tissue.

### The pharmacologic effects on tracheal contraction caused by methacholine

Adding the glucocorticoid, Kidsolone, to the basal tension had a negligible effect [Figure 1]. It resulted in no relaxation

Effect of Kidsolone on rat’s trachea

of the trachea when introduced after adding a constricting agent such as  $10^{-6}$  M of methacholine. As the concentration of Kidsolone increased from  $10^{-8}$  to  $10^{-5}$  M, it had no effect on contraction [Figure 2]. At  $10^{-8}$  M of Kidsolone, the tension was  $99.2 \pm 0.8\%$  of the control values. At  $10^{-6}$  and  $10^{-5}$  M of Kidsolone, the tensions were  $99.2 \pm 0.5\%$  and  $99.5 \pm 0.8\%$ , respectively [Table 1]. The difference in tension among the specimens treated with  $10^{-8}$  M of Kidsolone and  $10^{-6}$  or  $10^{-5}$  M of Kidsolone was not statistically significant. The total relaxation of the  $10^{-6}$  M methacholine–induced contracted tracheal strip was observed when adding  $10^{-4}$  M of xylocaine among the specimens treated with Kidsolone [Figure 2].

The pharmacologic effects on electrically induced tracheal contractions

Kidsolone could not inhibit EFS-induced spike contraction [Figure 3]. The peak tension of the tracheal strip evoked by EFS upon the addition of  $10^{-8}$  M Kidsolone was  $101 \pm 1.9\%$ , whereas at  $10^{-6}$  and  $10^{-5}$  M Kidsolone the peaks were  $102 \pm 2.4\%$  and  $103 \pm 1.4\%$ , respectively [Table 1]. The difference of tension among  $10^{-8}$  M Kidsolone and  $10^{-6}$  or  $10^{-5}$  M Kidsolone was not statistically significant.

DISCUSSION

Chronic obstructive airway disease, including asthma and chronic obstructive pulmonary diseases (COPD), has become one of the most prevalent diseases and is increasing throughout the world, particularly in developing countries.<sup>9</sup> Airway remodeling is a classic feature, which is characterized by changes in the airway tissue architecture, including increased extracellular matrix deposition and airway smooth muscle accumulation.<sup>10</sup> It is also characterized by variable airflow obstruction that is secondary to an allergic pattern of inflammation in the airways, which involves infiltration with inflammatory cells and the activation of resident mast cells and dendritic cells by allergens.<sup>11</sup> Structural cells, such as airway epithelial cells and smooth muscle cells, are important sources of inflammatory mediators as well as activated inflammatory cells. Multiple inflammatory mediators are involved, and many cytokines and chemokines orchestrate this complex chronic inflammation.

Major drug classes used in COAD encompass bronchodilators that act mainly by reversing airway smooth muscle contraction, and anti-inflammatory drugs, which suppress inflammation in the airways.  $\beta_2$ -agonists are by far the most effective bronchodilators for obstructive airway disease.<sup>1</sup> It can relax airway smooth muscle cells by activating the adenylyl cyclase via the stimulatory G-protein ( $G_s$ ).

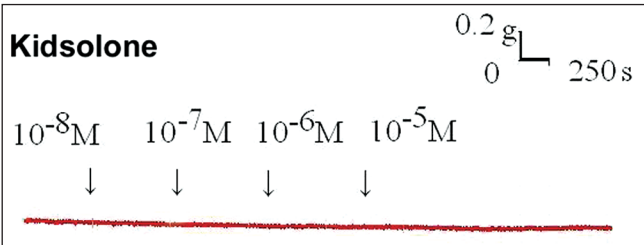


Figure 1. Tension changes in rat trachea after applying various Kidsolone concentrations. Kidsolone alone had a minimal effect on the basal tension of the trachea as the concentration increased. Original basal tension was 0.3 g

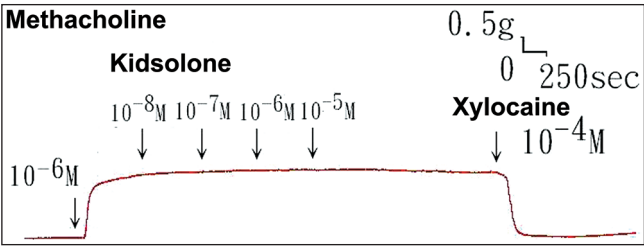


Figure 2. Original recording of the effects of Kidsolone on  $10^{-6}$  M of methacholine-induced contraction of rat trachea. Kidsolone had a minimal effect as the concentration increased. The total relaxation was observed when adding  $10^{-4}$  M of xylocaine among the specimens treated with Kidsolone

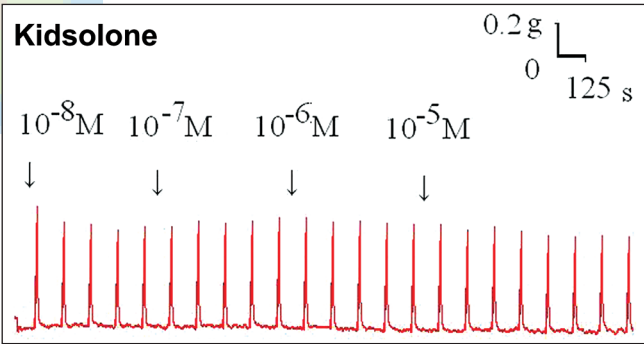


Figure 3. Original recording of effects of Kidsolone on electrically induced tracheal contractions was noted. Kidsolone had a minimal effect as the concentration increased

Table 1: Summary of Kidsolone effect on tracheal contractions

| n = 6              | Percentage of contraction induced by |         |
|--------------------|--------------------------------------|---------|
|                    | Methacholine (%)                     | EFS (%) |
| Control            | 100                                  | 100     |
| Kidsolone          |                                      |         |
| 10 <sup>-8</sup> M | 99.2±0.8                             | 101±1.9 |
| 10 <sup>-7</sup> M | 99.4±1.0                             | 103±1.9 |
| 10 <sup>-6</sup> M | 99.2±0.5                             | 102±2.4 |
| 10 <sup>-5</sup> M | 99.5±0.8                             | 103±1.4 |

EFS = Electrical field stimulation

This increases intracellular cAMP will lead to subsequent phosphorylation of several target proteins within the cell, leading to activation of myosin light chain phosphatase and inhibition of myosin light chain kinase and thus relaxation of airway smooth muscle.<sup>6</sup> In addition,  $\beta_2$ ARs are also directly coupled to calcium-activated potassium channels ( $K_{Ca}$ ) via  $G_s$ , so relaxation of airway smooth muscle may occur independently of an increase in cAMP. However, long-term agonist exposure can lead to  $\beta_2$ ARs desensitization by up-regulation of cAMP-specific phosphodiesterases and down-regulation of  $G_s$ .<sup>4</sup> Certain calcium-mobilizing contractile agonists, particularly muscarinic cholinergic agonists (e.g., acetylcholine [Ach]), directly inhibit adenylyl cyclase activity. Therefore, anti-cholinergics may have some additive bronchodilator effect.

Glucocorticoids are the most effective therapy for controlling COAD.<sup>12</sup> The inflammation of COAD is confined to the airways, so inhalational steroid therapy has been found to be the most effective treatment modality and largely avoids systemic side effects. Glucocorticoids can modulate the transcription of many inflammatory mediators in several cell types.<sup>13</sup> Glucocorticoid receptor dimers bind to glucocorticoid recognition elements to activate or to inhibit corticoid-sensitive genes via mechanisms known as trans-activation and trans-repression.<sup>6</sup> Genes that are switched on by glucocorticoids include genes encoding  $\beta_2$ AR and the anti-inflammatory proteins secretory leukoprotease inhibitor and mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1), which inhibits MAPK pathways.<sup>14,15</sup> These effects may contribute to the anti-inflammatory actions of glucocorticoids. The major action of glucocorticoids in treating COAD is to switch off multiple activated inflammatory genes that code for cytokines, chemokines, adhesion molecules, inflammatory enzymes, and receptors, including the suppression of  $T_H2$  cell and  $T_H2$  cytokines (interleukin-4 [IL-4], IL-5, and IL-13). These genes are switched on in the airways by proinflammatory transcription factors, such as nuclear factor-kappa B and activator protein-1, both of which are usually activated at sites of inflammation in asthma and COPD. In addition, *in vitro* studies have indicated that glucocorticoids inhibit proliferation of airway smooth muscle cells, indicating that they can also alter airway smooth muscle contractile properties.<sup>16</sup> Glucocorticoids reduce the production of extracellular matrix proteins and, therefore, inhibit airway smooth muscle remodeling.

In many patients, the two above-mentioned drug classes are co-administered because their combination can be more effective than either monotherapy. It is now recognized that there are beneficial molecular and biochemical interactions between  $\beta_2$ ARs and glucocorticoid-activated pathways.

Glucocorticoids increase transcription of the  $\beta_2$ -receptor gene, resulting in increased expression of cell surface receptors. In this way, glucocorticoids protect against the downregulation of  $\beta_2$ -receptors after long-term administration.<sup>17</sup> Glucocorticoids may also enhance the coupling of  $\beta_2$ -receptors to  $G_s$ , thus enhancing  $\beta_2$ -agonist effects and reversing the uncoupling of  $\beta_2$ -receptors that may occur in response to inflammatory mediators, such as IL-1 $\beta$ , through a stimulatory effect on G-protein receptor kinase-2.<sup>18</sup> Meanwhile, glucocorticoids can inhibit the production of proinflammatory cytokines, which have been shown to attenuate  $\beta_2$ -agonist-mediated relaxation of tracheal smooth muscle segments via enhanced expression of inhibitory G-proteins ( $G_i$ ). In airway smooth muscle cells, glucocorticoids and  $\beta_2$ -agonists also synergize to accelerate nuclear translocation of the glucocorticoid receptor and C/EBP $_{\alpha}$  (CCAAT/enhancer binding protein  $\alpha$ ), resulting in the inhibition of airway smooth muscle proliferation.<sup>19</sup>

However, it is unclear whether the benefits of glucocorticoids in COAD are merely based on anti-inflammatory properties, the genomic pathway, via the intracellular glucocorticoids receptor. The genomic effect of glucocorticoid is known to occur with a time lag of hours or even days. Research has also identified some stress-mediated responses of glucocorticoids that are much more rapid and take place in seconds or minutes, probably by the action at the membrane-level of cells to exert rapid nongenomic effects. These effects have been shown to modulate hormone secretion, neuronal excitability, carbohydrate metabolism, cell morphology, cell behavior, and other processes.<sup>20</sup> This action might also be important from a clinical point of view, as glucocorticoids induce short-term amelioration for COAD, rather than its long-term effects as anti-inflammatory and immunosuppressant agent in some chronic diseases. Sun *et al.* has shown that the high concentration of glucocorticoids could exert rapid spasmolytic effects on the contraction in guinea pig tracheal smooth muscle induced by histamine.<sup>21</sup> The authors conclude that glucocorticoids have nongenomic effects in airway smooth muscle cells because the application of RU486, the selective glucocorticoid receptors antagonist, and actidione, the blocker of protein synthesis, did not affect the spasmolytic effect of glucocorticoids. Rapid glucocorticoids actions are triggered by, or at least dependent on, membrane associated G-protein-coupled receptors and activation of downstream signaling cascades. However, in our study, using Kisdolone alone had a minimal effect on the basal tension of the trachea as the concentration increased. Meanwhile, Kisdolone resulted in no relaxation of the trachea smooth muscle which was presensitized by methacholine. However, the total relaxation of the contracted tracheal strip was observed when adding  $10^{-4}$  M of xylocaine.



The previously indicated Kisdolone along had no direct cholinergic or anti-cholinergic effect. The nongenomic effect of glucocorticoids seems to only influence the histamine-sensitized tracheal smooth muscle constriction at high concentration level. Wang *et al.* also confirm this view-point and show that glucocorticoids can synergize with  $\beta_2$ -agonist to relief the muscle-constricting effect of histamine, only when its concentration is above a threshold.<sup>5</sup> These rapid actions independent of the genome might be transduced by pleiotropic signaling pathways. Downstream from the putative membrane receptors, various signaling pathways have been implicated in the rapid actions of glucocorticoids in different cell types, and  $G_q$ -phospholipase C-protein kinase C is one of them. Those changes in these signal molecules probably have clinical therapeutic effect only while  $\beta_2$ -agonist and glucocorticoids are co-administrated. Therefore, solely use of glucocorticoids without  $\beta_2$ -agonist in treating acute asthma or COPD attack may be suboptimal since our finding confirms that glucocorticoids had no direct anti-cholinergic effects *in vitro*. Co-administration of the  $\beta_2$ -agonist and glucocorticoids in acute COAD attack patients remains the best treating strategy.

Electrical field stimulation is a common experimental tool activating the nerve terminals within the tissue to be tested and inducing the release of endogenous neurotransmitters, thereby triggering the smooth muscle to contract. In this study, EFS-induced spike contraction of the tracheal smooth muscle was believed to be from the stimulation of parasympathetic innervation. Therefore, EFS-induced contraction of the trachea did not decrease as the Kisdolone concentration was increased. These findings suggest that Kisdolone could not antagonize the parasympathetic innervation responsible for trachea smooth muscle contraction, and the effect of Kisdolone on COAD may be indirectly.

Our study was simple and effective. An intact tracheal ring was an important component of our technique. The isolated tracheal preparations used in our experiments were excised from rats without damaging the endothelium or smooth muscle. Therefore, it is reasonable to assume the tracheal responses to test agents in our study are comparable with those observed after applying an inhaler to the trachea during the COAD attack. However, since this was an *in vitro* study, there are reservations as to its comparability with an *in vivo* situation in humans. In the *in vivo* situation, the response might be much more complicated than that in the *in vitro* situation. Therefore, the results of our experiments still should be interpreted within the context of the test materials used. Further investigation is needed to elucidate the precise molecular mechanism of the glucocorticoids action and define the downstream targets of its pathway.

## CONCLUSION

In conclusion, this study indicates Kisdolone had no cholinergic or anti-cholinergic effect, nor did it have an effect on tracheal basal tension. The Kisdolone also could not antagonize the parasympathetic innervation responsible for trachea smooth muscle contraction. The immediate effect of Kisdolone on COAD may be indirectly. Therefore, solely use of inhalation Kisdolone without  $\beta_2$ -agonist in treating acute asthma or COPD attack may be more suboptimal than co-administration of them.

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