

Synergistic effects of fluticasone propionate and salmeterol on *in vitro* T-cell activation and apoptosis in asthma

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Background: In asthma T cells are characterized by an increased activation state and by reduced apoptosis. **Objective:** Because the clinical efficacy of inhaled corticosteroids combined with long-acting β_2 -agonists has been widely demonstrated in asthma, we studied, *in vitro*, the effect of fluticasone propionate (FP) and salmeterol alone and in combination on the activation and apoptosis of peripheral blood T cells (PBTs), on the expression of phosphorylated nuclear factor κ B inhibitor ($I\kappa B\alpha$), and on the nuclear translocation of glucocorticoid receptor (GR) in PBTs from asthmatic subjects.

Methods: Apoptosis was evaluated on the basis of annexin V binding, whereas the expression of caspases 8 and 3 and phosphorylated $I\kappa B\alpha$, as well as the nuclear translocation of the GR, were evaluated by means of Western blot analysis.

Results: FP alone increases and salmeterol alone does not affect T-cell apoptosis. The combination of FP and salmeterol significantly increases PBT apoptosis in comparison with FP alone. FP at the lower concentration, when combined with salmeterol, is equivalent to FP at the higher concentration in inducing PBT apoptosis. The synergy in the induction of cell apoptosis is associated with more efficient activation of caspases 8 and 3. FP plus salmeterol is also able to synergistically reduce the expression of phosphorylated $I\kappa B\alpha$, thus limiting nuclear factor κ B activation. The synergy was related to an increased nuclear translocation of the GR.

Conclusion: This study shows that the combination of FP and salmeterol is able to control PBT activation in asthmatic patients more efficiently than FP alone and with a lower concentration of steroids. (*J Allergy Clin Immunol* 2004;114:1216-23.)

Key words: Glucocorticoid, salmeterol, apoptosis, T cells

Abbreviations used

FP: Fluticasone propionate
GR: Glucocorticoid receptor
ICS: Inhaled corticosteroid
 $I\kappa B$: Inhibitor of nuclear factor κ B
LABA: Long-acting β_2 agonist
NF- κ B: Nuclear factor κ B
PBT: Peripheral blood T cell
PDTC: Pyrrolidinedithiocarbamic acid
p $I\kappa B\alpha$: Phosphorylated inhibitor of NF- κ B
PKC: Protein kinase C
PMA: Phorbol myristate acetate

T cells play a central role in the airway inflammation seen in asthma.^{1,2} Their accumulation at the site of inflammation is related to an increased recruitment from the peripheral blood and prolonged survival.³ Cell apoptosis is one of the mechanisms involved in the control of T-cell homeostasis,⁴ causing the deletion of autoreactive T cells.⁵ Most T cells in the airways of untreated asthmatic patients are not apoptotic.⁶ Moreover, in patients with asthma, peripheral blood T cells (PBTs) are characterized by an increased activation state and by reduced apoptosis.⁷

A persistent activation of the transcription nuclear factor κ B (NF- κ B)/inhibitor of NF- κ B ($I\kappa B$) system might represent one of the mechanisms by which T-cell apoptosis is reduced.⁸ This might be associated with the activation of the $I\kappa B\alpha$ kinases that phosphorylate $I\kappa B\alpha$, the most predominant NF- κ B inhibitor. The phosphorylation of $I\kappa B\alpha$ generates, in turn, the detachment of $I\kappa B\alpha$ from NF- κ B, as well as $I\kappa B\alpha$ degradation itself. As a result of these phenomena, NF- κ B translocates into the nucleus and activates the transcription of target genes.⁹

The efficacy of the combination of inhaled corticosteroids (ICSs) with long-acting β_2 -agonists (LABAs) in the therapy of asthma has been widely demonstrated.¹⁰ A possible explanation for this efficacy is represented by a synergistic interaction between ICSs and LABAs at a molecular level. In a model of fibroblasts and vascular smooth muscle cells, LABAs upregulate the activity of ICSs by promoting the translocation of glucocorticoid receptor (GR) into the nuclei of these cells.¹¹

We have previously demonstrated that fluticasone propionate (FP) is able to induce peripheral blood T-cell

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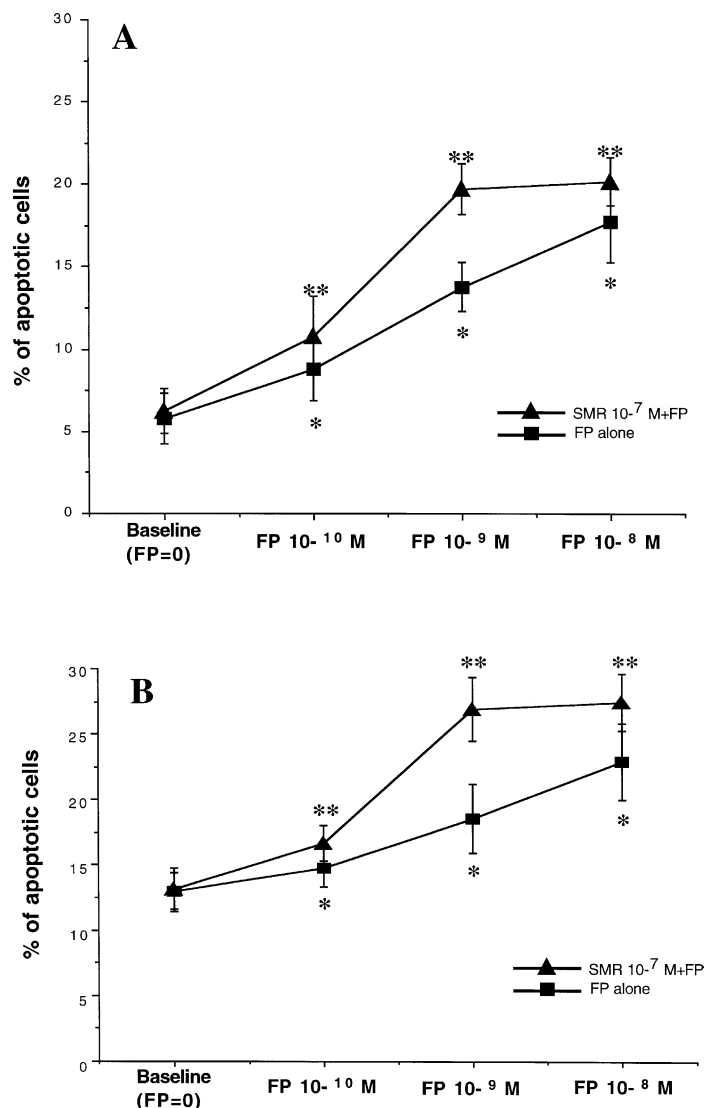


FIG 1. FP and salmeterol (SMR) in combination synergistically induce apoptosis of resting peripheral T cells (n = 10). PBTs were isolated and cultured with and without FP and salmeterol alone or in combination for 24 (A) and 72 (B) hours. T-cell apoptosis was evaluated on the basis of annexin V binding and expressed as the percentage of annexin V-positive cells. **P* < .05 compared with baseline. ***P* < .05 compared with FP alone.

apoptosis in asthma⁷ and that steroids are able to reduce the NF-κB activation in PBTs.¹² Despite this, the effects of the combination of ICSs and LABAs on T cells has not yet been firmly established.

The aims of this study were to assess (1) whether FP and salmeterol exert a synergistic effect on T-cell apoptosis; (2) whether FP and salmeterol exert a combined down-regulatory effect on NF-κB activation of T cells through reduced degradation of its endogenous inhibitor, IκBα; and (3) whether these synergistic effects are related to increased translocation of the GR from the cytosol to the nuclei of T cells.

METHODS

Subjects

We selected 15 patients with mild intermittent asthma (age, 9-13 years), according to the criteria of the American Thoracic Society. The diagnosis of asthma and the assessment of its severity were performed at study entry according to Global Initiative for Asthma guidelines.¹³ None of the patients received any corticosteroid treatment during the previous 2 weeks.

The study fulfilled the criteria of the ethics committee of our hospital, and all subjects, parents, or both provided informed consent.

TABLE I. Phenotype of annexin V–positive cells (24 hours of incubation, n = 5, mean ± SD)

	CD3	CD4	CD8
Baseline	9.8 ± 1.6	5.2 ± 0.7	4.5 ± 0.8
10 ⁻⁹ M FP	21.4 ± 5.6	12.2 ± 5	9 ± 2.5
10 ⁻⁹ M FP + 10 ⁻⁷ M salmeterol	24 ± 5	13.5 ± 4.7	10 ± 2

P < .05, FP plus salmeterol versus FP alone and versus baseline.

Isolation of peripheral blood lymphocytes

PBTs were obtained from 20 mL of heparinized blood from asthmatic patients, as previously described.¹⁴ The recovered PBT fraction was greater than 97% CD3⁺, as assessed by means of cytofluorimetric analysis.

PBT cultures

PBTs were incubated in the absence or presence of FP (10⁻⁸, 10⁻⁹, and 10⁻¹⁰ M; GlaxoSmithKline, London, United Kingdom), salmeterol (10⁻⁷ M; GlaxoSmithKline),^{15,16} or in combination with propranolol (10 μM, 30 minutes; Sigma Chemical Co, St Louis, Mo) before incubation with salmeterol.¹⁷ The concentration range of FP and the incubation times (24 and 72 hours) were previously shown to be optimal to induce cell apoptosis.⁷ Cell viability was assessed by means of trypan blue dye exclusion. In some experiments PBTs were cultured with anti-CD3 mAb (100 ng/mL; Dakopatts, Glostrup, Denmark) or with phorbol myristate acetate (PMA; 2–20 ng/mL; Sigma) for 24 hours, as previously reported.¹⁸ In other experiments PBTs were pretreated with Ac-IETD-CHO, an inhibitor of caspase 8 (1 hour, 60 μM; Pharmingen, San Jose, Calif) or with Ac-DEVD-CHO, an inhibitor of caspase 3 (1 hour, 60 μM; Pharmingen) and then cultured with FP, salmeterol, or both for 24 hours (37°C, 5% CO₂). PBTs were cultured with pyrrolidinedithiocarbamic acid (PDTC; 500 μM, 24 hours; Sigma), an inhibitor of NF-κB, to evaluate the role of NF-κB.¹² To evaluate pIκBα and GR nuclear translocation, PBTs were cultured (37°C, 5% CO₂) with FP, salmeterol, or both for 30 minutes.

PBT phenotype analysis and apoptosis detection

T-cell apoptosis was determined, as previously described,⁷ by evaluating annexin V expression. Apoptotic cells were identified by means of double or triple staining with an Annexin V–FITC kit (Bender MedSystem, Vienna, Austria) and anti-human Peridinin Chlorophyll Protein–CD3 (Becton Dickinson, San Jose, Calif) and anti-human phycoerythrin (RPE)–conjugated CD8, (Dakopatts Glostrup) or anti-human RPE–conjugated CD4 clone MT310 (Dakopatts). Cell apoptosis and the phenotype of PBTs were determined by using a FACS Escalibur Plus flow cytometer (Becton Dickinson). Phycoerythrin-conjugated (X-928, Dakopatts) and FITC-conjugated mouse anti-human IgG1 (X-927, Dakopatts) were used as negative controls.

Western blot analysis for caspases 3 and 8, phosphorylated inhibitor of nuclear factor κB (pIκBα) detection, and GR nuclear translocation

Western blot analysis for caspase 3 and 8 expression, for pIκBα detection, and for GR translocation was performed as previously

described.^{12,19} The cytoplasmic and nuclear protein fractions were separated by using a commercial kit (Pierce, Rockford, Ill) to study GR nuclear translocation. Fifty micrograms of total protein was subjected to SDS-PAGE on 4% to 12% gradient gels (Novex, San Diego, Calif), blotted onto nitrocellulose membranes, blocked with PBS containing 3% BSA and 0.1% Tween 20, and then probed with a polyclonal antibody directed against human procaspases 3 and 8 (Pharmingen and Becton Dickinson), with a polyclonal antibody directed against human GRα (Santa Cruz Biotechnology, Santa Cruz, Calif) or with a polyclonal antibody recognizing pIκBα (Santa Cruz Biotechnology). Detection was performed with an enhanced chemiluminescence system (NEN, Boston, Mass), followed by autoradiography. Negative controls were performed in the absence of primary antibody or including an isotype control antibody. β-actin (Sigma) was used as a housekeeping protein. Gel images were taken with an EPSON GT-6000 scanner and then imported to a National Institutes of Health Image analysis 1.61 program to determine band density. Data are expressed as arbitrary densitometric units corrected against the density of β-actin bands.

Statistical analysis

Data are expressed as mean counts ± SD. Statistical analysis was performed with repeated-measures ANOVA with nonparametric Kruskal-Wallis analysis and ANOVA with the Bonferroni Dunn correction. A *P* value of less than .05 was accepted as statistically significant.

RESULTS

FP and salmeterol in combination increase apoptosis in resting PBTs

FP significantly increased PBT apoptosis in a dose-dependent manner when compared with baseline values, whereas salmeterol alone did not affect spontaneous PBT apoptosis at either tested time point. Interestingly, the combined presence of FP at all the tested concentrations and salmeterol was able to significantly increase PBT apoptosis at both time points. Moreover, FP at the concentration of 10⁻⁹ M when combined with salmeterol was not statistically different from FP at the concentration of 10⁻⁸ M in inducing PBT apoptosis (Fig 1). Propranolol blocked FP- and salmeterol-induced T-cell apoptosis (data not shown). Both CD4⁺ and CD8⁺ cells composed the apoptotic CD3 population (Table I).

FP and salmeterol in combination synergistically increase anti-CD3–activated PBT apoptosis

The addition of 10⁻⁹ M FP to PBTs stimulated with anti-CD3 mAb significantly (*P* < .05) increased their apoptosis in comparison with the presence of anti-CD3 mAb alone. As expected, the addition of salmeterol alone did not exert any additional proapoptotic effects in anti-CD3–stimulated cultures. Nonetheless, the combination of FP and salmeterol was able to significantly increase anti-CD3–induced apoptosis compared with levels seen in the presence of FP alone (Fig 2).

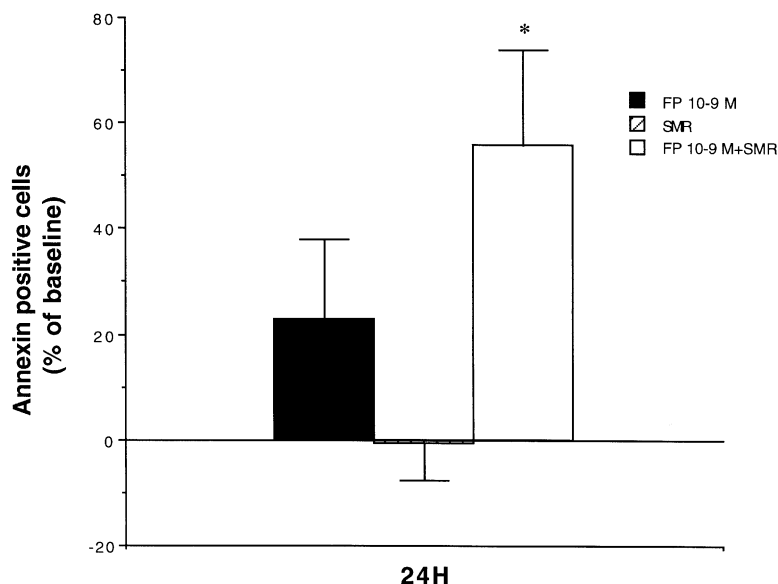


FIG 2. FP and salmeterol (SMR) in combination synergistically induce apoptosis of CD3-activated peripheral T cells ($n = 7$). * $P < .05$ compared with FP alone. PBTs were stimulated with anti-CD3 in the presence and absence of FP (10^{-9} M) and salmeterol (10^{-7} M) alone or in combination for 24 hours. Data are expressed as the percentage of control values (anti-CD3-stimulated cells). * $P < .05$ compared with FP alone.

FP and salmeterol in combination synergistically increase caspase activation

The presence of FP alone significantly decreased the levels of procaspase 8, whereas, as expected, the presence of salmeterol alone did not affect this level. Moreover, the presence of FP and salmeterol in combination further reduced the levels of procaspase 8 (Fig 3, A and C). With regard to procaspase 3, when PBTs were cultured in the presence of FP alone, a significant decrease of procaspase 3 levels occurred. Salmeterol alone did not affect the level of procaspase 3. The presence of FP and salmeterol in combination was able to further decrease the level of procaspase 3 (Fig 3, B and C).

FP and FP-salmeterol proapoptotic activity is dependent on caspase activation

We performed experiments with PMA as an activator of protein kinase C (PKC) and as an agent capable of blocking caspase 8 activation.²⁰ The addition of FP alone or in combination with salmeterol to PMA-stimulated PBTs was not able to induce apoptosis at either concentration of PMA (Fig 4, A). The presence of PMA alone induced T-cell apoptosis (data not shown). To gain insight into the impairment of FP and FP-salmeterol proapoptotic activity in the presence of PMA, we evaluated the levels of procaspases 8 and 3 in FP, salmeterol, and FP-salmeterol PMA-stimulated cultures. Interestingly, procaspase 8 and 3 activation in PMA-stimulated PBTs cultured with FP and with the FP-salmeterol combination was blocked (Fig 4, B). In addition, the inhibition of caspase 8 and 3 activation by Ac-IETD-CHO and Ac-DEVD-CHO

blocked both the proapoptotic activity of FP and that of the FP-salmeterol combination (Fig 5).

FP and salmeterol in combination decrease I κ B α phosphorylation

The inhibition of NF- κ B by PDTC was able to significantly increase PBT apoptosis (baseline, 19 ± 4 ; PDTC, 33 ± 9 ; Wilcoxon $P < .05$). In addition, FP at both tested concentrations was able to reduce I κ B α phosphorylation more efficiently than salmeterol. Moreover, the combination of FP and salmeterol at both tested FP concentrations was able to further reduce I κ B α phosphorylation when compared with the effects exerted by FP or salmeterol alone (Fig 6).

FP and salmeterol in combination increase the translocation of the GR from the cytosol to the nucleus of PBTs

Finally, we tested whether the synergistic activities exerted by FP and salmeterol in combination might be related to an increased nuclear translocation of the GR in PBTs. FP at the lesser and greater tested concentrations was able to dramatically decrease and completely abrogate the presence of GR in the cytosol, respectively. Moreover, the addition of salmeterol to FP at the lesser concentration was also able to abrogate the presence of the GR in the cytosol. Consequently, the decrease or the complete abrogation of the presence of the GR in the cytosol was paralleled by an increased presence of the GR in the nucleus (Fig 7), demonstrating that the addition of salmeterol to FP increases glucocorticoid activities

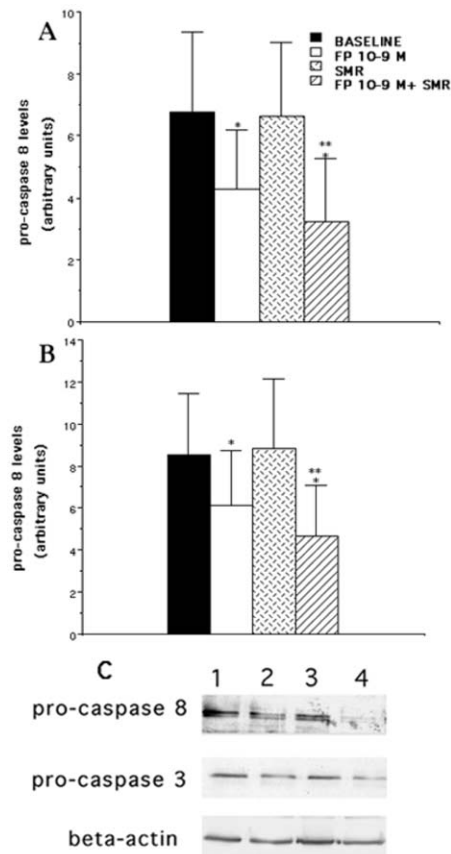


FIG 3. FP and salmeterol (SMR) in combination synergistically reduce caspase 8 and caspase 3 expression ($n = 7$). **A** and **B**, Densitometric analysis of procaspases 8 and 3, respectively. $*P < .05$ compared with baseline. $**P < .05$ compared with FP alone. **C**, Western blot analysis of procaspases 8 and 3 and β -actin expression by PBTs. Lane 1, Baseline; lane 2, 10^{-9} M FP; lane 3, 10^{-7} M salmeterol; lane 4, 10^{-9} M FP plus 10^{-7} M salmeterol.

through a more effective translocation of the GR into the nucleus.

DISCUSSION

A main goal of asthma therapy is to achieve optimal control of airway inflammation. ICSs are the mainstay of asthma management and are extremely effective inhibitors of T-cell activation⁷ and T_H2 cytokine production.²¹

Previous reports demonstrated the clinical utility of combined use of glucocorticoids and LABAs in the improvement of patient symptoms and airflow limitation.¹⁰ This study shows, for the first time, a synergistic proapoptotic effect of FP and salmeterol on resting and activated T cells isolated from the peripheral blood of asthmatic patients, the basis of which seems to be due to an increased GR nuclear translocation and enhanced inhibition of $I\kappa B\alpha$ phosphorylation. These effects are crucial because they provide an effective control of the T cell–related inflammation, a key component of asthma pathogenesis. The persistence of T-cell activation in asthma might be related to reduced T-cell apoptosis,³

generating, in turn, prolonged release of inflammatory soluble factors amplifying both asthma-associated inflammation and airway damage.²

We have previously demonstrated that in asthmatic patients FP is able to increase cell apoptosis of both resting and IL-2–stimulated PBTs.⁷ We now show that this FP-mediated effect on resting PBTs is increased by the addition of salmeterol, suggesting that the synergistic effects on asthma symptoms might be related, at least in part, to their ability to reduce T-cell survival. Both $CD4^+$ and $CD8^+$ PBTs are sensitive to both FP and the FP-salmeterol combination, suggesting that this proapoptotic effect is not restricted to a subtype of $CD3^+$ cells. Despite this, a great proportion of PBTs remains resistant to FP- and FP-salmeterol–induced apoptosis and is consistent with our previous observations.⁷ This is likely related to the expression of survival markers (ie, bcl-2) exerting a protective effect against apoptosis. On a speculative level, the relatively limited proapoptotic effect of FP and salmeterol on $CD3^+$ cells might prevent a massive depletion of these cells during the pharmacologic treatment, avoiding potential impairment of host defenses.

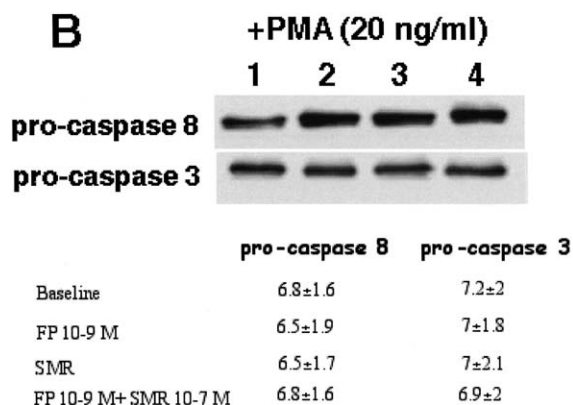
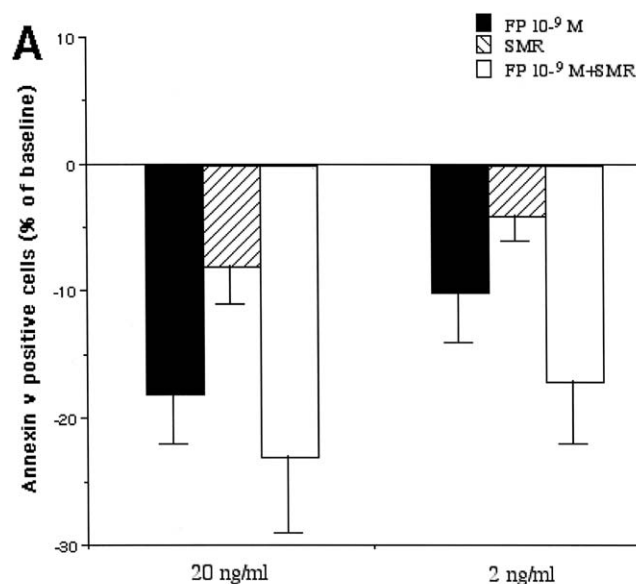


FIG 4. PMA blocks the apoptosis and the caspase 8 and 3 activation of PBTs (n = 5). PBTs were stimulated with PMA and FP (10⁻⁹ M) and salmeterol (SMR; 10⁻⁷ M) alone or in combination for 24 hours. **A**, PBT apoptosis expressed as the percentage of PMA-stimulated cells. *P* < .05 versus PMA alone. **B**, Western blot analysis of procaspases 8 and 3 by PMA-stimulated PBTs. Lane 1, Baseline; lane 2, 10⁻⁹ M FP; lane 3, 10⁻⁷ M salmeterol; lane 4, 10⁻⁹ M FP plus 10⁻⁷ M salmeterol. Table, Densitometry of procaspases 3 and 8.

Moreover, the level of PBT apoptosis induced by FP at lower concentrations in combination with salmeterol is similar to the levels seen with higher concentrations of FP alone. This supports the notion that FP and salmeterol combination therapy should permit the physician to use a lower concentration of steroid in the therapy of asthma, thereby reducing potential side effects. Additionally, the ability of propranolol to reverse the increase of PBT apoptosis induced by FP-salmeterol confirms that this phenomenon is mediated by β_2 receptor activation.

To evaluate whether these effects were also exerted in activated T cells, we stimulated PBTs from asthmatic patients with an anti-CD3 mAb. It has been demonstrated that the stimulation of the T-cell receptor/CD3 complex leads to T-cell activation and to a reduced responsiveness to dexamethasone in murine T-cell hybridomas.¹⁸ Our

findings that FP is able to increase the anti-CD3-stimulated T-cell apoptosis differently than the effects exerted by dexamethasone on T-cell hybridomas confirms that FP is a more effective steroid in exerting a proapoptotic activity on CD3-stimulated T cells.²² Another possible explanation of this different response might also be related to the use of a different T-cell target. Similar to the effects exerted on resting T cells, the addition of salmeterol to FP was able to significantly increase the anti-CD3-stimulated PBT apoptosis in comparison with both FP and salmeterol alone, suggesting that the proapoptotic effects of combined treatment also occur on activated PBTs.

Several studies report that sequential cleavage and activation of caspases are important mechanisms in most apoptosis models and that glucocorticoids are able to induce apoptosis through the activation of caspases 8

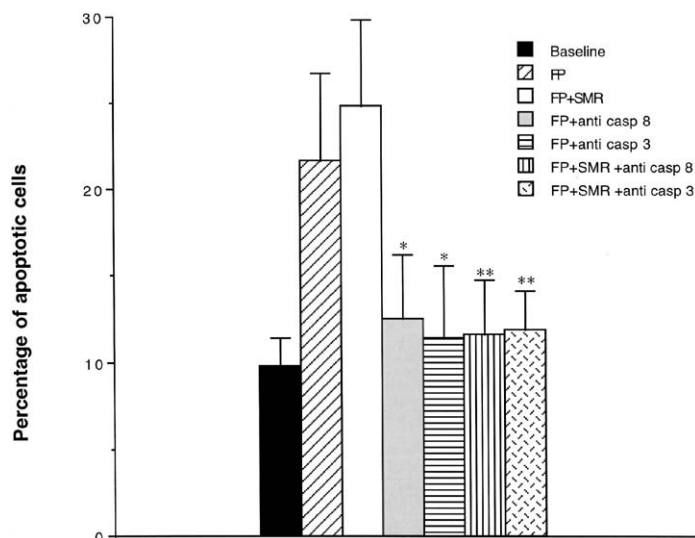


FIG 5. Inhibitors of caspases 8 and 3 block the apoptosis of PBTs ($n = 5$). PBTs were incubated with an inhibitor of caspase 8 (Ac-IETD-CHO) and with an inhibitor of caspase 3 (Ac-DEVD-CHO) and then cultured with FP (10^{-9} M) and salmeterol (SMR; 10^{-7} M) either alone or in combination for 24 hours. T-cell apoptosis was expressed as the percentage of annexin V– positive cells. * $P < .05$ compared with 10^{-9} M FP. ** $P < .05$ compared with 10^{-9} M FP plus 10^{-7} M salmeterol.

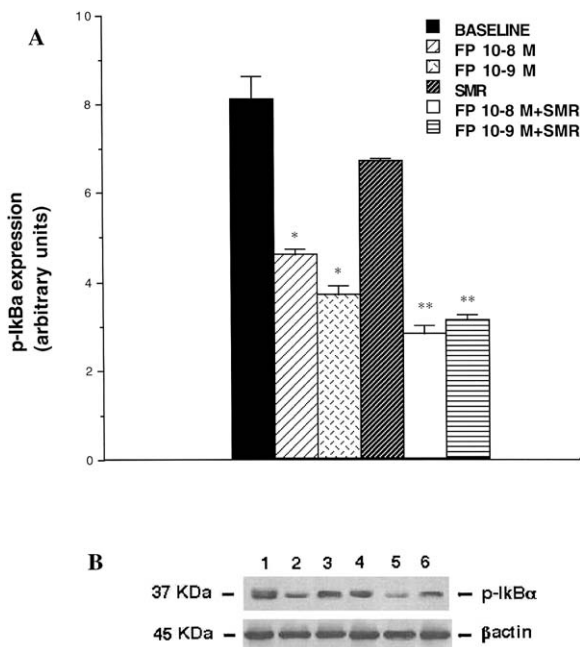


FIG 6. FP and salmeterol (SMR) in combination reduce synergistically phosphorylated IκBα expression ($n = 7$). **A**, Densitometric analysis of pIκBα. * $P < .05$ compared with the baseline. ** $P < .05$ compared with FP. **B**, Western blot analysis of pIκBα and β-actin expression by PBTs. Lane 1, Baseline; lane 2, 10^{-8} M FP; lane 3, 10^{-9} M FP; lane 4, 10^{-7} M salmeterol; lane 5, 10^{-8} M FP plus 10^{-7} M salmeterol; lane 6, 10^{-9} M FP plus 10^{-7} M salmeterol.

presence of FP, as well as that of FP plus salmeterol, is associated with the decreased levels of procaspases 8 and 3 because of their activation in PBTs.²³

To evaluate whether the activation of caspases was a crucial step in PBT apoptosis induced by FP and by FP plus salmeterol, we stimulated PBTs with PMA, which promotes PKC activation and inhibits caspase 8 cleavage and activation.²⁰ In this regard we also blocked caspase 8 and 3 activation with specific inhibitors in concomitant cultures. Our findings that the proapoptotic effects of FP and of FP plus salmeterol were inhibited by the presence of PMA and by specific inhibitors of caspases 8 and 3 strongly support the hypothesis that activation of caspases 8 and 3 is a crucial step in the proapoptotic activity of FP. Moreover, it is also conceivable that the reduced response to FP in PMA-pretreated PBTs might be linked to PKC activation, which in turn leads to increased GR turnover and GR downregulation.^{24,25} Another route related to the modulation of apoptosis by glucocorticoids is through NF-κB, a transcription factor induced by proinflammatory cytokines.^{26,27} A persistent activation of the NF-κB/IκB system might represent one of the mechanisms by which PBT apoptosis is reduced.⁸ Although there is some controversy about the role of this transcription factor in cell apoptosis, most data support an NF-κB antiapoptotic action.²⁸ In addition, it has been shown that cells that are naturally resistant to TNF-induced apoptosis become susceptible when transfected with an expression vector for IκBα, which inactivates NF-κB.²⁶ Consistent with these findings, we observed that the presence of FP and, to a greater extent, the combined presence of FP and salmeterol reduced the expression of phosphorylated IκBα, which, in turn, leads to an increased IκBα concentration and inhibition of NF-κB activity.

and 3. In particular, recent work demonstrated that the activation of the caspase 8–caspase 3 pathway is involved in the dexamethasone-induced apoptosis of thymocytes.²³ Accordingly, we demonstrated, for the first time, that the

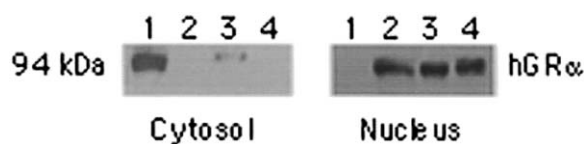


FIG 7. FP and salmeterol in combination increase nuclear translocation of the GR ($n = 5$). Western blot analysis of GR nuclear translocation by PBTs incubated with FP (10^{-8} and 10^{-9} M), salmeterol (10^{-7} M), and FP (10^{-9} M) and salmeterol (10^{-7} M) in combination is shown. Lane 1, Baseline; lane 2, 10^{-8} M FP; lane 3, 10^{-9} M FP; lane 4, 10^{-9} M FP plus 10^{-7} M salmeterol.

Finally, we evaluated the effect of FP and of the FP-salmeterol combination on the nuclear translocation of GR. Glucocorticoids act through interaction with a cytoplasmic GR that functions as a transcription factor in the nucleus. Dexamethasone-induced apoptosis is mRNA and protein synthesis dependent, requiring dexamethasone-GR interaction and the consequent GR nuclear translocation.²⁹ Consistent with this, we demonstrated that the PBT apoptosis induced by FP is associated with nuclear translocation of GR and that a further increase in translocation is observed when FP is combined with salmeterol. These findings suggest that the synergistic effects on PBT apoptosis and on the phosphorylation process of I κ B α might occur at the level of the nuclear translocation of the GR. In contrast to Eickelberg et al,¹¹ salmeterol alone did not induce nuclear translocation of the GR. Importantly, it must be noted, this apparent discrepancy might be due to the lower β_2 receptor expression in PBTs compared with that seen in fibroblasts and vascular smooth muscle cells.³⁰

In conclusion, this study shows that the combination of FP and salmeterol is able to control PBT activation in asthmatic patients more efficiently than FP alone and with a lower concentration of steroid by increasing apoptosis and by inhibiting the NF- κ B of PBT and that this synergy is related to an increased nuclear translocation of the GR.

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