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To cite this article: Ming-Kai Tsai, Yi-Ching Lin, Ming-Yii Huang, Min-Sheng Lee, Chang-Hung Kuo, Po-Lin Kuo, Ching-Hsiung Lin & Chih-Hsing Hung (2017): The effects of asthma medications on reactive oxygen species production in human monocytes, Journal of Asthma, DOI: 10.1080/02770903.2017.1339798

To link to this article: <http://dx.doi.org/10.1080/02770903.2017.1339798>



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Accepted author version posted online: 11 Jul 2017.
Published online: 11 Jul 2017.



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The effects of asthma medications on reactive oxygen species production in human monocytes

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ABSTRACT

Objective: Asthma is a chronic inflammatory airway disease induced by many environmental factors. The inhalation of allergens and pollutants promotes the production of reactive oxygen species (ROS) leading to airway inflammation, hyper-responsiveness, and remodeling in allergic asthma. The effects of asthma medications on ROS production are unclear. The present study investigated the anti-ROS effects of current asthma medications including inhaled corticosteroid (ICS; budesonide and fluticasone), leukotriene receptor antagonist (LTRA; montelukast), long-acting β_2 agonists (LABAs; salmeterol and formoterol), and a new extra-LABA (indacaterol). **Methods:** The human monocyte cell line THP-1 cells were pre-treated with different concentrations of the asthma medications at different time points after hydrogen peroxide (H_2O_2) stimulation. H_2O_2 production was measured with DCFH-DA by flow cytometry. **Results:** Montelukast, fluticasone, and salmeterol suppressed H_2O_2 -induced ROS production. Indacaterol enhanced H_2O_2 -induced ROS production. Budesonide and formoterol alone had no anti-ROS effects, but the combination of these two drugs significantly suppressed H_2O_2 -induced ROS production. **Conclusions:** Different asthma medications have different anti-ROS effects on monocytes. The combination therapy with LABA and ICS seemed not to be the only choice for asthma control. Montelukast may also be a good supplemental treatment for the poorly controlled asthma because of its powerful anti-ROS effects. Our findings provide a novel therapeutic view in asthma.

ARTICLE HISTORY

Received 30 November 2016
Revised 18 May 2017
Accepted 4 June 2017

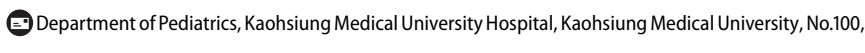
KEYWORDS

Indacaterol; inhaled corticosteroid (ICS); leukotriene receptor antagonist (LTRA); long-acting β_2 agonist (LABA); montelukast

Introduction

Environmental factors are important for asthma. Much evidence supports that the inhalation of aggravating compounds such as allergens and pollutants could increase the production of reactive oxygen species (ROS), which would lead to oxidative stress-induced damage to the respiratory system in allergic asthma [1–3]. Airborne particulate matter (PM) components from fossil fuel combustion can also induce oxidative stress initiated by ROS [4]. Although human lungs have a potent antioxidant system, excessive oxidative and nitrative stress leads to an imbalance of oxidants/antioxidants.

Asthma medications reduce airway inflammation and relieve asthma symptoms. Leukotriene modifiers, long-acting β_2 -adrenoreceptor agonists (LABAs), and inhaled corticosteroids (ICSs) are common long-term asthma control medications. Leukotriene receptor antagonists (LTRA), such as montelukast, would prevent provoking asthma responses, ameliorate asthma symptoms, improve lung function, and reduce β_2 -agonist use in patients with persistent asthma [5, 6]. β_2 adrenoreceptor agonists are used widely as bronchodilators in treatment of asthma and have important anti-inflammatory effects on eosinophils and neutrophils [7, 8]. Formoterol and

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salmeterol are two inhaled long-acting β_2 -adreno-receptor agonists (LABAs) widely used for the treatment of asthma and chronic obstructive pulmonary disease (COPD). These two common LABAs were reported to have inhibitory effects on the expression of pro-inflammatory cytokines. Formoterol could suppress lipopolysaccharide (LPS)-induced IL-6 expression in a mouse model [9]. Salmeterol could suppress LPS-induced TNF- α production in THP-1 cells [10] and it also reduces the IgE-dependent TNF- α production in human skin mast cells [11]. Indacaterol, an extra-LABA, is new breakthrough for LABA and can be a monotherapy for COPD, but not for asthma. Indacaterol is able to induce a rapid and long-lasting relaxation of airway smooth muscles via a prolonged activation of β_2 -receptors, and provides a persistent bronchodilation due to the prolonged competitive blockade of M₃ muscarinic receptors [12, 13]. ICS is known to be effective as a maintenance medication in persistent asthma. The clinical usefulness of fixed-dose maintenance therapy with ICS/LABA combination inhalers, such as fluticasone/salmeterol and budesonide/formoterol, has been established though the long-term anti-inflammatory effects of these two inhalers are limited [14].

ROS production is an important pathophysiology of asthma; however, the effects of asthma medications on ROS production are still unclear. Monocytes play an important role in asthma pathogenesis. Monocytes of asthmatic patients were shown to produce eminent amounts of reactive nitrogen species, and exhibited higher activities of nitric oxide synthase and total free radicals, the feature that was closely related to asthma severity [15]. THP-1 is a human leukemia monocytic cell line, which has been a common model to estimate modulation of monocyte and macrophage activities [16]. In the present study, we aimed to investigate whether these common asthma medications would suppress ROS productions on THP-1 cells and THP-1 derived macrophages. Our findings broaden the knowledge of anti-ROS effects by treatment with common controller medications in asthma.

Methods

Cell culture

THP-1 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin B at 37°C and 5% CO₂ in a humidified incubator. The cells were re-suspended in fresh media at a concentration of 1×10^5 /mL, 1 mL/well in 24 wells plates or 1×10^6 /mL,

2 mL/well in 6 well plates overnight for attachment. For macrophage polarization, THP-1 cells were cultured with phorbol 12-myristate 13-acetate (20 ng/mL) for 2 days. Hydrogen peroxide (H₂O₂, 400 μ M) (Sigma Chemical, MO, USA) was then added with or without pretreatment of the cells with montelukast, formoterol, salmeterol, fluticasone, or budesonide or with combination of salmeterol/fluticasone and budesonide/formoterol for 30 minutes, 1 hour, and 2 hours. The cells were then collected at 30-minute time point after H₂O₂ stimulation for ROS production assay.

Measurement of H₂O₂ production

H₂O₂ is a main type of ROS. H₂O₂ levels could be measured with DCFH-DA by a flow cytometry [17, 18]. DCFH-DA is an oxidation-sensitive fluorescent probe. Intracellular H₂O₂ or low-molecular-weight peroxides can oxidize DCFH-DA to the highly fluorescent 20, 70-dichlorofluorescein (DCF). Following treatment of asthma medications, the medium was aspirated and cells were incubated with 1 mM DCFH-DA in PBS at 37.8°C for 15 minutes, and cells were then washed twice with serum-free medium for flow cytometry assay by immediate DCF detection. The generation of H₂O₂ was measured by FAC-SCalibur flow cytometer (Becton Dickinson, San Jose, CA). The cells gated on flow cytometry were more than 90% of H₂O₂-stimulated THP-1 cells.

Statistical analysis

We used SPSS 19th edition for Windows for statistical analysis. Data are presented as means $\pm$ SD. Significant differences were assessed using the Mann Whitney test. *p* Values less than 0.05 were considered statistically significant.

Results

The cell viability on flow cytometry between with and without asthmatic medication pretreatment

In the present study, the relevant drug concentrations of the asthmatic medications are mimicked the in vivo situation at the airway level (10^{-10} – 10^{-6} M formoterol and 10^{-8} M budesonide) [19]. The montelukast concentrations we used in the present study are in the range of 10^{-7} – 10^{-5} M, in agreement with cysteinyl leukotriene type 1 receptor antagonist activity that has been demonstrated in previous studies [6, 20]. All concentrations of the asthmatic medications in the present study had no cytotoxic effects on THP-1 cells, and the percentages of the cell viability on flow cytometry were similar between

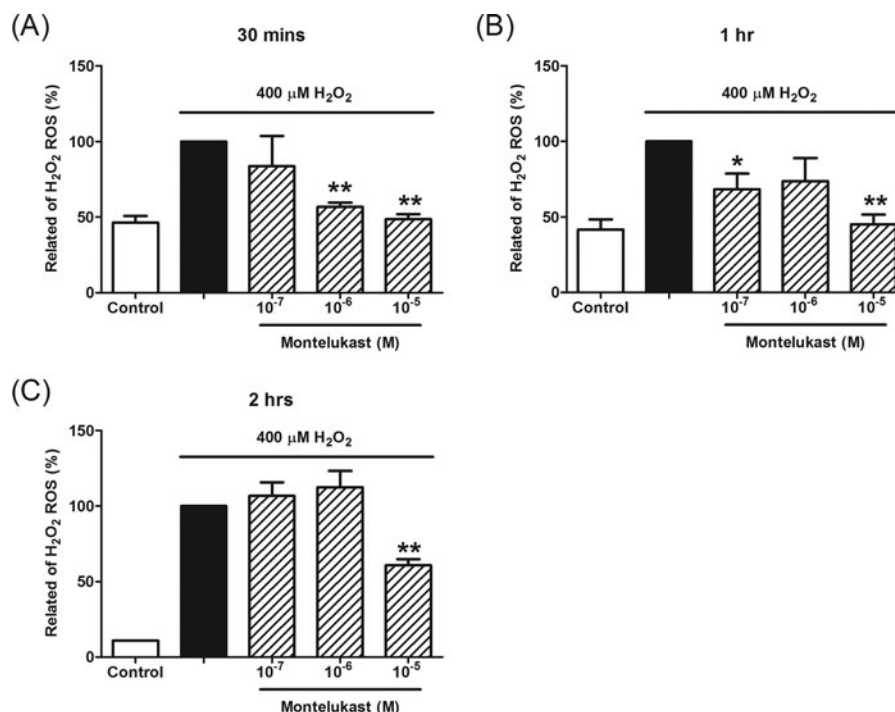


Figure 1. Montelukast suppressed H₂O₂-induced ROS production in THP-1 cells. Montelukast significantly suppressed H₂O₂-induced ROS expression in THP-1 cells at (A) 30-minute, (B) 1-hour, and (C) 2-hour time points. The bars represent the means $\pm$ standard deviations from three individual experiments. The black bar represents the cells under H₂O₂ stimulation without any asthmatic medication pretreatment. * $p < 0.05$ and ** $p < 0.01$ between H₂O₂ stimulation with and without montelukast pretreatment.

with and without asthmatic medication pretreatment in H₂O₂-stimulated THP-1 cells (data not shown).

Montelukast suppressed H₂O₂-induced ROS production in THP-1 cells

H₂O₂ alone could induce ROS production in THP-1 cells at 30-minute, 1-hour, and 2-hour time points (Figure 1A–C). The pretreatment of montelukast (10⁻⁶ M to 10⁻⁵ M) for 30 minutes significantly suppressed H₂O₂-induced ROS expression in THP-1 cells (Figure 1A). Montelukast suppressed ROS production in THP-1 cells at 1 hour after H₂O₂ stimulation even at lower concentrations (10⁻⁷ M and 10⁻⁵ M) (Figure 1B). Montelukast suppressed ROS production in THP-1 cells at 2-hour time point after H₂O₂ stimulation at only higher concentration (10⁻⁵ M) (Figure 1C).

Salmeterol but not formoterol suppressed H₂O₂-induced ROS production in THP-1 cells

Next, we evaluated another common asthma medication LABA's effects on ROS production. Interestingly, salmeterol alone could reduce H₂O₂-induced ROS production in THP-1 cells at only 2-hour time point, but has no obvious effects at 30-minute and 1-hour time points (Figure 2A–C). The pretreatment of another LABA

formoterol (10⁻⁹ M to 10⁻⁷ M) had no effects on the suppression of ROS production in THP-1 cells at 30-minute, 1-hour, and 2-hour time points (Figure 2D–F).

Indacaterol enhanced H₂O₂-induced ROS production in THP-1 cells

We further evaluated extra LABA's effects on ROS production. Interestingly, indacaterol alone enhanced H₂O₂-induced ROS production in THP-1 cells at 30-minute and 1-hour time points (Figure 3A and B). The pretreatment of indacaterol (10⁻⁹ M to 10⁻⁷ M) had no effects on ROS production in THP-1 cells at 2-hour time point (Figure 3C).

Fluticasone but not budesonide suppressed H₂O₂-induced ROS production in THP-1 cells

We also evaluated effects of another common asthma medication, ICS, on ROS production. Fluticasone alone could suppress H₂O₂-induced ROS production in THP-1 cells at 2-hour time point, but has no obvious effects at 30-minute and 1-hour time points (Figure 4A–C). The pretreatment of another ICS budesonide (10⁻⁹ M to 10⁻⁷ M) had no effects on the suppression of ROS production in THP-1 cells at 30-minute, 1-hour, and 2-hour time points (Figure 4D–F).

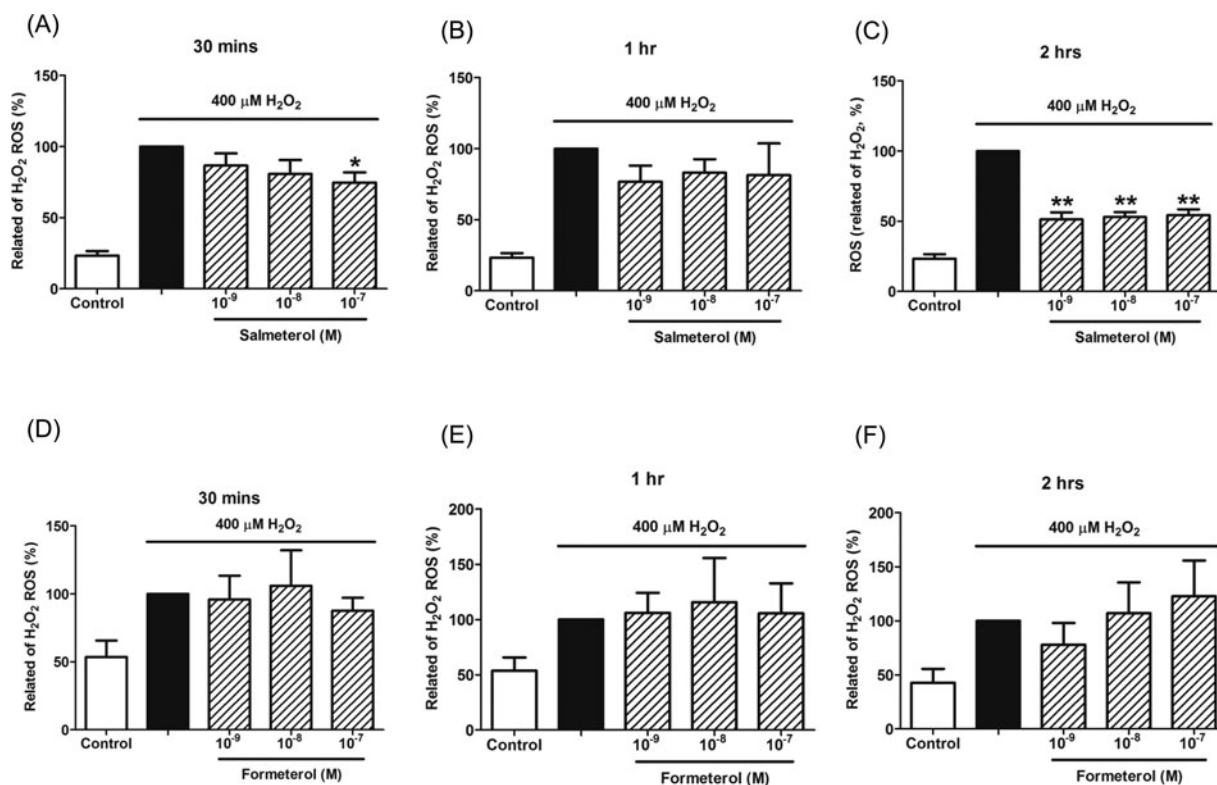


Figure 2. The effects of salmeterol and formoterol on H₂O₂-induced ROS production in THP-1 cells. The higher dose of salmeterol (10⁻⁷ M) could significantly reduce H₂O₂-induced ROS production in THP-1 cells at 30-minute (A), but no effects at 1-hour time point (B). All three concentrations of salmeterol (10⁻⁹ M to 10⁻⁷ M) could significantly reduce H₂O₂-induced ROS production in THP-1 cells at 2-hour time point (C). Formoterol could not significantly suppress H₂O₂-induced ROS expression in THP-1 cells at 30-minute (D), 1-hour (E), and 2-hour (F) time points. The bars represent the means $\pm$ standard deviations from three individual experiments. The black bar represents the cells under H₂O₂ stimulation without any asthmatic medication pretreatment. * $p < 0.05$ and ** $p < 0.01$ between H₂O₂ stimulation with and without salmeterol or formoterol pretreatment.

Salmeterol/fluticasone and formoterol/budesonide suppressed H₂O₂-induced ROS production in THP-1 cells

Since different ICS and LABA have diverse effects on ROS production in THP-1 cells, we further evaluated the effects of ICS plus LABA on ROS production. Interestingly, salmeterol/fluticasone combination could significantly reduce H₂O₂-induced ROS production in THP-1 cells at 2-hour time point, but has no obvious effects at 30-minute and 1-hour time points (Figure 5A–C). The pretreatment of another ICS and LABA combination formoterol/budesonide also suppressed H₂O₂-induced ROS production in THP-1 cells at 2-hour time point, but has no obvious effects at 30-minute and 1-hour time points (Figure 5D–F).

The effects of asthma medications on H₂O₂-induced ROS production in THP-1-derived macrophages

We differentiated the THP-1 cells into macrophage-like cells with phorbol ester phorbol 12-myristate 13-acetate

(PMA) and then tested the effects of asthma medications on H₂O₂ production. At 30-minute, 1-hour, or 2-hour time point after H₂O₂ stimulation, only montelukast had significantly suppressive effects on H₂O₂ production in THP-1-derived macrophage (Figure 6).

Discussion

Oxidative stress has been determined to be important in the pathogenesis of allergic asthma. Exposure to environmental antigens can stimulate overproduction of ROS that augments bronchial hyper-responsiveness and inflammation [1, 21]. ROS and reactive nitrogen species (RNS), such as hydrogen peroxide and nitric oxide, are important mediators of natural physiological processes. Inflammatory cells that are recruited to the asthmatic airways have an exceptional capacity for producing a variety of highly reactive ROS and RNS that lead to tissue damage and chronic airways inflammation. The useful redox-based therapy to attenuate levels of ROS presents a potential strategy to alleviate oxidative stress-induced airway inflammation in patients with asthma. In the present

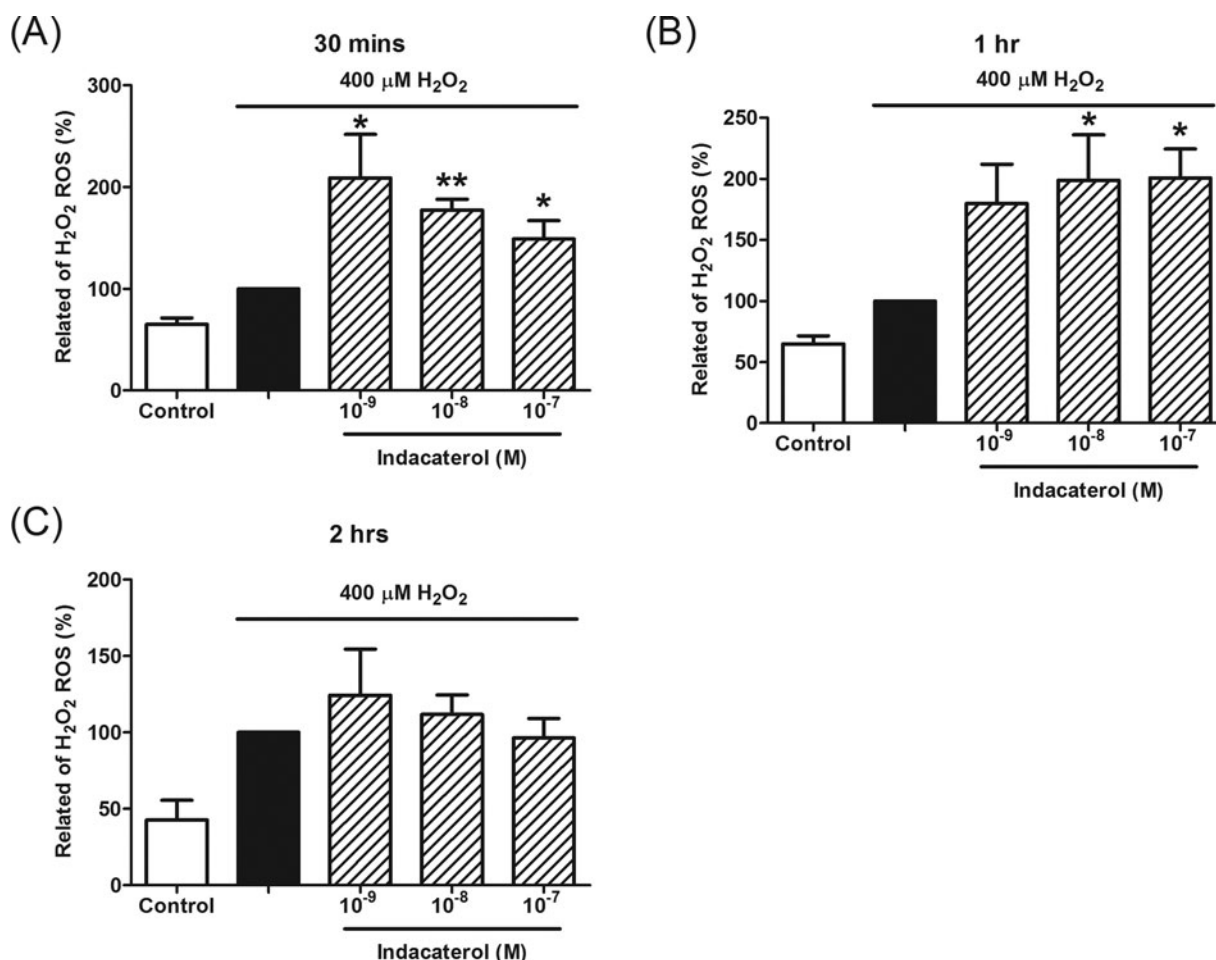


Figure 3. The effects of indacaterol on H₂O₂-induced ROS production in THP-1 cells. Indacaterol could not significantly reduce H₂O₂-induced ROS production in THP-1 cells at 30-minute (A), but significantly enhanced H₂O₂-induced ROS production at 1-hour (B) and 2-hour (C) time points. The bars represent the means $\pm$ standard deviations from three individual experiments. The black bar represents the cells under H₂O₂ stimulation without any asthmatic medication pretreatment. * $p < 0.05$ and ** $p < 0.01$ between H₂O₂ stimulation with and without indacaterol pretreatment.

study, we investigated the anti-ROS effects of several common asthma medications in THP-1 cells. The scheme of our findings is summarized in Table 1.

According to the Global Initiative for Asthma (GINA) guidelines, ICS, LABA, and LTRA are the main controllers for asthma patients. Controller medications should have enough anti-inflammation properties because asthma is an airway inflammatory disease. LTRA or ICS monotherapy is the appropriate therapeutic option for the treatment of step 2 asthma. Montelukast and inhaled budesonide provide acceptable asthma control, whereas budesonide inhalation is favored in children aged 2–8 years with mild persistent asthma [22]. In our present study, we compare the efficacy of LTRA (montelukast) and ICS (budesonide and fluticasone) in ROS production in monocytes. Montelukast and fluticasone, but not budesonide, seem to be able to obviously decrease ROS production in monocytes. Fluticasone seemed to be superior to budesonide in the suppression of ROS production. The cysteinyl

leukotrienes LTC₄, LTD₄, and LTE₄ induce many pathophysiological changes in the lungs of asthma patients, including airflow obstruction, mucus secretion, and inflammatory cell infiltration. It has been reported that LTD₄ stimulation of a Gi/o protein-coupled cysteinyl leukotriene receptor CysLT₁-R triggers the transactivation of the epidermal growth factor receptor through the intervention of phosphatidylinositol-3-OH-kinase and ROS [23]. Montelukast blocks the action of LTD₄ on the CysLT₁-R in the lungs. Some studies suggest that oral montelukast is not inferior to budesonide in young children with mild persistent asthma in terms of control of symptoms [22, 24]. The suppressive effects of montelukast on ROS production might be through the completion of CysLT₁-R binding.

The LABAs are globally used in combination with ICS in moderate-to-severe adult asthma. However, the LABAs are limited in use in pediatric asthma under the age of five. Some studies showed that salmeterol lacks protective

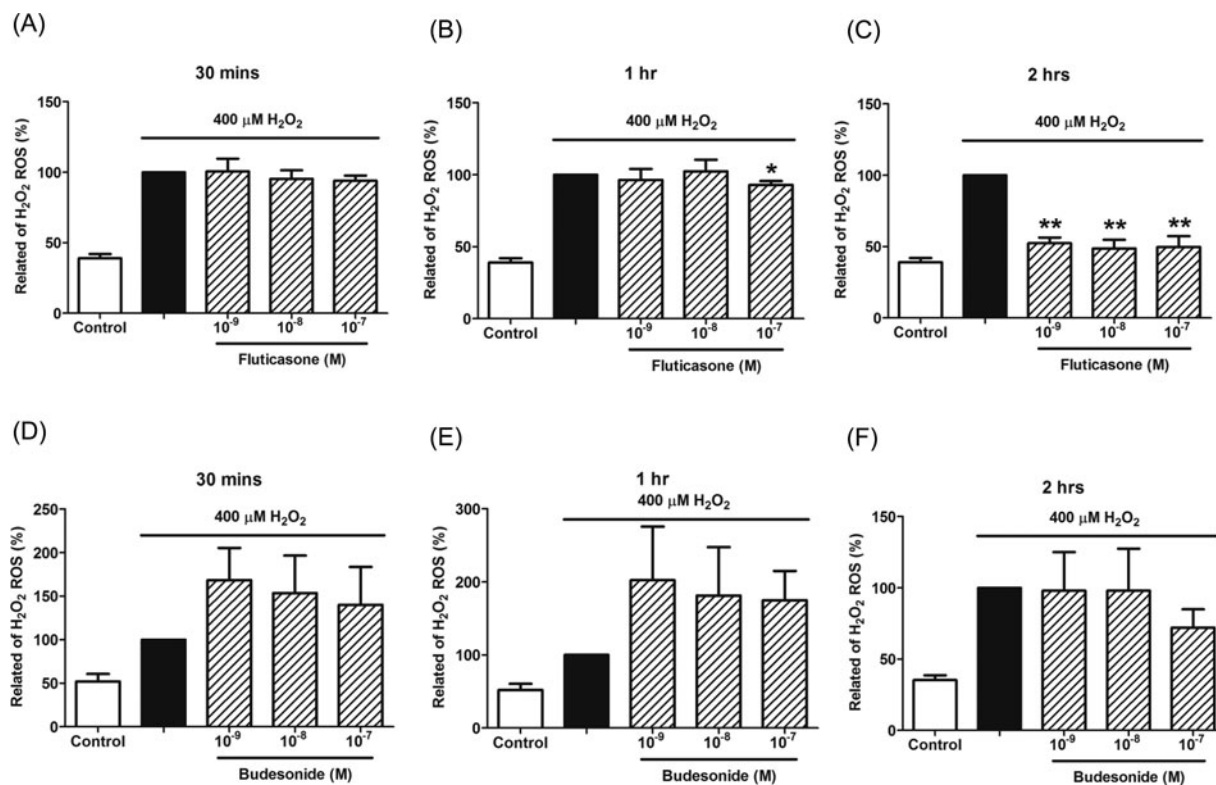


Figure 4. The effects of fluticasone and budesonide on H₂O₂-induced ROS production in THP-1 cells. Fluticasone was not able to significantly reduce H₂O₂-induced ROS production in THP-1 cells at 30-minute time point (A), but a higher concentration of salmeterol (10⁻⁷ M) significantly suppressed H₂O₂-induced ROS production at 1-hour time point (B). Fluticasone could significantly reduce H₂O₂-induced ROS production in THP-1 cells at 2-hour time point (C). Budesonide could not significantly reduce H₂O₂-induced ROS production in THP-1 cells at 30-minute (D), 1-hour (E), and 2-hour (F) time points. The bars represent the means $\pm$ standard deviations from three individual experiments. The black bar represents the cells under H₂O₂ stimulation without any asthmatic medication pretreatment. * p < 0.05 and ** p < 0.01 between H₂O₂ stimulation with and without fluticasone or budesonide pretreatment.

effects on pediatric asthma [25]. In the present study, salmeterol but not formoterol suppressed ROS production in monocyte. Indacaterol greatly enhanced H₂O₂-induced ROS. These findings suggest that different LABAs have different properties. Only salmeterol, but not formoterol or indacaterol could suppress ROS production effectively in monocytes. Unlike salmeterol, indacaterol does not alter the fluidity of the cell membrane, and its long-lasting bronchodilating effect is due to a high affinity of the lipophilic tail of the molecule for the so-called lipid rafts [26]. The “masking effects” while mono-using LABAs in pediatric patients and “increasing the chance of a severe exacerbation” have been concerned [27]. The monotherapy of LABAs has been labeled with “black box” warnings because of reports of the occurrences of severe asthma exacerbations in some patients with asthma, and even with some associated deaths several years ago [28].

ROS production is elicited by several stimuli, such as immunoglobulins and cytokines [29]. It has been previously reported that eosinophils migrate into tissues and release toxic granule proteins and ROS, which lead to tissue damage during the allergic-induced inflammatory process [30]. ICSs pose strongly anti-inflammatory

properties in asthma. In the present study, fluticasone but not budesonide has the suppressive effects on ROS production in monocytes. ICS is generally combined with LABA as an asthma controller. The combination of ICS with LABA is the main therapy for moderate-to-severe asthma. Two fixed combination inhalers (salmeterol/fluticasone and formoterol/budesonide) are increasingly used as a convenient controller in patients with persistent asthma. Our data showed that formoterol/budesonide combination suppressed ROS production in monocytes, even formoterol or budesonide alone has no effects. These findings of anti-ROS effects are compatible with the clinical therapeutic response in asthma.

Macrophages are relevant to the environment of the lung in asthma, and the circulating monocytes are the source of alveolar macrophages. In the present study, we also differentiated the THP-1 cells into macrophage-like cells with PMA and then tested the effects of asthma medications on H₂O₂ production. We found that only montelukast had significantly suppressive effects on H₂O₂ production in THP-1-derived macrophages (Figure 6). The findings between THP-1 cells and THP-1-derived

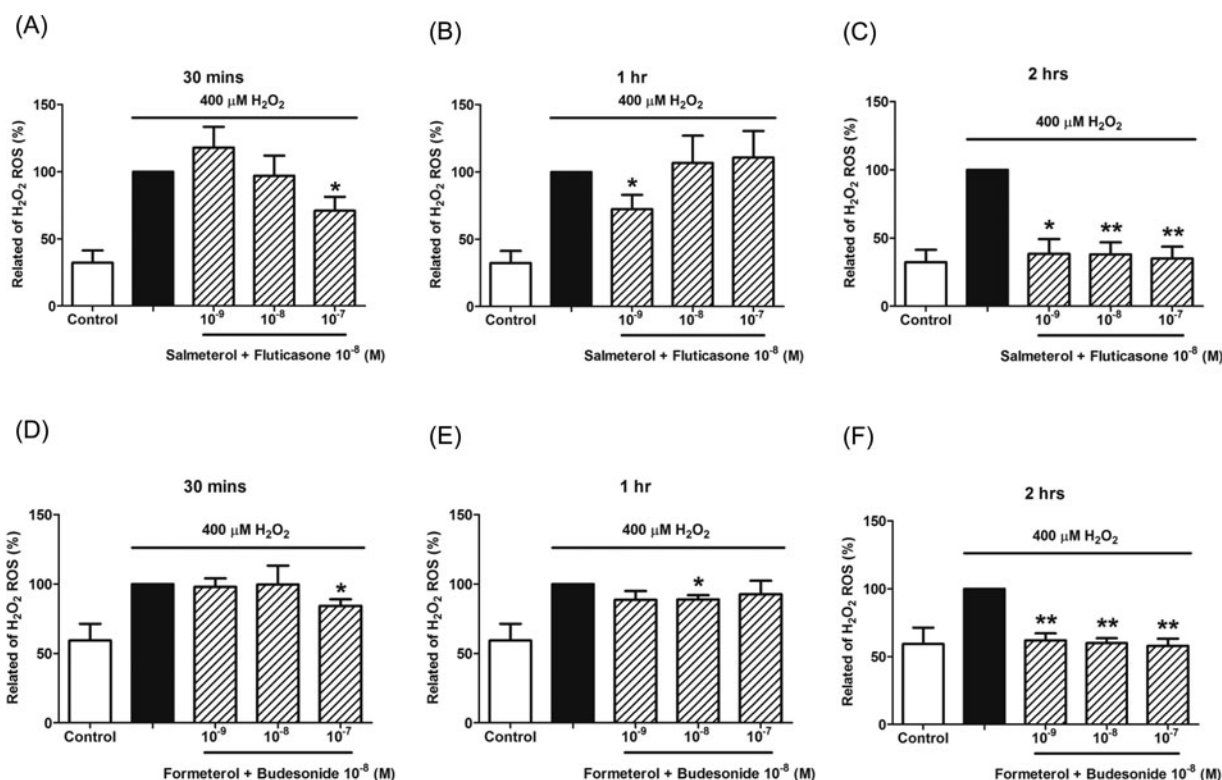


Figure 5. The effects of salmeterol/fluticasone and formoterol/budesonide combination on H₂O₂-induced ROS production in THP-1 cells. Salmeterol/fluticasone combination had no obvious effects at 30-minute (A) and 1-hour (B) time points. However, the pretreatment of salmeterol/fluticasone combination for 2 hours could reduce H₂O₂-induced ROS production in THP-1 cells (C). The pretreatment of formoterol and budesonide has no obvious effects at 30-minute (D) and 1-hour time points (E). (F) The pretreatment of formoterol and budesonide for 2 hours suppressed H₂O₂-induced ROS production in THP-1 cells. The bars represent the means $\pm$ standard deviations from three individual experiments. The black bar represents the cells under H₂O₂ stimulation without any asthmatic medication pretreatment. * $p < 0.05$ and ** $p < 0.01$ between H₂O₂ stimulation with and without asthmatic medication pretreatment.

macrophages might imply that the anti-ROS effects of asthma medications are different between human monocytes and macrophages. Although THP-1 human monocytic leukemia cell line is widely used as an appropriate substitute to human primary monocytes for

studying in vitro [16], the response of THP-1 cells may not always reflect the true physiological response of human primary monocytes. In the present study, we focus the anti-ROS effects of asthma medications only in monocytes/macrophages. However, it is not only

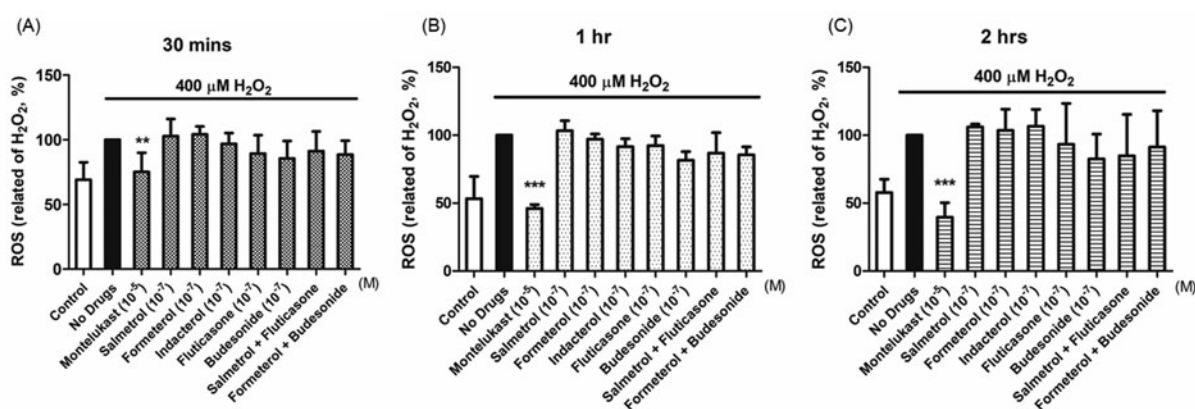


Figure 6. The effects of asthma medications on H₂O₂-induced ROS production in THP-1-derived macrophages. Montelukast significantly suppressed H₂O₂-induced ROS expression in THP-1-derived macrophages at 30-minute (A), 1-hour (B), and 2-hour (C) time points. The bars represent the means $\pm$ standard deviations from at least four individual experiments. The black bar represents the cells under H₂O₂ stimulation without any asthmatic medication pretreatment. ** $p < 0.01$ and *** $p < 0.001$ between H₂O₂ stimulation with and without asthmatic medication pretreatment.

Table 1. The scheme of anti-ROS effects of asthma medication in THP-1 cells.

Drugs/Time	LTRA	LABA			ICS		LABA/ICS	
	Montelukast	Salmeterol	Formeterol	extra LABA Indacaterol	Fluticasone	Budesonide	Salmeterol + Fluticasone	Formeterol + Budesonide
30 minutes	↓	↓	n.s	↑	n.s	n.s	↓	↓
1 hour	↓	n.s	n.s	↑	n.s	n.s	↓	↓
2 hour	↓	↓	n.s	n.s	↓	n.s	↓	↓

LTRA, leukotriene receptor antagonists; LABA, β 2-adrenoreceptor agonists; ICS, inhaled corticosteroids; n.s: no significance.

monocytes/macrophages but also other immune cells, such as T lymphocytes, eosinophils, mast cells and dendritic cells, that are involved in the pathological processes of asthma. The immune cells in vivo always work in cross talk with other cells as a network. Research on the confirmation of the interaction between these immune cells might be required in the future.

Conclusions

In the present study, we found that different asthma medications have different anti-ROS effects on monocytes. Both montelukast and the combination of ICS/LABA had anti-ROS effects. The combination therapy with LABA and ICS may not be the only choice for asthma control. Montelukast may also be a good supplemental treatment for the poorly controlled asthma because of its powerful anti-ROS effects. This study provides a novel therapeutic view in asthma.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

Funding

The study was supported by a grant from Research Center for Environmental Medicine, Kaohsiung Medical University, Taiwan (KMU-TP104A06), National Science Council (NSC 104-2314-B-037-070-MY3 and 103-2314-B-037-013) of the Republic of China, Kaohsiung Medical University Hospital Research Foundation (KMUH102-2T04 and KMUH104-4M58) and grants from Kaohsiung Municipal Hsiao-Kang Hospital (KMHKH-103-013 and KMHKH-104-003).

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