

Figure 2. Effect of ligands for TLRs on IL-8 release and effect of bafilomycin on poly(I:C)-induced IL-8 release in BEAS-2B cells. (A) BEAS-2B cells were treated with various concentrations of poly(I:C). (B) The cells were treated with 10 μg/ml poly(I:C) or vehicle in the presence of various concentrations of bafilomycin, an inhibitor of TLR3 that blocks endosomal H⁺-ATPase activity. After 24 hours, supernatants were harvested and assayed for IL-8 by enzyme-linked immunosorbent assay (ELISA). The data are expressed as mean values ± SEM for three to four separate experiments. *P < 0.05, **P < 0.01 compared with the values of control. (C–E) BEAS-2B cells were treated with various concentrations of (C) lipopolysaccharide (LPS), a ligand for TLR4; (D) R837, a ligand for TLR7; or (E) R848, a ligand for TLR7/8. After 24 hours, supernatants were harvested and assayed for IL-8 by ELISA.

were added to each well. The plates were incubated for 1 hour at 37°C, after which the MTT solution was discarded. Fifty microliters of DMSO were added to each well. The product was quantified at 550 nm with a microplate reader.

Measurement of IL-8 Using Enzyme-Linked Immunosorbent Assay

IL-8 was measured in the supernatants using enzyme-linked immunosorbent assay (ELISA; R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. The lower detection limit was 16 pg/ml.

Detection of NF-κB (p65) and IRF-3 by Nuclear Extraction and Immunoblotting

Cells were treated with 10 μg/ml poly(I:C) in the presence or absence of 100 μM H₂O₂ for 0 to 120 minutes. After washing with HBSS, cells were homogenized in cell lysis buffer to obtain the nuclear fraction using Nuclear Extraction Kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Samples were separated by electrophoresis and blotted on a PVDF membrane (Bio-Rad). The following antibodies were used for detection of the target proteins: mouse monoclonal anti-NF-κB p65 antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-IRF-3 antibody (1:500 dilution; Santa Cruz Biotechnology), or mouse monoclonal anti-lamin A/C antibody (1:400 dilution; Santa Cruz Biotechnology). Peroxidase-conjugated appropriate secondary antibodies were used. The following detection and visualization procedures were performed the same as for TLR3 immunoblotting.

NF-κB p65 DNA Binding Activity

To assess NF-κB p65 DNA binding activity, nuclear fraction was obtained after 60 minutes of incubation with poly(I:C) or vehicle and evaluated by NF-κB p65 transcription factor assay kit, which is based on the method of ELISA (Cayman Chemical Co., Ann Arbor, MI) accord-

ing to the manufacturer’s instructions. Briefly, equal amounts of nuclear extract were added on 96-well plates which immobilized a specific double-stranded DNA sequence containing the NF-κB response element on the bottom. After incubation and washing, the plates were incubated with anti-human NF-κB p65 primary antibody. Then a goat anti-rabbit HRP-conjugated secondary antibody was used. After developing, the plates were read at 450 nm with a microplate reader.

Knockdown of IRF-3 by siRNA

Cells were seeded in 6-well plates in complete media. At 50% confluence, transfection with siRNA was performed. In one tube, 40 μl of lipofectamine 2000 (Invitrogen Life Technologies) were mixed gently with 1.5 ml Opti-MEM medium (Invitrogen Life Technologies) and incubated for 5 minutes at room temperature. In a separate tube, 975 pmol of either nontargeting or IRF-3 siRNA (SMART pool plus; Dharmacon, Lafayette, CO) were mixed gently with 1.5 ml Opti-MEM medium. These siRNA and lipofectamine solutions were then combined, gently mixed, and incubated for 20 minutes at room temperature. After incubation, 2 ml of DMEM were added to obtain a final volume of 5 ml (final concentration of siRNAs = 200 nM), which was added to each dish. Cells were then incubated at 37°C for 6 hours. After that, media were changed to serum-free Keratinocyte Basal Medium with growth factor and further cultured for 24 hours. The siRNA-treated cells were then used to assess the role of the IRF-3 on IL-8 release in the presence of 10 μg/ml poly(I:C) or to assess IRF-3 expression by immunoblotting.

Statistical Analysis

Data were expressed as means ± SEM. GraphPad Prism (GraphPad Software Inc., San Diego, CA) was used to perform all statistical tests. Experiments with multiple comparisons were evaluated by one-way ANOVA followed by Bonferroni’s test to adjust for multiple comparisons. A paired two-group Student’s *t* test was used for single comparisons. Probability values of less than 0.05 were considered significant.

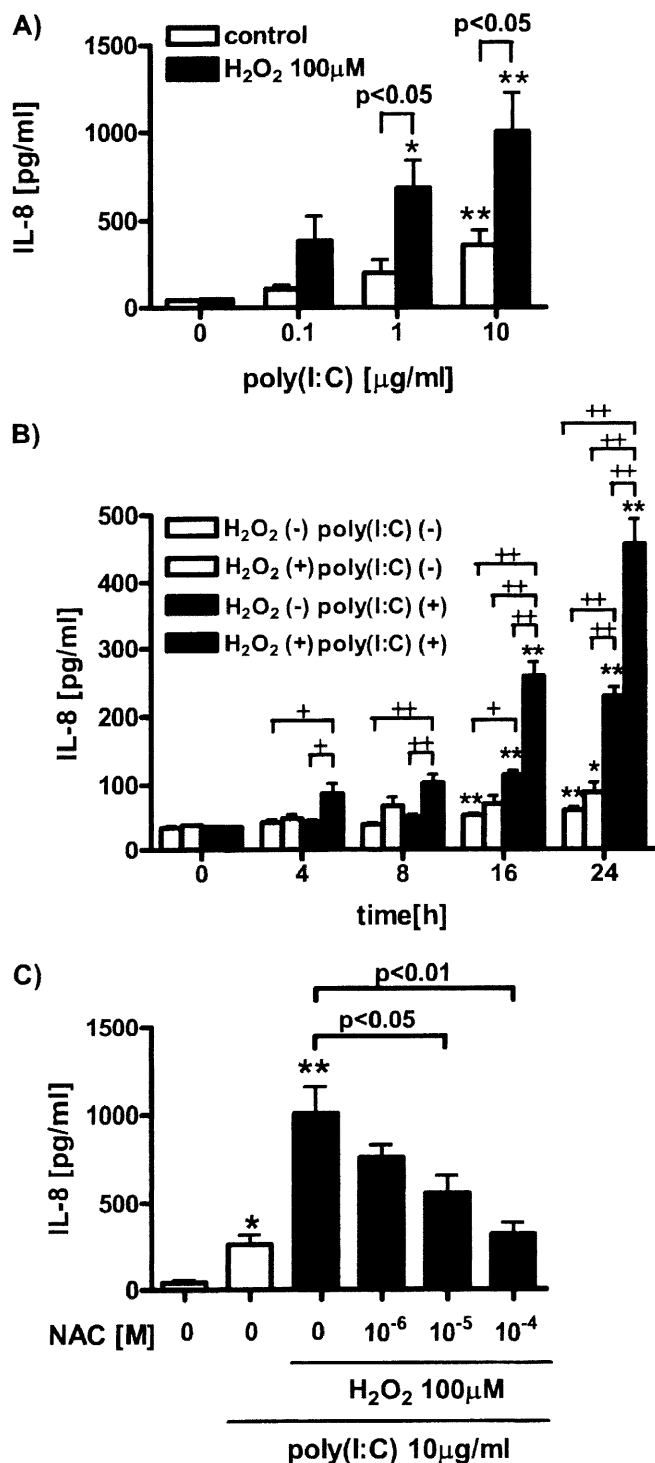


Figure 3. Effect of H₂O₂ on IL-8 release and effect of N-acetylcysteine (NAC) on H₂O₂-augmented IL-8 release in poly(I:C)-treated cells. (A) BEAS-2B cells were treated with 100 µM H₂O₂ or vehicle 30 minutes before the treatment with various concentrations of poly(I:C). After 24 hours, supernatants were harvested and assayed for IL-8. (B) Cells were treated with 100 µM H₂O₂ or vehicle 30 minutes before the treatment with 10 µg/ml poly(I:C). At various time points after the incubation, supernatants were harvested and assayed for IL-8. (C) Various concentrations of NAC were added 2 hours before H₂O₂ treatment, and then cultured in the presence of 10 µg/ml poly(I:C). After 24 hours, supernatants were harvested and assayed for IL-8. The data are expressed as mean values ± SEM for four to six separate experiments. *P < 0.05, **P < 0.01 compared with the values of vehicle-treated cells; +P < 0.05, ++P < 0.01 compared with the values of each group.

2B cells by immunocytochemistry (Figure 1A). TLR3 was detected at approximately 110 kD by immunoblotting in BEAS-2B cells and HBEpC as previously reported (Figure 1B). Next, we investigated the effect of the TLR3 ligand, poly(I:C) on the release of IL-8 from BEAS-2B cells. Poly(I:C) significantly increased the release of IL-8 from the epithelial cells in a dose-dependent manner (Figure 2A). TLR3 has been reported to exist in the endosome and to require an acidic environment for its activation. To confirm whether the effect of poly(I:C) was mediated through TLR, we used bafilomycin, which is an inhibitor of endosomal acidification. Bafilomycin dose-dependently inhibited the poly(I:C)-induced IL-8 release (Figure 2B). We examined the effect of other TLR ligands, including LPS (TLR4), R837 (TLR7), and R848 (TLR7/8), on the release of IL-8. LPS (Figure 2C), R837 (Figure 2D), or R848 (Figure 2E) had no effect on the release of IL-8 from the epithelial cells.

Effect of H₂O₂ on the Release of IL-8 from BEAS-2B Cells after Poly(I:C) Stimulation

To elucidate the effect of oxidative stress on the IL-8 release, the cells were treated with H₂O₂ in the current study. Because treatment with less than 200 µM H₂O₂ did not affect the cell viability and the release of IL-8 (see Figures E1A and E1B in the online supplement), we used H₂O₂ at the concentration of 100 µM or 150 µM in the current study. One hundred micromoles or 150 µM H₂O₂ significantly potentiated the release of IL-8 in the presence of poly(I:C) in a time- and concentration-dependent manner (Figures 3A, 3B, and E1B). The potentiation by H₂O₂ was significantly inhibited by pretreatment with NAC in a concentration-dependent manner (Figure 3C).

Effect of H₂O₂ on the Nuclear Translocation of NF-κB and IRF3 Induced by Poly(I:C)

To explore the mechanism for the H₂O₂-augmented IL-8 release in the poly(I:C)-treated cells, the effect of H₂O₂ on the translocation of NF-κB and IRF-3 into the nucleus was evaluated. Treatment with H₂O₂ caused a small but significant increase in NF-κB p65 translocation into the nucleus (Figures 4A–4D). However, H₂O₂ significantly potentiated NF-κB p65 translocation into the nucleus in the presence of poly(I:C) in a time- and concentration-dependent manner (Figures 4A–4D). The potentiation of NF-κB translocation was not observed after 24 hours of H₂O₂ treatment (Figure E2). H₂O₂ also significantly potentiated NF-κB DNA binding activity in the presence of poly(I:C) (Figure 4E). Furthermore, pretreatment with MG132 significantly inhibited the H₂O₂-augmented IL-8 release in the poly(I:C)-treated cells (Figure 4F).

RESULTS

Expression of TLR3 on Human Bronchial Epithelial Cells and Effect of TLR3 Ligand, Poly(I:C) on IL-8 Release

To confirm whether human bronchial epithelial cells express TLR3, we first examined the expression of TLR3 by immunocytochemistry and immunoblotting. TLR3 was detected in BEAS-

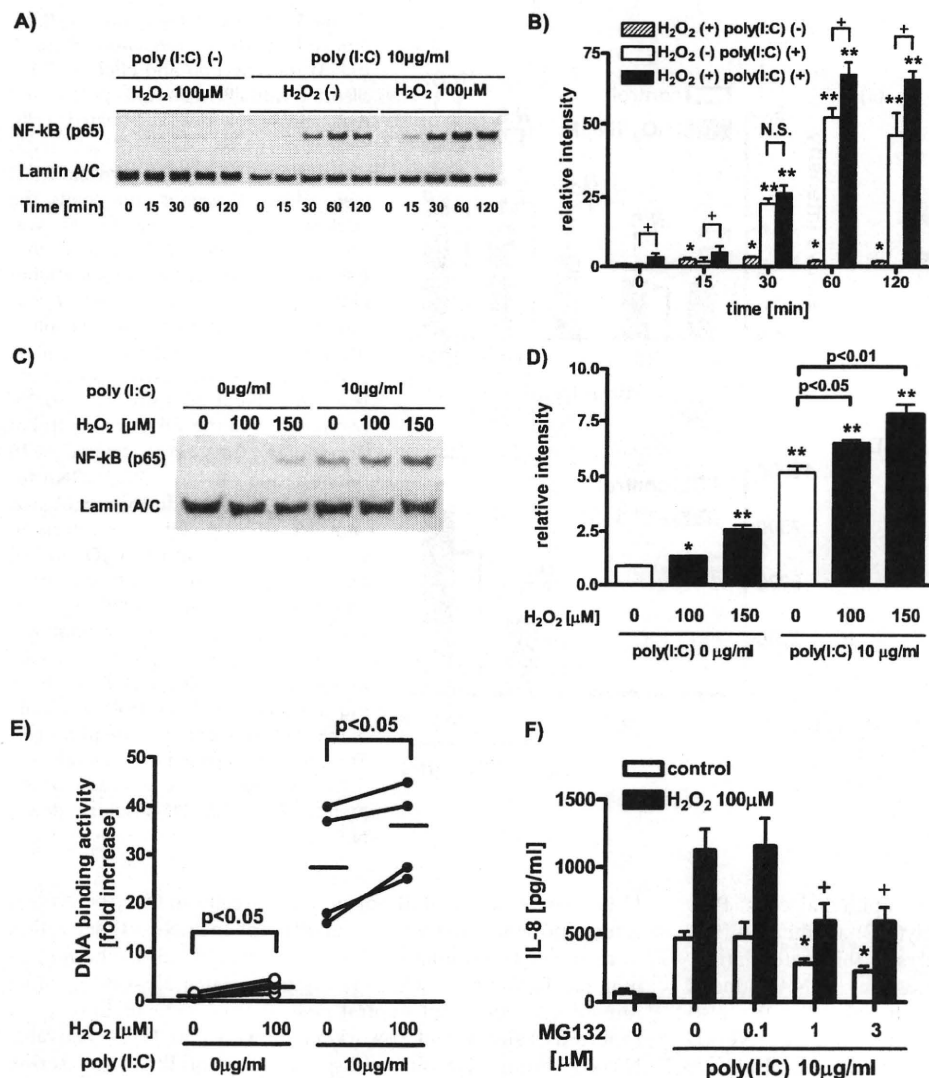


Figure 4. Effect of H₂O₂ on poly(I:C)-induced nuclear factor (NF)-κB translocation into nucleus and DNA binding activity and effect of MG132 on H₂O₂-augmented IL-8 release in poly(I:C)-treated cells. (A) Cells were treated with 100 μM H₂O₂ or vehicle 30 minutes before the treatment with 10 μg/ml poly(I:C). At various time points, the nuclear fraction of cell lysates was obtained. NF-κB p65 translocation into the nucleus was evaluated by immunoblotting. (B) Each band intensity was assessed by densitometry. Relative intensity was calculated by dividing the NF-κB band intensity by the appropriate lamin A/C band intensity. *P < 0.05, **P < 0.01 compared with the values of vehicle-treated cells at 0 minutes; +P < 0.05 compared with the values of each group. (C, D) Dose-dependent effect of H₂O₂ on poly(I:C)-induced NF-κB translocation was assessed by immunoblotting (C). The relative intensity of each band was assessed by densitometry (D). *P < 0.05, **P < 0.01 compared with the values of vehicle-treated cells. (E) NF-κB DNA binding activity was evaluated by ELISA. (F) Cells were treated with 10 μg/ml poly(I:C) with 100 μM H₂O₂ or vehicle in the presence of MG132. After 24 hours, the supernatants were assayed for IL-8 by ELISA. *P < 0.05, +P < 0.05 compared with the values of vehicle-pretreated poly(I:C)-treated cells. The data are expressed as mean values ± SEM for three to six separate experiments.

Ten micrograms per milliliter of poly(I:C) significantly enhanced IRF-3 translocation into the nucleus (Figures 5A and 5B). However, pretreatment with H₂O₂ caused no potentiation of IRF-3 translocation into the nucleus in the poly(I:C)-treated cells (Figures 5A and 5B). Since there is no available inhibitor for IRF-3, siRNA, for IRF3 was used to estimate the inhibitory effect on the release of IL-8. Although treatment with siRNA eliminated the IRF-3 protein (Figure 5C), IRF-3 knockdown did not affect the H₂O₂-augmented IL-8 release in the poly(I:C)-treated cells (Figure 5D).

Effect of H₂O₂ on the Expression of TLR3

To explore another mechanism of the H₂O₂-potentiated IL-8 release in the poly(I:C)-treated cells, the effect of H₂O₂ on the expression of TLR3 in the epithelial cells was evaluated. Treatment with 100 μM H₂O₂ or 10 μg/ml poly(I:C) alone did not affect the expression of TLR3 (Figure 6A). However, combination of poly(I:C) and H₂O₂ significantly increased the TLR3 expression in a time-dependent manner (Figures 6A and 6B).

Effect of Dexamethasone on the Potentiated IL-8 release by H₂O₂ and TLR3 Expression in BEAS-2B Cells

Because systemic steroid has been reported to be effective for exacerbations of COPD (35, 36), we investigated the effect of

steroid on the H₂O₂-potentiated IL-8 release in the poly(I:C)-treated cells. A quantity of 10⁻⁷ to 10⁻⁶ M DEX significantly inhibited the poly(I:C)-augmented IL-8 release in the presence or absence of H₂O₂ (Figure E3). At 10⁻⁶ M DEX, there was still a significant difference in the poly(I:C)-augmented IL-8 release between the H₂O₂-treated and vehicle-treated groups (Figure E3). Treatment with DEX did not affect the H₂O₂-potentiated TLR3 expression in the poly(I:C)-treated cells (Figure 6C).

Effect of H₂O₂ and Poly(I:C) on the Release of IL-8, NF-κB Translocation, and TLR3 Expression in HBEpC

We confirmed the potentiation by H₂O₂ on the IL-8 release, NF-κB translocation, and TLR3 expression in HBEpC. Treatment with H₂O₂ slightly but significantly augmented IL-8 release in the poly(I:C)-treated cells (Figure 7A). H₂O₂ also significantly potentiated the poly(I:C)-augmented NF-κB p65 translocation and TLR3 expression in the poly(I:C)-treated cells as well as in BEAS-2B cells (Figures 7B and 7C).

DISCUSSION

In the current study, we demonstrated the expression of TLR3 on BEAS-2B cells and HBEpC and that a synthetic dsRNA,

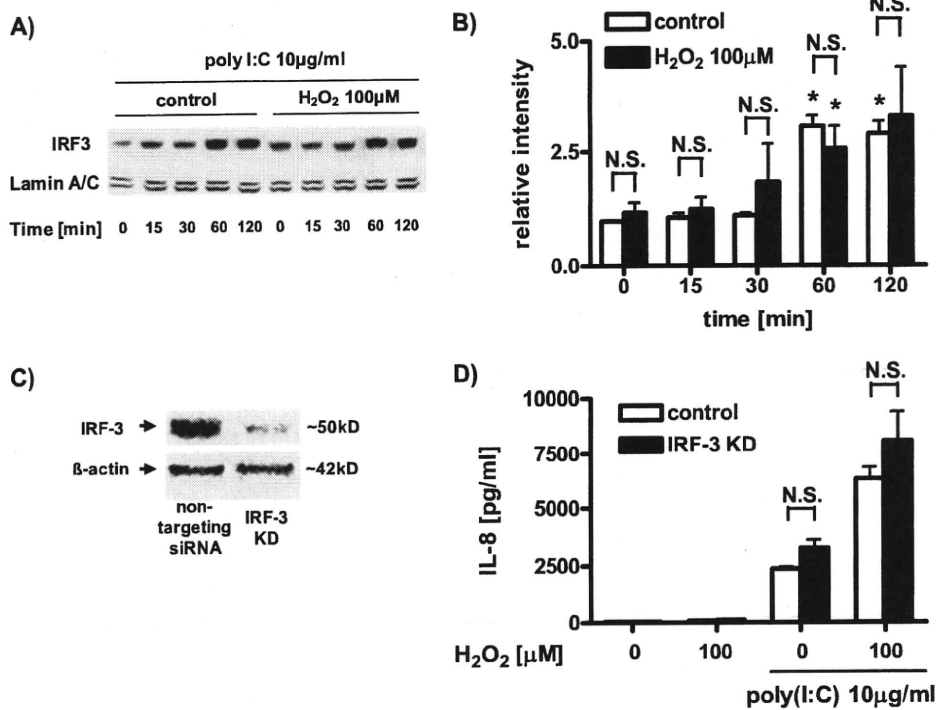


Figure 5. Effect of H₂O₂ on poly(I:C)-induced interferon regulatory factor-3 (IRF-3) translocation and effect of IRF-3 silencing with siRNA on H₂O₂-potentiated IL-8 release in poly(I:C)-treated cells. Cells were treated with 100 μM H₂O₂ or vehicle and then 10 μg/ml poly(I:C) were added. At various time points, the nuclear fraction of cell lysates was obtained. (A) IRF-3 translocation into the nucleus was evaluated by immunoblotting. (B) Each band intensity was assessed by densitometry. Relative intensity was calculated by dividing the IRF-3 band intensity by each appropriate lamin A/C band intensity. **P* < 0.05; compared with the values of control at 0 minutes. Cells were treated with nontargeting siRNA or IRF-3 siRNA for 6 hours. Cells were further incubated with 10 μg/ml poly(I:C) or vehicle in the presence of 100 μM H₂O₂ for 24 hours. Supernatants were harvested and assayed for IL-8. (C) IRF-3 expression was evaluated by immunoblotting. The amount of IRF-3 was assessed by densitometry. (D) Effect of IRF-3 silencing with siRNA on H₂O₂-potentiated IL-8 release from poly(I:C)-treated cells. The data are expressed as mean values ± SEM for three to six separate experiments. IRF-3 KD, IRF-3 knock down; N.S., not significant.

poly(I:C), induced IL-8 release from the epithelial cells. Pretreatment with H₂O₂ potentiated the poly(I:C)-augmented IL-8 release, and the antioxidant NAC reversed this potentiation, suggesting that oxidative stress potentiates the dsRNA-induced IL-8 release from human bronchial epithelial cells. We also showed that H₂O₂ potentiated the poly(I:C)-induced NF-κB translocation, not IRF-3, into the nucleus and NF-κB DNA binding activity, and suppression of NF-κB by MG132 inhibited the H₂O₂-potentiated IL-8 release in the poly(I:C)-treated cells. Furthermore, treatment with H₂O₂ plus poly(I:C) increased the TLR3 expression compared with the basal condition. These H₂O₂-mediated potentiations of the TLR3 responses were also confirmed in primary human airway epithelial cells. These data suggest that oxidative stress potentiates the poly(I:C)-augmented IL-8 release in airway epithelial cells through NF-κB activation, and this potentiation might be partly explained by the increased TLR3 expression.

During exacerbations of COPD, levels of IL-8 in sputum and exhaled breath condensate were elevated (28, 30, 31) and neutrophil infiltration was augmented in the airways (32, 33). Although the mechanism for this enhanced neutrophilic inflammation has not been fully elucidated yet, our findings may explain the mechanism. Generally, greater amounts of oxygen radicals are produced during exacerbations compared with the stable condition (12, 13). When viral infections occur, TLR3-mediated IL-8 release might further increase in the airways and consequently lead to excessive neutrophil accumulation.

In the current study, H₂O₂ augmented the poly(I:C)-induced translocation of NF-κB p65 into the nucleus and suppression of NF-κB by MG132 inhibited the H₂O₂-augmented IL-8 release in the poly(I:C)-treated cells. These results suggest that the augmentation of IL-8 release by H₂O₂ is due to the potentiation of poly(I:C)-induced NF-κB activation. In previous studies,

H₂O₂ has been reported not only to directly induce the NF-κB transcriptional activity (37), but also enhance NF-κB activation in response to proinflammatory cytokines (38, 39). The molecular mechanisms by which H₂O₂ potentiates NF-κB signaling remain uncertain, but several possible mechanisms have been reported. Takada and coworkers showed that H₂O₂ activates NF-κB through tyrosine phosphorylation of IκBα and serine phosphorylation of the p65 subunit of NF-κB (39). Others have also reported that H₂O₂ activates NF-κB via phosphorylation of serine residues in the IκB kinases (IKKs) (40). Recently, H₂O₂ has been reported to prolong nuclear localization of NF-κB in proinflammatory cytokine-activated cells by suppressing the negative regulation by IκBα and other proteases (41). In our current study, H₂O₂-augmented NF-κB p65 translocation to the nucleus in poly(I:C)-treated cells continued until 120 minutes compared with the control, which might be consistent with the previous study (41). We showed that poly(I:C) induced the translocation of IRF3 into the nucleus, but H₂O₂ did not enhance this translocation. Further silencing of IRF-3 did not affect the potentiation of IL-8 release. Together, potentiation of IL-8 release is mainly mediated via NF-κB but not IRF-3 signaling.

In the present study, we showed that the combination of poly(I:C) and H₂O₂ significantly enhanced TLR3 expression in airway epithelial cells. This result suggests that oxidative stress might affect the regulation of TLR3 expression. Previous reports have shown that poly(I:C) augments TLR3 expression in epithelial cells through a type I IFN-dependent mechanism (21, 42). Oxidative stress also has been reported to enhance TLR3 expression in adult human astrocytes (43). These reports might support the current results. Recently, increased TLR3 expression has been observed in airway epithelial cells from patients with acute respiratory distress syndrome in which

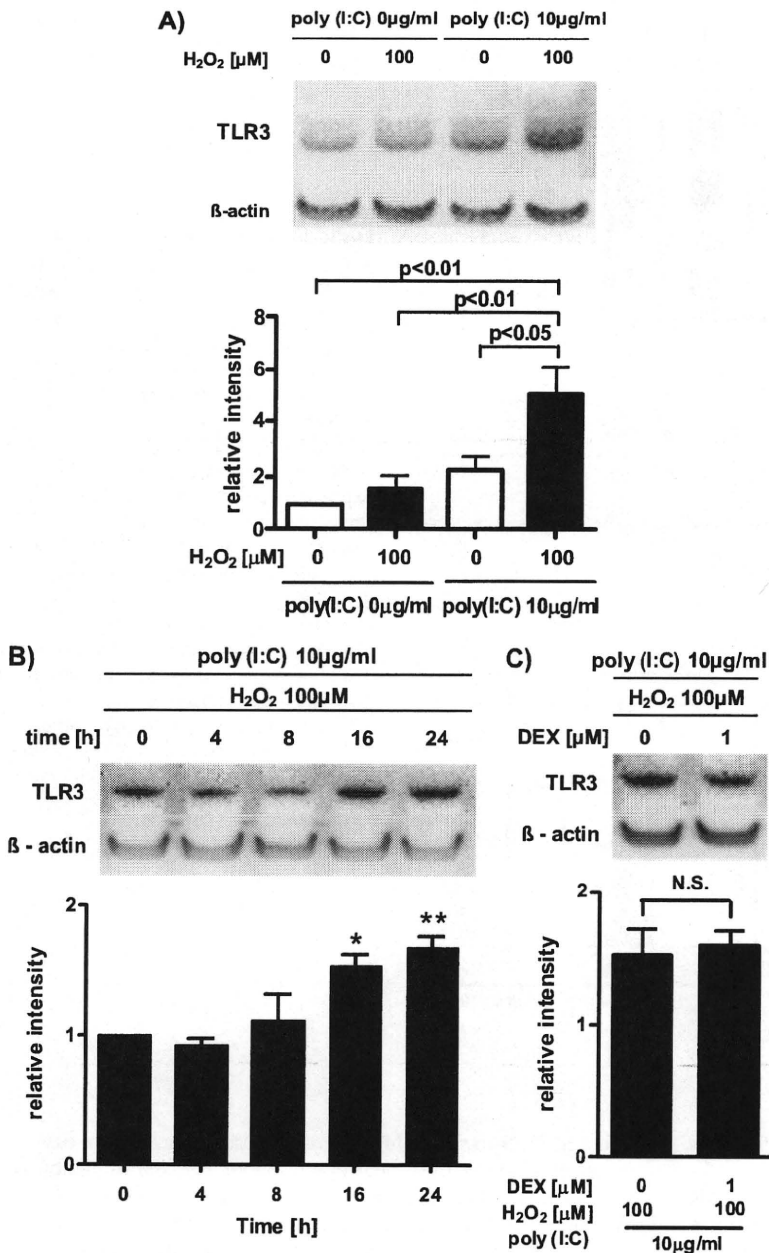


Figure 6. Effect of H₂O₂ on the expression of TLR3 and effect of dexamethasone (DEX) on H₂O₂-augmented TLR3 expression in poly(I:C)-treated cells. (A) Cells were treated with 100 μM H₂O₂ or vehicle and then 10 μg/ml poly(I:C) were added. After 24 hours, whole cell lysates were obtained. The expression of TLR3 was evaluated by immunoblotting. Each band intensity was assessed by densitometry. Relative intensity was calculated by dividing the TLR3 band intensity by each appropriate β-actin band intensity. (B) Cells were treated with 100 μM H₂O₂ and then 10 μg/ml poly(I:C) were added. At various time points, whole cell lysates were obtained and TLR3 expression was evaluated by immunoblotting. (C) Cells were treated with 1 μM DEX or vehicle 30 minutes before the treatment with 100 μM H₂O₂ and 10 μg/ml poly(I:C). After 24 hours, whole cell lysates were obtained. The expression of TLR3 was evaluated by immunoblotting. The data are expressed as mean values ± SEM for four to five separate experiments.

airway cells are exposed to a hyperoxic condition (44), suggesting that oxidative stress might modulate TLR3 expression. However, there have not been any studies to clarify the mechanism of this modulation in epithelial cells. In the current study, DEX did not affect the H₂O₂-potentiated TLR3 expression. This might suggest that steroid-sensitive signals such as NF-κB are not involved in the mechanism of H₂O₂-potentiated TLR3 expression. However, this mechanism remains unclear and further studies are needed.

In the current study, we investigated the time course of potentiation by H₂O₂ in IL-8 release and TLR3 expression to examine whether potentiation in the IL-8 release by H₂O₂ is due to up-regulation of TLR3 expression in the poly(I:C)-treated cells. While the TLR3 expression was potentiated by H₂O₂ at 16 hours or later, potentiation of the IL-8 release occurred at 4 to 8 hours or later. These results suggest that the

potentiation of the IL-8 release by H₂O₂ was mainly mediated by the potentiation of NF-κB activation. However, at 16 hours or later, the enhanced TLR3 expression might contribute to the potentiation of IL-8 release by H₂O₂.

During acute exacerbations, patients with COPD usually are treated with steroids, and steroids have been reported to improve the severity of exacerbations (35, 36). In this study, DEX dose-dependently inhibited the poly(I:C)-induced IL-8 release from epithelial cells. The inhibition of the IL-8 release by DEX in the H₂O₂-pretreated and poly(I:C)-exposed cells was less than in the vehicle-pretreated and poly(I:C)-exposed cells, suggesting that oxidative stress reduces the effect of steroid on the IL-8 release. The acquisition of steroid resistance under oxidative stress has been reported to occur in macrophages and epithelial cells via inactivation of histone deacetylase 2 (HDAC2) (34, 45). This inactivation of HDAC2

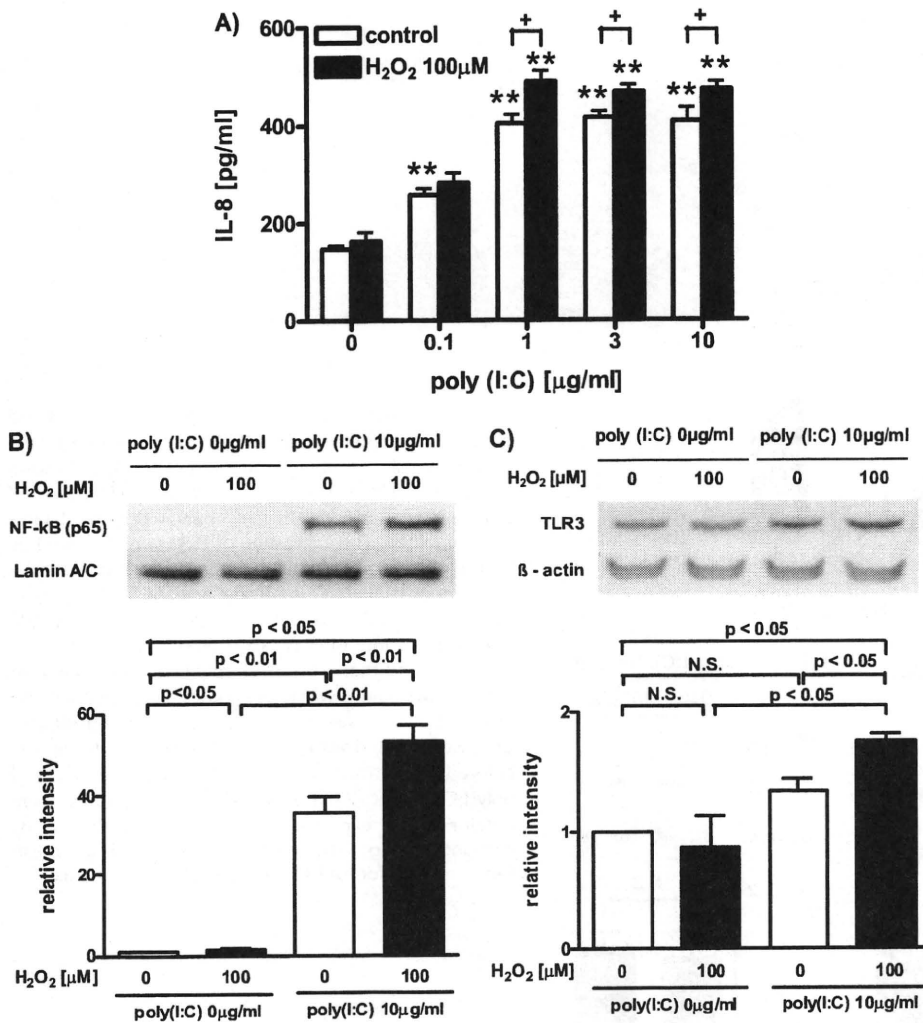


Figure 7. Effect of H₂O₂ on IL-8 release, poly(I:C)-induced NF- κ B translocation and TLR3 expression in poly(I:C)-treated primary human bronchial epithelial cells (HBEpC). Four different strains of primary cells were treated with 100 μM H₂O₂ or vehicle 30 minutes before the treatment with 10 $\mu\text{g/ml}$ poly(I:C). (A) After 24 hours, supernatants were harvested and assayed for IL-8. The data are expressed as mean values \pm SEM for four separate experiments. (B) After 60 minutes, the nuclear fraction of cell lysates was obtained. NF- κ B p65 translocation into the nucleus was evaluated by immunoblotting. (C) After 24 hours, whole cell lysates were obtained. Expression of TLR3 was evaluated by immunoblotting. The data are expressed as mean values \pm SEM for four separate experiments. ** $P < 0.01$ compared with the values of vehicle-treated cells; + $P < 0.05$ compared with the values of each group.

by oxidative stress might explain our result that DEX was less effective in the H₂O₂-treated cells.

Recently, several reports have shown the role of TLR activation under oxidative stress. Chen and colleagues have reported that the production of cytokines and chemokines such as TNF- α , IL-6, and IL-8 is increased in alveolar macrophages from smokers after poly(I:C) stimulation compared with those from nonsmokers (46). In a murine model, depletion of TLR3 was demonstrated to inhibit the smoking-enhanced airway inflammation and remodeling after influenza virus infection (47). This result suggests that oxidative stress from cigarette smoke might enhance the TLR3 signaling, which is consistent with our results.

In a recent report, Zmijewski and coworkers have shown that H₂O₂ has an anti-inflammatory effect and prevents the activation of NF- κ B (48), which is inconsistent with the result of our current study. They demonstrated that a catalase inhibitor, aminotriazole, increased the production of reactive oxygen species in murine neutrophils and attenuated LPS-induced acute lung injury in a murine model. This effect was due to the alleviation of NF- κ B activity and proinflammatory cytokine production in neutrophils. The discrepancy from our current results might be explained as follows. First, we administered H₂O₂ exogenously to the airway epithelial cells, whereas they evaluated the role of endogenously

produced H₂O₂ by the inhibition of catalase in the reactive oxygen species production from neutrophils. Moreover, the inhibition of catalase might affect the antioxidant system, including glutathione and superoxide dismutase. Second, they evaluated the role of catalase inhibition in NF- κ B signaling and proinflammatory cytokine production in murine neutrophils. We assessed the role of H₂O₂ in NF- κ B signaling and IL-8 production in human bronchial epithelial cells. Differences in the species and cell type might have affected the results. Third, we assessed TLR3 signaling in the current study. They showed the effect of catalase inhibition on lung inflammation induced by LPS treatment. In general, LPS stimulates TLR4; therefore, the discrepancies in the results might be due to the difference in activated TLRs.

In the current study, TLR4, TLR7, and TLR7/8 ligands did not enhance IL-8 release from BEAS-2B cells. Previously, Sha and colleagues have shown that 1 $\mu\text{g/ml}$ LPS treatment slightly potentiated the IL-8 release from the cells (49). Generally, this concentration appears quite high. In the current study, we used 100 ng/ml LPS to stimulate IL-8 release in BEAS-2B cells. Therefore, the lack of augmentation in IL-8 release by LPS could be due to the difference in the LPS concentration. In a recent report, Koff and coworkers have shown that TLR1/2, TLR5, and TLR6/2 ligands augment IL-8 release, albeit to a lesser extent than TLR3 stimulation in human bronchial

epithelial cells (50). Therefore, not only TLR3 ligand but also other TLR ligands could stimulate IL-8 release in human bronchial epithelial cells. The aim of this study was to clarify the role of TLR3 activation induced by viral infection under oxidative stress. Therefore, we investigated only TLR3 signaling, not other TLRs.

There are several limitations in the current study. First, H₂O₂ potentiated IL-8 production in the poly(I:C)-treated cells through the activation of NF- κ B. However, it remains unclear whether the potentiation of H₂O₂ in NF- κ B activation is synergistic or not. Second, TLR3 was up-regulated by the H₂O₂ plus poly(I:C) treatment for 24 hours in epithelial cells in the current study. However, the role of the up-regulated TLR3 in the IL-8 release could not be fully elucidated. Because TLR3 expression was augmented at 16 hours or later by treatment with H₂O₂ and poly(I:C), the augmentation of the expression may affect the IL-8 release.

In conclusion, we showed that oxidative stress potentiates the poly(I:C)-induced IL-8 release from airway epithelial cells through the augmentation of NF- κ B signaling and that this potentiation might be partly explained by the enhancement of TLR3 expression. These results suggest that oxidative stress augments the neutrophilic inflammation in the airways of patients with COPD during viral-induced exacerbations. Modulation of this pathway may be a therapeutic target for exacerbations of COPD.

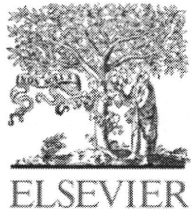
Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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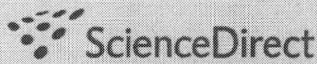
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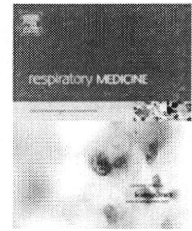
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Tiotropium 5 µg via Respimat and 18 µg via HandiHaler; efficacy and safety in Japanese COPD patients

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Summary

Background and objectives: To compare the efficacy and safety of tiotropium inhaled via Respimat[®] Soft Mist Inhaler, a multidose propellant-free inhaler and HandiHaler[®], a single-dose dry powder inhaler, in a phase 2 study of Japanese COPD patients.

Methods: Patients with FEV₁ ≤ 70% predicted, FEV₁/FVC ≤ 70% and a smoking history of >10 pack-years received tiotropium once daily via Respimat[®] (5 µg) and HandiHaler[®] (18 µg) for 4 weeks each in a randomised, double-blind, double-dummy, two-way crossover study. Lung function, adverse events, pharmacokinetics and safety were assessed.

Results: Of 184 patients screened, 134 were evaluable. The trough FEV₁ response on Day 29 showed Respimat[®] to be non-inferior to HandiHaler[®] (mean treatment difference, 0.008 L; 95% CI, -0.009 to +0.024 L; *p* < 0.001). Peak and average FEV₁ and FVC responses on Day 1 and Day 29 were very similar for the two treatments. Tiotropium plasma levels and excretion kinetics showed a similar profile of systemic exposure for the two formulations of tiotropium. Adverse events were reported by similar numbers of patients on each treatment, i.e. 27.9 and 30.6% in the Respimat[®] and HandiHaler[®] groups, respectively.

Conclusions: In Japanese patients with COPD, tiotropium Respimat[®] 5 µg and tiotropium HandiHaler[®] 18 µg showed a similar profile of efficacy, safety and pharmacokinetics.

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Introduction

The anticholinergic agent tiotropium (Spiriva[®]) is a potent and long-acting bronchodilator whose clinical benefits when used chronically for the maintenance treatment of COPD have been established in several clinical studies.^{1–4} When taken as a once daily inhalation, tiotropium improves lung function, reduces dyspnoea, reduces the incidence of exacerbations and improves health-related quality of life in patients with COPD.^{5–10} It also reduces hyperinflation^{11,12} and improves exercise tolerance.^{12,13}

Tiotropium is the first long-acting anticholinergic approved for the treatment of COPD. It was first introduced in capsule form for inhalation via the HandiHaler[®], a single-dose dry powder inhaler (DPI), and this form of tiotropium was given marketing approval in Japan in 2004. Recently, tiotropium has been introduced in a new inhaler device, the Respimat[®] Soft Mist Inhaler, a novel, multidose, propellant-free inhaler that delivers a fine particle fraction of over 65%.¹⁴ The Respimat[®] inhaler generates a fine aerosol cloud that is generated over a longer period and moves more slowly than the aerosol from a pressurised metered-dose inhaler (pMDI). Because the aerosol cloud lasts 4–10 times longer than a pMDI aerosol,¹⁵ the Respimat[®] inhaler offers the potential for easier co-ordination of inhalation with actuation of the inhaler. Deposition studies have shown that a higher proportion of the emitted dose from the Respimat[®] inhaler is delivered to the lungs than from a pMDI or from a multidose DPI.^{16,17}

The results of a European phase II dose-ranging study on tiotropium in COPD patients showed that daily doses of 5–20 µg inhaled from the Respimat[®] inhaler were more effective than placebo in improving trough FEV₁ and other lung function measures after 3 weeks' treatment, and that steady-state urinary excretion of tiotropium with the 5 µg daily dosage from the Respimat[®] inhaler was similar to that with the 18 µg daily dosage from HandiHaler[®].¹⁸ Furthermore, in a pooled analysis of two crossover studies of identical design in American and European COPD patients, tiotropium Respimat[®] 5 µg was similar to tiotropium HandiHaler[®] 18 µg with respect to lung function improvement, pharmacokinetic profile and safety after 4 weeks' treatment.¹⁹ On the basis of these results, a tiotropium dose of 5 µg from the Respimat[®] inhaler was considered to be comparable to an 18 µg dose from the HandiHaler[®] in American and European patients with COPD. The objective of the current study was to compare the same two treatments in Japanese COPD patients, to investigate whether the comparability also applies in this population. The effects of the two inhalers were compared using measurements of clinical efficacy, pharmacokinetic, tolerability and safety.

Methods

Study design

This phase 2 clinical trial used a randomised, double-blind, double-dummy, 2-way crossover design, and was conducted in 27 outpatient centres in Japan. The trial was carried out in compliance with principles laid down in the Declaration

of Helsinki and in accordance with both the International Conference on Harmonisation (ICH) Harmonised Tripartite Guideline for GCP and Japanese GCP. The protocol and all amendments were approved by the local institutional review board, and all participants gave written informed consent. The trial is registered as NCT00292448 on ClinicalTrials.gov registry.

Patients

Japanese men or women were eligible for study entry if they were aged 40 years or older, had COPD (FEV₁ of no more than 70% predicted normal and ratio of FEV₁ to FVC of no more than 70%), and were current or ex-smokers (smoking history of >10 pack-years). Predicted normal FEV₁ values were calculated according to the standard formula for Japanese individuals.²⁰ To enter the second treatment period (Period 2), a patient's baseline FEV₁ reading at the start of that period had to be within ±15% of his or her reading at the start of the first treatment period. Patients were excluded if they had a history of asthma or allergic or atopic disease or, during the month before the screening visit, had received any specific treatment for such diseases (e.g. disodium cromoglycate, leukotriene antagonists, or antihistamines). Other exclusion criteria included a history of arrhythmias, or myocardial infarction in the previous year, or heart failure requiring hospital treatment in the previous 3 years, or treatment with beta-blockers during the month before the screening visit.

Treatments and crossover procedure

After the initial visit for patient screening, all participants entered a 4-week screening period during which they were instructed to practice inhalation from the Respimat[®] inhaler and HandiHaler[®], using placebo versions of both test inhalers; patients also received instruction on correct preparation and inhalation technique for each inhaler. At the end of this period, patients meeting the entry criteria were randomly assigned in a 1:1 ratio to either regimen A or B for a 4-week period (Period 1):

Regimen A = tiotropium 5 µg inhaled via the Respimat[®] inhaler (two puffs of 2.5 µg each) plus placebo capsule inhaled via HandiHaler[®], both given once daily in the morning.

Regimen B = placebo inhaled via the Respimat[®] inhaler (two puffs) plus tiotropium 18 µg capsule inhaled via HandiHaler[®], both given once daily in the morning.

In both groups, the Respimat[®] inhaler was used first and HandiHaler[®] second (within 3 min of each other). After Period 1, patients entered a 4-week wash-out period (no study treatment), and then restarted study treatment for a further 4 weeks (Period 2), receiving whichever Regimen (A or B) they did not receive in the first period. For both treatment periods and the wash-out period, the allowable variance in duration was ±7 days.

The following concomitant medications were permitted at any time during the study provided the dosage was stable: inhaled short-acting beta-agonists (SABAs), which could also be used as rescue medication except on days when lung function tests were performed; oral or inhaled

corticosteroids; theophylline, and mucolytics. During the screening period and wash-out period only, the following additional medications were permitted (again at stable dosages only): inhaled long-acting beta-agonists (LABAs), oral and transdermal beta-agonists and inhaled short-acting anticholinergics.

Assessments

Pulmonary function tests (PFTs) were performed by spirometry at the screening visit to assess patients against screening criteria. Testing was repeated at the start and end (Day 1 and Day 29) of Periods 1 and 2 for efficacy measurements; on these occasions, tests were done 10 min before dosing of study medication, and 1, 2 and 3 h post-dose. At each time point, the highest FEV₁ and FVC values were recorded (from at least 3 attempts). The administration of other bronchodilators in the 24 h before the PFT was restricted to avoid confounding. For each patient, the baseline FEV₁ on Day 1 of Period 2 was required to be within $\pm 15\%$ of the baseline FEV₁ on Day 1 of Period 1. If this requirement was not met, the patient had up to two additional opportunities to comply, each within 7 days of the previous PFT.

To measure steady-state pharmacokinetics, blood and urine samples were taken at the end of both treatment periods (Day 29). Blood was taken pre-dose (no more than 1 h prior to dosing), then 10 min, 1.5 h and 4 h after dosing. For each patient, urine samples were combined into two separate collection periods, for the first 2 h after dosing and for the period 2–4 h after dosing. Plasma and urine levels of tiotropium were assayed by HPLC plus tandem mass spectrometry (HPLC–MS/MS) system, with a lower limit of quantification of 2.5 pg/ml in plasma samples and 10 pg/ml in urine samples. Steady-state kinetics were described by the parameters AUC _{τ ,ss} and Ae_{0–4,ss}, i.e. the area under the tiotropium concentration-time curve over the dosing interval τ (24 h) and the amount of tiotropium in the urine in the 4 h after dosing.

Tolerability was assessed by adverse event monitoring from screening visit to 30 days after the last dose of study treatment. Any adverse event occurring after the first dose of a study treatment and up to 30 days after the last dose of the treatment was assigned to that treatment, as long as the next study treatment had not started.

At the screening visit and at the end of Periods 1 and 2, patients underwent physical examination and ECG, and had blood and urine sampled for haematology, urinalysis, and routine blood chemistry. Vital signs were measured at all visits (screening, and start and end of Periods 1 and 2).

Endpoints and statistical analysis

The Full Analysis Set (FAS), which consisted of the two treatment periods where baseline data and post-treatment data were available, was used for the analyses. The primary efficacy endpoint was the trough FEV₁ response, i.e. the difference between pre-dose FEV₁ on Day 1 of the treatment period and the pre-dose value on Day 29 of the same period. Using an analysis of covariance (ANCOVA) with terms for period, treatment and patient as fixed effects, and baseline FEV₁ as a covariate, the null hypothesis tested

was that trough FEV₁ response with tiotropium Respimat[®] was inferior to that with tiotropium HandiHaler[®]. Assuming that the smallest clinically meaningful difference between treatments in this measure was 0.05 L, the null hypothesis could be rejected, i.e. tiotropium Respimat[®] would be non-inferior to tiotropium HandiHaler[®], if the lower 95% confidence limit (CL) for the difference between treatments (tiotropium Respimat[®] minus tiotropium HandiHaler[®]) was greater than -0.05 L.

Assuming a standard deviation in paired differences of trough FEV₁ of 0.12 L, 78 patients would need to complete the study to detect a difference in mean trough FEV₁ response between the two study treatments of at least 0.05 L with a significance level of 2.5% (one-sided) and 95% statistical power.

Secondary efficacy endpoints included the peak and average bronchodilator responses (measured as both FEV₁ and FVC) on Day 1 and Day 29. For both test days, peak response was the difference between the highest value recorded during the post-dose PFTs and the period baseline; average response was the area under the response-time curve during the first 3 h after dosing (AUC_{0–3h}). Estimates for peak and average responses were adjusted for patient, period and baseline value. Trough FVC response after 4 weeks, derived in the same way as trough FEV₁, was an additional efficacy endpoint. Analysis of all secondary efficacy endpoints was done using ANCOVA in the same way as for the primary endpoint, except that a two-tailed 95% confidence interval (CI) around the mean estimate was derived.

Analysis of tolerability, safety and pharmacokinetics data were done for all patients who had received at least one dose of study medication (the safety population).

Results

Patient disposition and screening characteristics

Of 184 patients enrolled at the screening visit, 157 met the criteria for entry to the study. In all, 134 patients completed the study (full analysis set), and 157 received at least one dose of study medication (safety population). A diagram of patient flow through each stage of the study is shown in Fig. 1.

At the screening visit, the great majority (98.1%) of COPD patients were men, and the mean age of the sample was 70.2 years (Table 1). Most were ex-smokers (77.1%). Although the average time since COPD diagnosis was 5.8 years, mean lung function measures at screening were an FEV₁ of 43.1% predicted normal and FEV₁/FVC ratio of 41.9%, and the majority of patients (66.9%) had severe or very severe COPD, i.e. Stage III or IV as defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines.²¹ These data, together with baseline values of FEV₁ and FVC for the two treatment groups, are shown in Table 1.

Efficacy

Primary endpoint

Graphs of change in mean FEV₁ in the 3 h after inhalation of tiotropium from each test inhaler show that on Day 1 and

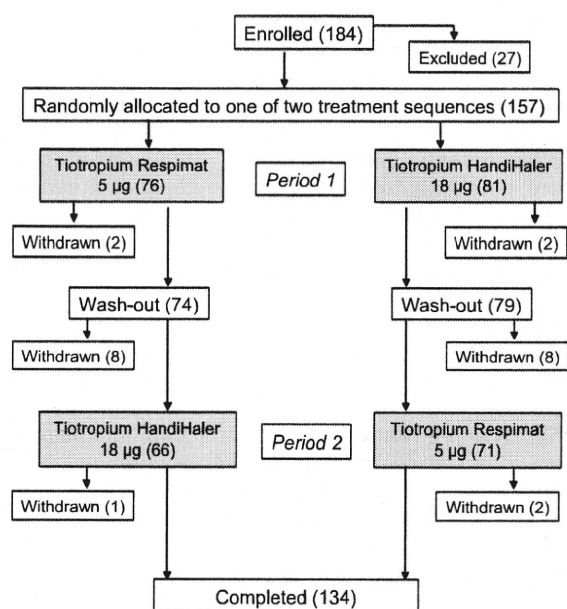


Figure 1 Patient flow through study.

Day 29, the bronchodilator responses to tiotropium were very similar for the Respimat[®] inhaler and HandiHaler[®] (Fig. 2a and b). Compared with the Day 1 baseline (pre-dose value), the mean pre-dose (trough) FEV₁ on Day 29 increased by 0.109 L and 0.101 L for the Respimat[®] inhaler and HandiHaler[®], respectively. The mean difference in trough FEV₁ response between the inhalers was 0.008 L (95% CI, -0.009 to +0.024 L). As the lower CI was greater than -0.05 L, tiotropium Respimat[®] was non-inferior to tiotropium HandiHaler[®] ($p < 0.001$; Table 2).

In addition, the 95% CI for the mean estimated difference in trough FEV₁ between tiotropium Respimat[®] and tiotropium HandiHaler[®] lay entirely within the range of -0.05 L to +0.05 L, indicating very similar performance of the two inhalers according to this efficacy measure.

Secondary efficacy measures

The peak and average (AUC_{0-3h}) FEV₁ responses to tiotropium Respimat[®] were very similar to those to tiotropium HandiHaler[®] on Day 1 and Day 29, with no statistically significant differences between the two inhalers (Table 3).

The changes in FVC in the 3 h after inhalation of tiotropium from each test inhaler also show very similar responses for the two inhalers (Fig. 2c and d). On Day 29, the adjusted mean trough FVC value was higher than the pre-dose value on Day 1 by 0.213 L and 0.217 L for the Respimat[®] inhaler and HandiHaler[®] respectively; the difference between inhalers in trough FVC response (-0.004 L) was not significant ($p = 0.84$) (Table 4). For peak FVC responses and AUC_{0-3h} FVC responses on Day 1 and Day 29, there were also no significant differences between the two inhalers (Table 4).

Pharmacokinetics

Data from 153 patients was available for pharmacokinetic analysis. Steady-state plasma concentration-time profiles

Table 1 Characteristics of study participants at screening ($n = 157$ who took at least one dose of study medication) and spirometry measures at baseline (combined data for Periods 1 and 2; $n = 147$ for both treatments).

	Mean value (standard deviation)	Range
Values at screening visit		
Age, y	70.2 (7.5)	43–87
Men/women	154/3	–
Ex-smokers/ smokers, n	121/36	–
Smoking history, pack-years	60.4 (30.0)	20–150
Time since COPD diagnosis, y	5.8 (5.0)	0.1–38.0
FEV ₁ , L	1.171 (0.383)	0.35–2.21
FEV ₁ , % predicted	43.1 (13.3)	11–79
FVC, L	2.795 (0.646)	1.17–4.37
FEV ₁ /FVC ratio, %	41.9 (9.7)	19.8–69.7
GOLD stage, n (%):		
I	0 (0)	–
II	52 (33.1)	–
III	81 (51.6)	–
IV	24 (15.3)	–
Values at baseline		
FEV ₁ , L		
Tiotropium Respimat [®] 5 µg	1.072 (0.386)	0.37–2.11
Tiotropium HandiHaler [®] 18 µg	1.096 (0.381)	0.37–2.10
FVC, L		
Tiotropium Respimat [®] 5 µg	2.614 (0.621)	1.31–4.12
Tiotropium HandiHaler [®] 18 µg	2.662 (0.656)	1.21–4.40

for tiotropium were similar for the two inhalers (Fig. 3), and steady-state tiotropium plasma exposures (AUC_{τ,ss}) were 94.4 and 89.6 pg/h/ml (geometric mean values, $n = 128$) for the Respimat[®] inhaler and HandiHaler[®] respectively, giving an adjusted mean ratio for the Respimat[®] inhaler to HandiHaler[®] of 105.60%. For steady-state urinary excretion of unchanged tiotropium in the first 4 h after dosing, the respective values of Ae_{0-4,ss} were 342 and 341 ng ($n = 128$), giving an adjusted mean ratio of 102.22%.

The 90% CIs of the adjusted mean ratios (Respimat[®]: HandiHaler[®]) were 98.00 and 113.78% for AUC_{τ,ss} and 92.50 and 112.96% for Ae_{0-4,ss}; both of these lay within the interval of 80–125%, thus meeting the criterion for therapeutic equivalence for orally administered drugs. For inhaled drugs, this finding indicates comparable systemic exposure with the two inhalers.

Tolerability and safety

Adverse events are summarised in Table 5. The number of adverse events reported during treatment with tiotropium

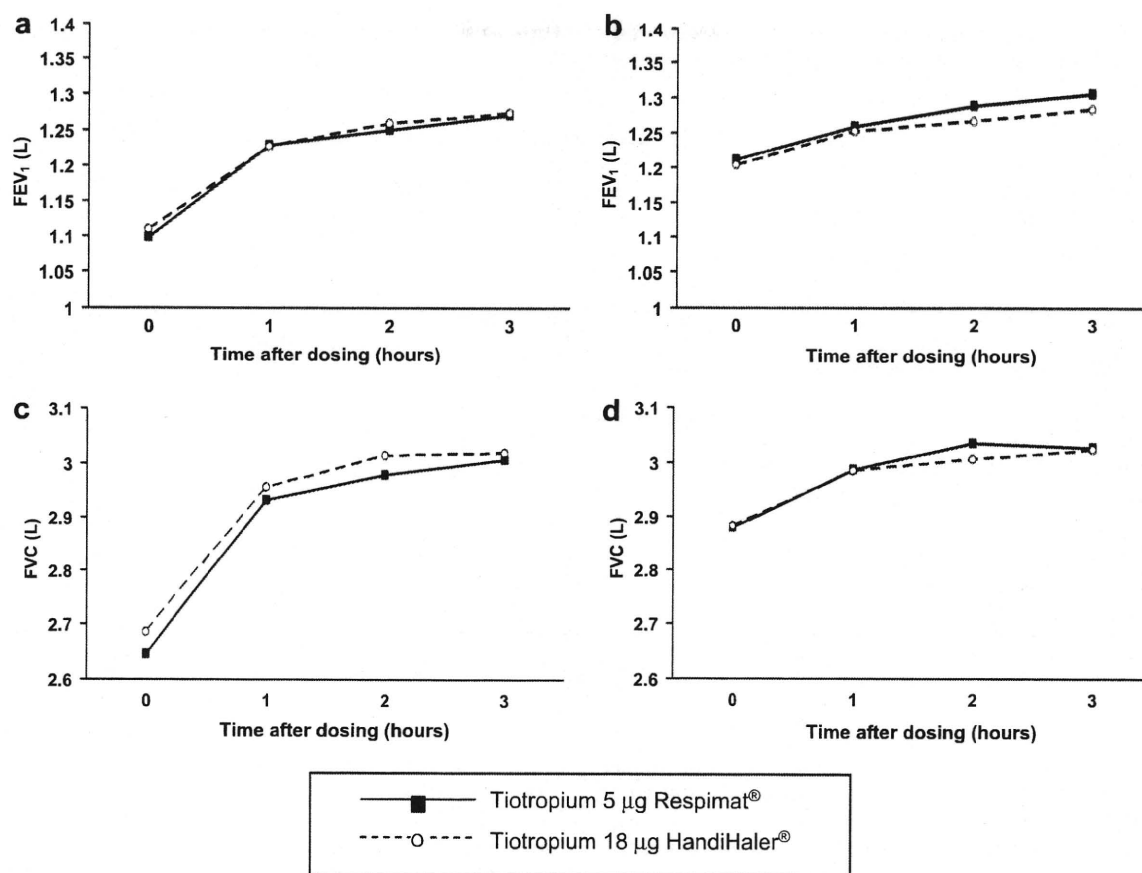


Figure 2 Changes in lung function parameters in the 3 h after dosing in full analysis set ($n = 134$): mean FEV_1 on Days 1 and 29 (a and b); mean FVC on Days 1 and 29 (c and d).

Respimat[®] (45; 30.6%) was similar to that with tiotropium HandiHaler[®] (41; 27.9%). The number of patients reporting adverse events considered by the investigator to be related to study treatment was low (4 [2.7%] and 8 [5.4%] patients during Respimat[®] and HandiHaler[®] treatment periods respectively). Three patients withdrew from the treatment phase of the study as a result of adverse events.

In all, 11 serious adverse events (SAEs) were reported by ten patients during the two treatment periods, none of which were considered to be related to study treatment. The 11 SAEs that occurred (five with Respimat[®] and six with HandiHaler[®]) were bacterial bronchitis, COPD exacerbation (4 events), haemorrhoids, oesophageal carcinoma, pneumonia,² pneumothorax and rheumatoid arthritis. No deaths occurred during the study.

Table 2 Non-inferiority analysis for trough FEV_1 increase (change in pre-dose FEV_1 from Day 1 to Day 29) in the full analysis set. Mean increases are adjusted for effects of patient, period and baseline FEV_1 .

	N	Adjusted mean increase, L (SE)	Mean difference, L (SE)	95% CL	<i>p</i> value for non-inferiority ^a
Tiotropium Respimat [®] 5 µg	134	0.109 (0.006)	—	0.097, 0.120	—
Tiotropium HandiHaler [®] 18 µg	134	0.101 (0.006)	—	0.089, 0.113	—
Difference, Respimat minus HandiHaler [®]	134	—	0.008 (0.009)	-0.009, 0.024	$p < 0.001$

Abbreviations: CL, confidence limits; SE, standard error.

^a One-sided ANCOVA for non-inferiority.

Table 3 Summary of peak and average (AUC_{0-3h}) FEV₁ responses on Day 1 and Day 29 in the full analysis set. Mean responses are adjusted for effects of patient, period and baseline FEV₁ on Day 1.

	Tiotropium Respimat [®] 5 µg (n = 134)	Tiotropium HandiHaler [®] 18 µg (n = 134)
Peak FEV ₁ , Day 1, L		
Adjusted mean response (95% CL)	0.186 (0.175, 0.196)	0.189 (0.179, 0.199)
Difference, Respimat [®] minus HandiHaler [®] (95% CL)		-0.003 (-0.018, 0.011)
p value for difference between inhalers ^a		0.6481
Peak FEV ₁ , Day 29, L		
Adjusted mean response (95% CL)	0.220 (0.208, 0.232)	0.205 (0.193, 0.217)
Difference, Respimat [®] minus HandiHaler [®] (95% CL)		0.015 (-0.002, 0.032)
p value for difference between inhalers ^a		0.0925
FEV ₁ AUC _{0-3h} , Day 1, L		
Adjusted mean response (95% CL)	0.119 (0.111, 0.127)	0.122 (0.114, 0.130)
Difference, Respimat [®] minus HandiHaler [®] (95% CL)		-0.003 (-0.014, 0.009)
p value for difference between inhalers ^a		0.6358
FEV ₁ AUC _{0-3h} , Day 29, L		
Adjusted mean response (95% CL)	0.166 (0.155, 0.177)	0.151 (0.140, 0.162)
Difference, Respimat [®] minus HandiHaler [®] (95% CL)		0.015 (-0.001, 0.030)
p value for difference between inhalers ^a		0.0679

Abbreviations: CL, confidence limits; SE, standard error.

^a Two-sided ANCOVA.

In the listing of adverse events by preferred term, the most common events were nasopharyngitis (22 patients) and COPD exacerbation (10 patients). Dry mouth was reported by 5 patients. These and other common events, i.e. those occurring in 2% of more of patients on either inhaler, are listed in Table 5.

No clinically important drug-related changes were noted in the assessments of vital signs, ECG and laboratory test variables.

Discussion

In this study, we have shown that a daily tiotropium dose of 5 µg inhaled via the Respimat[®] inhaler was similar to a dose of 18 µg inhaled from HandiHaler[®] in terms of pulmonary function improvement and pharmacokinetics when both were given for a period of 4 weeks. As well as producing an acute bronchodilator effect after each daily dose, 4 weeks' treatment with tiotropium inhaled from both test inhalers was associated with an improvement in airway calibre as shown by an increase of roughly 100 ml in trough (pre-dose) FEV₁ over the period from Day 1 to Day 29. On the basis of this efficacy measure, tiotropium Respimat[®] was demonstrated to be non-inferior to tiotropium HandiHaler[®] at the doses studied in this trial ($p < 0.001$).

The similar clinical performance of tiotropium Respimat[®] 5 µg and tiotropium HandiHaler[®] 18 µg was confirmed by other spirometry measurements. The trough FVC at Day 29 was roughly 200 ml higher than the corresponding value on Day 1 with both inhalers and for this measure, as well as for peak and average (AUC_{0-3h}) FEV₁ and FVC results, differences between the two inhalers were not statistically significant. Our efficacy findings are in line with the results of a dose-ranging study that compared lung function after 3 weeks'

treatment with tiotropium HandiHaler[®] 18 µg and a range of tiotropium doses from the Respimat[®] inhaler.¹⁸ They also agree closely with a comparison done in European and North American COPD patients who received two different daily doses of tiotropium from the Respimat[®] inhaler (5 and 10 µg) and tiotropium 18 µg from HandiHaler[®], each for 4 weeks in two crossover studies of identical design.¹⁹ Pooled analysis of those studies demonstrated non-inferiority of tiotropium Respimat[®] 5 µg to tiotropium HandiHaler[®] 18 µg for the primary efficacy variable of trough FEV₁, and also showed trough FEV₁ responses to be statistically significantly higher with tiotropium Respimat[®] 5 µg, although the difference was small and of questionable clinical relevance.

Until now, no studies have been published on tiotropium pharmacokinetics in Japanese individuals (COPD patients or volunteers). Average tiotropium plasma concentrations and urinary excretion of tiotropium at steady-state (Day 29) were very similar for tiotropium Respimat[®] and tiotropium HandiHaler[®]. When adjusted mean ratios (Respimat[®]: HandiHaler[®]) were calculated for both parameters, the associated 90% CIs were within the interval of 80–125%, which meets the criterion for demonstrating therapeutic equivalence for orally administered drugs. The pooled analysis of the twin crossover studies mentioned above¹⁹ also found that systemic exposures for tiotropium Respimat[®] 5 µg and tiotropium HandiHaler[®] 18 µg were similar, although urinary excretion of tiotropium was 26% higher in the tiotropium Respimat[®] 5 µg arm than in the tiotropium HandiHaler[®] 18 µg arm.

The number and type of adverse events reported in the current study did not indicate any difference in the tolerability of tiotropium Respimat[®] and tiotropium HandiHaler[®], and safety assessments (vital signs, ECG and laboratory test values) did not suggest any adverse responses to either of the study treatments. These results are in line with those

Table 4 Summary of trough, peak and average (AUC_{0-3h}) FVC responses, in the full analysis set. Mean responses are adjusted for effects of patient, period and baseline FVC on Day 1.

	Tiotropium Respimat [®] 5 µg (n = 134)	Tiotropium HandiHaler [®] 18 µg (n = 134)
Trough FVC, L		
Adjusted mean increase, Day 29 minus Day 1 (95% CL)	0.213 (0.186, 0.241)	0.217 (0.190, 0.245)
Difference, Respimat [®] minus HandiHaler [®] (95% CL)	-0.004 (-0.044, 0.036)	
p value for difference between inhalers ^a	0.8384	
Peak FVC, Day 1, L		
Adjusted mean response (95% CL)	0.383 (0.359, 0.407)	0.409 (0.386, 0.433)
Difference, Respimat [®] minus HandiHaler [®] (95% CL)	-0.026 (-0.060, 0.008)	
p value for difference between inhalers ^a	0.1278	
Peak FVC, Day 29, L		
Adjusted mean response (95% CL)	0.424 (0.398, 0.450)	0.411 (0.385, 0.437)
Difference, Respimat [®] minus HandiHaler [®] (95% CL)	0.013 (-0.024, 0.049)	
p value for difference between inhalers ^a	0.4973	
FVC AUC_{0-3h}, Day 1, L		
Adjusted mean response (95% CL)	0.250 (0.230, 0.269)	0.272 (0.252, 0.291)
Difference, Respimat [®] minus HandiHaler [®] (95% CL)	-0.022 (-0.050, 0.006)	
p value for difference between inhalers ^a	0.1219	
FVC AUC_{0-3h}, Day 29, L		
Adjusted mean response (95% CL)	0.326 (0.303, 0.350)	0.316 (0.292, 0.339)
Difference, Respimat [®] minus HandiHaler [®] (95% CL)	0.010 (-0.023, 0.044)	
p value for difference between inhalers ^a	0.5414	

Abbreviations: CL, confidence limits; SE, standard error.

^a Two-sided ANCOVA.

reported from the pooled crossover results in European and US patients.¹⁹ In our study, nasopharyngitis, COPD exacerbation and dry mouth were the three most common adverse events, which is consistent with previous experience from clinical trials of tiotropium.²²

Taken together, the results of our study show that the Respimat[®] inhaler is a more efficient inhaler than HandiHaler[®]. A daily dose of only 5 µg produces similar efficacy and pharmacokinetics to a dose more than threefold higher from

HandiHaler[®], and tolerability and safety profiles are similar. Deposition studies have shown that a higher proportion of the dose emitted from the Respimat[®] inhaler is delivered to the lungs compared with pMDIs and a multidose DPI.^{16,17} In a clinical trial of COPD patients, this allowed the nominal dose of bronchodilator from a pMDI to be reduced by 50% while maintaining efficacy and safety.²³

Although the protocol of our study allowed inclusion of men and women, the full analysis set included only 3 women.

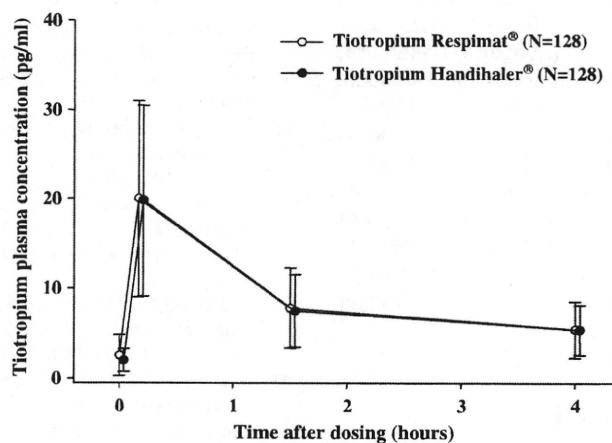
**Figure 3** Change in arithmetic mean plasma concentrations of tiotropium after dosing on Day 29. Error bars show standard deviations.

Table 5 Summary of adverse events during study. Events that occurred in at least 2% of patients in either group are listed by preferred term.

Number of patients reporting (n, %)	Tiotropium Respimat [®]	Tiotropium HandiHaler [®]
All patients	147 (100)	147 (100)
Any adverse event	45 (30.6)	41 (27.9)
Nasopharyngitis	13 (8.8)	9 (6.1)
COPD exacerbation	6 (4.1)	4 (2.7)
Dry mouth	2 (1.4)	3 (2.0)
Diarrhoea	1 (0.7)	3 (2.0)
Rash	0 (0)	3 (2.0)
Any drug-related adverse event ^a	4 (2.7)	8 (5.4)
Adverse events leading to discontinuation	1 (0.7)	2 (1.4)
Serious adverse events	4 (2.7)	6 (4.1)

^a As judged by the investigator.

This is not unexpected, firstly because the smoking rate in Japan is much higher in men than in women, and secondly because Japanese women are generally reluctant to enrol in clinical trials, not only in COPD but also in other diseases. Although placebo inhalers were used in our study to conceal the identity of the active inhaler, there was no all-placebo arm in the study, in contrast to the Respimat[®]–HandiHaler[®] comparison studies in Europe and US patients. In that analysis however, all three active treatment arms were found to be associated with significantly better lung function than placebo.¹⁹

In conclusion, tiotropium Respimat[®] 5 µg was shown to have similar efficacy and safety as tiotropium HandiHaler[®] 18 µg when used for the treatment of Japanese patients with COPD, and the 90% confidence intervals of AUC and Ae ratios (Respimat[®]: HandiHaler[®]) lay within the interval of 80–125%.

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Conflict of interest statement

M.I. has previously served on scientific advisory boards of GlaxoSmithKline KK, Nippon Boehringer Ingelheim, Novartis

Pharma KK and AstraZeneca KK. He has received lecture fees from GlaxoSmithKline KK, AstraZeneca KK and Nippon Boehringer Ingelheim and unrestricted grants from GlaxoSmithKline KK and Nippon Boehringer Ingelheim. TF is an employee of Nippon Boehringer Ingelheim, the sponsors of the trial, and contributed to study design, data interpretation, manuscript review and the collective decision to submit the manuscript for publication. Y.F. has previously served on scientific advisory boards of GlaxoSmithKline KK, Nippon Boehringer Ingelheim, Novartis Pharma KK, AstraZeneca KK, Kyorin KK, Abbot Japan and Ohtsuka pharma. He has received lecture fees from GlaxoSmithKline KK, AstraZeneca KK, Nippon Boehringer Ingelheim, Abbot Japan and Ohtsuka pharma and unrestricted grants from Nippon Boehringer Ingelheim.

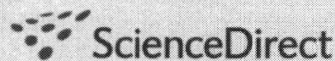
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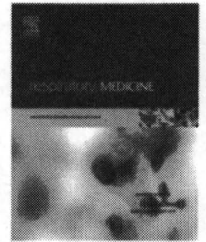
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Additive effects of transdermal tulobuterol to inhaled tiotropium in patients with COPD

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KEYWORDS

Chronic obstructive pulmonary disease;
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Pulmonary function;
Quality of life;
Transdermal tulobuterol

Summary

Background: The current mainstream treatment for COPD is bronchodilators alone or in combination. The effects of a β_2 -agonist, tulobuterol, administered transdermally, have been reported to last for 24 h. However, there are no reports on the efficacy of tulobuterol combined with an anticholinergic. In this study, we investigated the efficacy and safety of transdermal tulobuterol combined with inhaled tiotropium in COPD.

Methods: After a 2-week run-in period, 103 stable COPD patients aged ≥ 40 years were randomized into two groups: inhaled tiotropium (18 μg , Tio group) or transdermal tulobuterol (2 mg) combined with inhaled tiotropium (18 μg , Tio + Tulo group) for 8 weeks. Primary endpoints were pulmonary function and severity of dyspnea. The St. George's Respiratory Questionnaire (SGRQ) score was a secondary endpoint.

Results: In both groups, FEV₁ and FVC as well as dyspnea improved significantly after 8 weeks. In a comparison of both groups, percentage changes in IC and morning and evening peak expiratory flow were significantly greater in the Tio + Tulo group than in the Tio group. In addition, significant improvement in SGRQ score was observed in the Tio + Tulo group only. The risk of

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adverse events related to the study drugs was not increased.

Conclusion: In COPD patients, additional administration of transdermal tulobuterol to inhaled tiotropium produced significant benefits in dyspnea and SGRQ score as well as pulmonary function. These benefits may be due to a reduction in pulmonary hyperinflation resulting from improvement of peripheral airflow obstruction through tulobuterol via the systemic circulation.

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Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disease that occurs as a result of inhalation of harmful particles, such as those in cigarette smoke. There is some concern that the number of COPD patients will increase with the aging of the population. The Global Initiative for Chronic Obstructive Lung Disease (GOLD) recommends the use of long-acting bronchodilators, such as anticholinergics, β_2 -agonists, and methylxanthines, for the management of stable COPD patients.¹ In addition, the combined use of bronchodilators with different mechanisms of action results in greater potency and fewer adverse effects, and is therefore recommended rather than increasing the dose of a single agent when symptoms are not well controlled by monotherapy.

Tiotropium, which has been available in Japan since 2004, is an anticholinergic that is inhaled once daily. Its efficacy has been demonstrated in large-scale clinical studies.^{2–10} In a recent study in which the use of all drugs for COPD treatment except inhaled anticholinergics was allowed, the efficacy and safety of tiotropium were investigated in 5993 COPD patients over 4 years, in comparison with a placebo. In this study, tiotropium was demonstrated to improve pulmonary function and quality of life (QOL), and to reduce the risk of exacerbations in COPD and related hospitalizations.¹¹ Based on such evidence, inhaled tiotropium is regarded as a new therapeutic option for COPD in addition to β_2 -agonists and methylxanthines.

Transdermal tulobuterol, which is frequently used for COPD treatment, was developed in Japan as the world's first long-acting β_2 -agonist in a patch formulation. This formulation of tulobuterol was designed to maintain drug levels at constant effective concentrations over a 24-h period when applied once daily.^{12,13} Administered this way, tulobuterol exerts its effect through the systemic circulation and provides a lower maximum blood concentration, resulting in fewer systemic adverse effects, such as palpitation and tremor, than oral formulations. In addition, transdermal tulobuterol avoids the first-pass effect and is expected to cause fewer adverse effects, such as gastrointestinal symptoms. To date, transdermal tulobuterol has been launched in Japan, Korea, and China. The efficacy of this drug in COPD patients was investigated in a randomized comparative study using inhaled salmeterol, which is used for the treatment of COPD.¹⁴ That study demonstrated that, compared with inhaled salmeterol, transdermal tulobuterol has an equivalent effect of improving pulmonary function and is significantly superior in improving sleep scores and QOL.¹⁴

These findings suggest that the combined use of two long-acting once-daily bronchodilators, transdermal tulobuterol and inhaled tiotropium, with different mechanisms of action, is an excellent treatment for COPD patients whose symptoms are not well controlled by monotherapy. However, there are few reports on the efficacy and safety of combination therapy with these two drugs, and the benefits of such a combination therapy have not been fully confirmed. Therefore, we conducted a clinical study to verify the efficacy and safety of the combined use of transdermal tulobuterol and inhaled tiotropium in COPD patients.

Methods

Subjects

The study was conducted in patients aged ≥ 40 years with a clinical diagnosis of COPD who had the following conditions: FEV₁/FVC $< 70\%$ 15–60 min after inhalation of a short-acting β_2 -agonist, with FEV₁ 30–80% of the predicted value, in pulmonary function tests performed during the screening period; current or past smoking history; and relatively stable condition with persistent dyspnea. All subjects were out-patients. Their status did not change during the study period, and they remained out-patients. Before the implementation of the study, the study objectives were explained to patients and those who provided informed consent to participate in the study were enrolled.

The following patients were excluded: patients whose major symptom was bronchial asthma; patients on treatment with oral steroids; patients with respiratory failure on home oxygen therapy; patients with previous hypersensitivity to tulobuterol; patients with skin diseases, such as atopic dermatitis, who are considered unsuitable for treatment with transdermal tulobuterol; patients with concurrent hyperthyroidism, hypertension, heart disease, or diabetes mellitus, who are considered unsuitable for treatment with β_2 -agonists; patients with glaucoma; patients with dysuria associated with, for example, benign prostatic hyperplasia; patients with previous hypersensitivity to atropine and related substances or to tiotropium; patients who were or may have been pregnant, were breastfeeding, or intended to become pregnant during the study period; and other patients judged ineligible for the present clinical study by the attending physician.

The study complied with the Declaration of Helsinki of the World Medical Association. The protocol was approved by the ethics committees of the institutions involved.

Study design

The study was conducted as a multicenter parallel-group comparison study. The assignment of subjects was performed using a randomized subject assignment list prepared based on a random number table by a central registration center, a third party. After a 2-week run-in period, patients were randomized to either a group receiving inhaled tiotropium (one capsule of 18 µg) (the Tio group) or a group receiving transdermal tulobuterol (2 mg) in combination with inhaled tiotropium (one capsule of 18 µg) (the Tio + Tulo group), and treated for 8 weeks (Fig. 1). Tiotropium was inhaled using a designated inhalation device (HandiHaler), once daily between 7 a.m. and 9 a.m. Transdermal tulobuterol was applied to the chest, back, or upper arm, once daily, after bathing and before going to bed, by about 8 p.m.

Throughout the study period, the use of additional bronchodilators was not allowed, but the use of short-acting inhaled β_2 -agonists was allowed as necessary. A 2-week washout period was set for patients already on treatment with a long-acting β_2 -agonist or tiotropium. For patients who were on treatment with theophyllines, inhaled steroids, antiallergic agents, antihistamines, anti-tussives, expectorants, or anti-inflammatory enzyme preparations from before the start of the study, the concomitant use of these drugs was allowed. However, in principle, the dose and dosing regimen were not to be changed during the study period.

Pulmonary function tests and QOL assessment

The primary endpoints were pulmonary function and severity of dyspnea. For pulmonary function, spirometry was performed to determine FVC, FEV₁, %FEV₁, and IC at the start and end of study treatment. Spirometry was performed and measured between 9:00 am and noon. In addition, the morning and evening peak expiratory flows (PEFs) were measured using a peak flow meter (Mini-Wright, ATS scale; Matsuyoshi) every day. Trough values of peak flows were measured on getting up (before tiotropium inhalation) and before going to bed (before tulobuterol application). Severity of dyspnea was evaluated using the Medical Research Council dyspnea scale at the start and end of study treatment. As a secondary endpoint, QOL was evaluated using the St. George's Respiratory Questionnaire (SGRQ), which is a disease-specific QOL questionnaire, at the start and end of study treatment. In addition, for

safety, adverse events and abnormal laboratory values were evaluated.

Statistical procedures

Data are expressed as means \pm SD. For pulmonary function indices, comparison of mean changes from baseline was performed using the paired *t*-test, and between-group comparisons of percentage changes from baseline were performed using the Wilcoxon rank sum test. For PEFs, comparisons of percentage changes from baseline were performed using the Wilcoxon signed rank test, and between-group comparisons of percentage changes from baseline were performed using the Wilcoxon rank sum test. For QOL scores, comparison of mean changes from baseline were performed using the paired *t*-test, and between-group comparisons of changes from baseline were performed using Welch's *t*-test. The significance level was set at less than 5%.

Results

Subjects analyzed

One hundred and three patients were enrolled from 26 institutions in Japan. Of these, 50 and 53 patients were randomly assigned to the Tio group and the Tio + Tulo group, respectively. During the study period, 11 patients from the Tio group and nine patients from the Tio + Tulo group were withdrawn from the study. Reasons for withdrawal of these patients were failure to meet the pulmonary function test criteria (three of the Tio group and three of the Tio + Tulo group), no data available for the screening period (one of the Tio group), treatment violation during the screening period (two of the Tio group and two of the Tio + Tulo group), treatment non-compliance during the study period (one of the Tio group and one of the Tio + Tulo group), and treatment discontinuation owing to, for example, adverse drug reactions (four of the Tio group and three of the Tio + Tulo group). As a result, 83 patients (39 patients from the Tio group and 44 patients from the Tio + Tulo group) were evaluated for efficacy (Fig. 2).

The characteristics of the 83 subjects evaluated for efficacy are shown in Table 1. No differences between the treatment groups were noted in sex, age, height, weight, or smoking status. In addition, although theophyllines, expectorants, and short-acting β_2 -agonists were used as

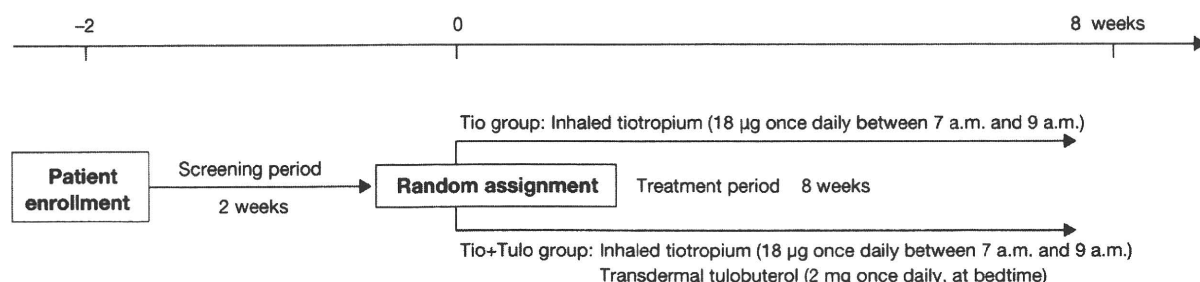


Figure 1 Study protocol.