Activation of PI3K-Akt pathway mediates antiapoptotic effects of β-adrenergic agonist in airway eosinophils

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1Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyushu University, Fukuoka; 2First Department of Internal Medicine, Kurume University School of Medicine, Fukuoka; and 3First Department of Internal Medicine, Mie University School of Medicine, Mie, Japan

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Machida, Kentaro, Hiromasa Inoue, Koichiro Matsumoto, Miyuki Tsuda, Satoru Fukuyama, Hiroshi Koto, Hisamichi Aizawa, Yasuko Kureishi, Nobuyuki Hara, and Yoichi Nakanishi. Activation of PI3K-Akt pathway mediates antiapoptotic effects of β-adrenergic agonist in airway eosinophils. Am J Physiol Lung Cell Mol Physiol 288:L680–L867, 2005. First published December 23, 2004; doi:10.1152/ajplung.00131.2004.—β-Adrenergic agonists reportedly decrease spontaneous apoptosis of peripheral blood eosinophils; however, its signaling pathway is unknown. Survival signals can be elicited by the activation of phosphatidylinositol 3-kinase (PI3K) and Akt, both of which are known to be potent regulators of apoptosis, and Akt in turn inactivates Forkhead transcription factors, including FKHR (Forkhead in rhabdomyosarcoma). We have investigated the effect of β-agonists on apoptosis of local eosinophils isolated from the airways and the involvement of PI3K, Akt, and FKHR in its survival signal. Eosinophils obtained from immunized mice by bronchoalveolar lavage after allergen provocation underwent apoptosis in a time-dependent manner. Incubation of eosinophils with isoproterenol or formoterol dose-dependently inhibited both spontaneous eosinophil apoptosis and apoptosis induced by Fas receptor activation. Incubation with cAMP or forskolin also inhibited eosinophil apoptosis. The PI3K inhibitors wortmannin and LY-294002 and an Akt inhibitor, 1-1,6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate, but not a mitogen-activated protein kinase inhibitor PD-98059, blocked isoproterenol-mediated eosinophil survival. Wortmannin also inhibited cAMP-mediated eosinophil survival. Isoproterenol rapidly induced phosphorylation of Akt and FKHR in eosinophils in a PI3K-dependent manner. These findings indicate that the PI3K-Akt-FKHR pathway conveys a critical survival signal induced by β-agonists in airway eosinophils.

granulocyte; protein kinase; lung; asthma

Eosinophilic inflammation in the airways is an important pathological feature of allergic asthma. The severity of asthma is correlated with the degree of airway eosinophilia (3). Eosinophils contribute to the pathogenesis of asthma by releasing inflammatory mediators and toxic products, including oxygen free radicals and major basic protein, which can damage the airway epithelium and increase airway hyperresponsiveness (46). Because blood and tissue eosinophilia are related to delayed apoptosis of these cells (48, 55), it is important to study antiapoptotic signals in eosinophils. Phosphatidylinositol 3-kinase (PI3K) and its downstream effector Akt (also called protein kinase B) regulate a diverse array of cellular events (5, 13), including cell survival of various types of cells, including neutrophils and monocytes (29, 31). The effect of Akt on cell-survival responses is mediated by the regulation of forkhead transcription factors [FKHR (forkhead in rhabdomyosarcoma), FKHR-L1 (FKHR-like 1), AFX (acute lymphocytic leukemia-1 fused gene from chromosome X)], now referred to as Foxo1, Foxo3, and Foxo4 (2, 6, 27). The role of PI3K in eosinophil survival is unclear. Although cytokines such as interleukin (IL)-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) inhibit apoptosis of human peripheral blood (24, 56) and murine lung (51) eosinophils, PI3K is reportedly not involved in GM-CSF-induced antiapoptotic signals in eosinophils (38). However, PI3K in eosinophils regulates the respiratory burst by opsonized particles, cytokine-mediated Fc receptor activation, and IL-5-induced mobilization from the bone marrow (4, 11, 40). Thus the PI3K pathway appears to play an important role in modulating eosinophil function.

The cAMP-dependent signaling transduction pathway also modulates apoptosis in a wide variety of cells. Specific activation of appropriate G-protein-coupled receptors, such as the β2-adrenergic receptor, followed by adenylate cyclase activation, leads to production of cAMP, leading in turn to activation of protein kinase A, which then phosphorylates several target proteins (15). The signaling mechanism used by cAMP to control apoptosis is expected to be complex and cell type specific. For example, in contrast to inducing apoptosis in thymocytes (37), CAMP delays apoptosis in neutrophils (35, 41, 43) and eosinophils (8, 22, 42, 57). Recent findings suggest that there is cross-talk between the cAMP-dependent signaling pathway and the PI3K pathway (7, 9, 30, 54). The molecular mechanisms underlying cAMP-mediated modulation of apoptosis in eosinophils remain to be elucidated.

β2-Adrenoceptor agonists are effective bronchodilators for relief of acute bronchospasm in asthma; however, their regular use is associated with increased airway hyperresponsiveness and allergen-induced airway response (10, 52). One possible mechanism underlying these adverse effects could be inhibition of apoptotic clearance of eosinophils, resulting in increased inflammatory responses (28). An increase in airway eosinophils was demonstrated after regular treatment with inhaled β2-agonist (1, 20, 34). β2-Agonists decrease spontaneous apoptosis of eosinophils isolated from human peripheral blood (28, 39). Different apoptotic responses have been re-
ported between peripheral blood eosinophils and lung eosino-
phils (45). Therefore, using eosinophils isolated from murine
airways, we directed this study at determining the effect of
β-agonists on apoptosis of eosinophils and the role of PI3K
and Akt in this signaling pathway. Few studies have examin-
ed the regulation of forkhead transcription factors in eosi-

nophils. We also studied the involvement FKHR, one of the possible
downstream targets of PI3K and Akt, in the survival signal
induced by β-agonists in eosinophils.

MATERIALS AND METHODS

Induction and purification of lung eosinophils. For induction
of lung eosinophilia, 6- to 8-wk-old male BALB/c mice were sensitized
by intraperitoneal injection of 10 μg of ovalbumin in 0.2 ml of alum
(Alu-Gel-S, Serva, Heidelberg, Germany) on day 0 and day 10. Mice
were then challenged with an aerosol of 5% ovalbumin for 20 min
daily between days 19 and 24, as reported previously (33). Forty-eight
hours after the final challenge, mice were anesthetized with pentobar-
bituric acid (100 mg/kg), and the tracheas were cannulated via
tracheostomy. Bronchoalveolar lavage was performed by three re-
petated washes with DMEM containing 5% fetal calf serum, mercap-
tetoethanol (50 μM), HEPES (10 mM), and penicillin-streptomycin
(100 U/ml and 0.1 mg/ml) at 25 cmH2O. The experimental protocol
was approved by the Committee on Animal Research (Faculty of
Medicine, Kyushu University, Fukuoka, Japan). The 48-h time point
was chosen because there is a peak in eosinophil infiltration (33) with
<1% neutrophils and <9% lymphocytes, thus allowing eosinophils to
be purified. The lavage fluid was collected and incubated for 30 min
to remove macrophages from the cell suspension by adherence to plastic.

Eosinophil cultures. Harvested cells (1 × 106 cells/well) were
incubated in 24-well plates either in culture medium alone or in the
presence of isoproterenol, formoterol, 8-bromoadenosine-cAMP (8-
Br-cAMP), forskolin, hamster anti-mouse Fas monoclonal antibody
(clone Jo-2), or GM-CSF. An inhibitor of adenylyl cyclase 2
5,5'-dideoxyadenosine (DDA) or pertussis toxin was added 30 min or 3 h,
respectively, before isoproterenol. To determine whether the activa-
tion of PI3K, Akt, or mitogen-activated protein kinase kinase
(MEK) is involved in the effect of isoproterenol on apoptosis, eosi-

nophils were pretreated with wortmannin, LY-294002, 1-L-6-hydroxy-
chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate, or
PD-98059 for 30 min and then exposed to isoproterenol.

Fig. 1. Effect of β-adrenoceptor agonists on spontan-
eous apoptosis of eosinophils in vitro. A: time
course of eosinophil viability. Eosinophils were
incubated for 48 h in culture medium alone and
analyzed by flow cytometry. Cellular staining, in-
strument settings, and gating procedures are de-
scribed in MATERIALS AND METHODS. Viable nonapop-
totic eosinophils were quantified as the percentage
of the total population of cells that were negative
for both annexin V-FITC and propidium iodide
(PI) (left). Early apoptotic cells were annexin V-
FITC positive (−) but PI negative (+). B: two-
color flow cytometry showing the kinetics of spon-
taneous eosinophil apoptosis. The numbers repre-
sent the percentage of cells in each quadrant.
Results are representative of 5 independent experi-
ments. C: mouse airway eosinophils obtained from
bronchoalveolar lavage (BAL) fluid were incubated
at 37°C in culture medium with varying concentra-
tions of isoproterenol (left) or formoterol (right).
After 24 h, the cells were harvested and incubated
with FITC-labeled annexin V and PI. Cells were
then assessed by flow cytometry on a FACS Calibur
and analyzed with the associated CellQuest soft-
ware. Data from 5,000 cells were analyzed for each
experiment. D: viable nonapoptotic eosinophils,
negative for both annexin V-FITC and PI, were
quantified as the percentage of the total population
of cells 24 h after incubation in medium alone
(Cont) or with isoproterenol (Iso, 10−8 M). Values
are means ± SE of 4 independent experiments, each
performed in duplicate. *P < 0.05 vs. control.
Assessment of apoptosis. Apoptosis was assessed by flow cytometry. Forward and side scatters were used to exclude cellular fragments and lymphocytes by flow cytometry, and the purity of eosinophils was confirmed to be more than 99% by cell sorting and staining cytospin preparations with Diff-Quik (Baxter, McGaw Park, IL). FACScan analysis was performed with an annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA) according to the manufacturer’s instructions. Briefly, after incubation, cells were harvested, washed, and resuspended in annexin V binding buffer. Cells were then stained with annexin V and propidium iodide (PI) at room temperature for 15 min in the dark. The samples were stored in the dark and analyzed by flow cytometry within 1 h. Annexin V-FITC and PI were analyzed by flow cytometry. Annexin V-FITC was analyzed with excitation and emission settings of 488 and 535 nm. PI was analyzed with excitation and emission settings of 488 and 755 nm. The exposure of phosphatidylserine to the outer leaflet of the plasma membrane has been shown to be a sensitive marker of early phases of apoptosis in eosinophils (53) and other cell types, and annexin V bind in a phosphatidylserine-specific manner. Viable cells with intact membranes exclude PI, whereas membranes of dead cells are permeable to PI, which binds to the cellular DNA. Cells that stained with PI were excluded from apoptosis. In mouse eosinophils, the annexin V-PI binding assay (annexin V+, PI−) for apoptosis had been confirmed by electron microscopic studies (16).

Western blotting. Phosphorylation of Akt or FKHR was assessed by Western blot analysis as described previously (32). Eosinophils were incubated under appropriate conditions and then were lysed in 200 µl of ice-cold lysis buffer [50 mM Tris·HCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% (vol/vol) Nonidet P-40, 0.1% SDS, 0.1% deoxycholic acid, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM Pefabloc, 10 mg/ml aprotinin, and 10 mg/ml leupeptin]. The unsolubilized material was removed by centrifugation for 10 min at 14,000 rpm. Samples were then boiled for 5 min with 2× SDS sample buffer. The boiled supernatants were electrophoresed on SDS-polyacrylamide gels and transferred to PVDF membrane. Membranes were blocked by 2-h incubation in Tris-buffered saline (10 mM Tris, pH 7.5, 100 mM NaCl) containing 0.1% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dry milk followed by 2-h incubation at room temperature with rabbit polyclonal anti-phospho-Akt-Ser 473, anti-Akt, anti-FKHR, anti-phospho-FKHR-Ser-256 antibodies (Cell Signaling Technology, Beverly, MA). The filters were washed extensively in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 before incubation for 1 h with anti-rabbit horseradish peroxidase-conjugated secondary antibodies. Membranes were then washed and developed with enhanced chemiluminescence substrate (Amersham, Little Chalfont, Buckinghamshire, UK).

Statistics. Results are expressed as means ± SE. Comparisons between groups were analyzed by ANOVA, and the significance of differences between values was assessed with Bonferroni correction. A P value of <0.05 was considered significant.

RESULTS

Spontaneous apoptosis of airway eosinophils. Double labeling of eosinophils with annexin V-FITC and PI enables differentiation between viable nonapoptotic cells (annexin V−, PI−), viable early apoptotic cells (annexin V+, PI−), and late apoptotic cells (annexin V+, PI+). PI uptake, annexin V-FITC staining, and trypan blue exclusion revealed that >98% of eosinophils were viable and nonapoptotic after purification. Annexin V-positive eosinophils were increased after incubation in medium alone, and viable nonapoptotic cells were decreased in a time-dependent manner (Fig. 1A, left). Approximately 38% of the cells were annexin V positive but PI negative after 24 h of culture (Fig. 1B, bottom right), and this fluorescence profile is compatible with early apoptosis. To confirm eosinophil apoptosis by morphological analysis, eosinophils were stained with Wright-Giemsa staining and examined under light microscopy. Classic apoptotic morphology was shown as nuclear and cytoplasmic condensation (data not shown). However, by 48 h, this profile changed to reflect the additional appearance of PI-positive, late-apoptotic cells in the cell population (Fig. 1B, top right). Annexin V-positive, PI-negative cells were visible at this stage, indicating additional cells were undergoing apoptosis. These findings indicate that eosinophils isolated from murine airways undergo spontaneous apoptosis. In the following experiments, the 24-h time point of
Incubation was used because it represents the peak of early apoptosis (Fig. 1A, right).

β-Adrenoceptor agonists inhibit spontaneous or anti-Fas-induced apoptosis of eosinophils. We then checked whether β-adrenoceptor agonists influence apoptosis of airway eosinophils. Isoproterenol inhibited spontaneous early apoptosis of eosinophils in vitro in a concentration-dependent manner after 24 h of incubation (Fig. 1C, left). The percentage of viable nonapoptotic cells (annexin V, PI) was increased after isoproterenol treatment (Fig. 1D). A long-acting β2-agonist, formoterol, inhibited early apoptosis of eosinophils dose dependently (Fig. 1C, right). At 48 h, 10 nM isoproterenol also inhibited early and late apoptosis (data not shown).

Cross-linking of the Fas receptor with anti-Fas antibody induces apoptosis of human and murine eosinophils in vitro (17, 36, 51). Therefore, we investigated the effect of β-adrenoceptor agonist on anti-Fas-induced eosinophil apoptosis. Incubation with hamster anti-mouse Fas monoclonal antibody (clone Jo-2, 500 ng/ml) increased eosinophil apoptosis significantly. Treatment with 10 nM isoproterenol inhibited anti-Fas-induced eosinophil apoptosis significantly (Fig. 2A). Incubation with the isotype-matched control antibody did not have any significant effect on eosinophil apoptosis. Treatment with 1 μM formoterol also inhibited anti-Fas-induced eosinophil apoptosis (data not shown).

Effect of cAMP elevating agents on eosinophil apoptosis. β-Adrenoceptor agonists increase intracellular cAMP by activating adenylate cyclase via G-protein-coupled receptor in eosinophils. To investigate whether an increase in cAMP affects eosinophil apoptosis, we studied the effects of forskolin, a direct adenylate cyclase activator, and 8-Br-cAMP, a membrane-permeable cAMP. Spontaneous apoptosis of eosin-phils were preincubated for 30 min with wortmannin (Wort; 10⁻⁸ M), LY-249002 (LY; 10⁻⁶ M), or PD-98059 (PD; 10⁻⁶ M) before stimulation with isoproterenol (10⁻⁸ M) or 8-bromoadenosine-cAMP (cAMP; 10⁻⁴ M). After additional 24-h incubation, apoptosis was analyzed by flow cytometry. B: eosinophil apoptosis was analyzed 24 h after incubation of GM-CSF (1 ng/ml) with or without 30-min preincubation with wortmannin. All values represent means ± SE of 2 independent experiments, each performed in triplicate. *P < 0.05.
oophils at 24 h was significantly inhibited by forskolin and 8-Br-cAMP (Fig. 2B). We also analyzed the effect of DDA, an inhibitor of adenylate cyclase, on the antiapoptotic action of isoproterenol. Preincubation of eosinophils with DDA blocked the antiapoptotic effects of isoproterenol (Fig. 2B). In some cell types, β2-adrenoceptors can also couple to Gi protein as well as to Gs protein (47). It has been reported that stimulation of β2-adrenoceptors inhibits apoptosis via a Gi-coupled pathway (12). Therefore, we assessed the effect of pertussis toxin on the antiapoptotic action of isoproterenol. Preincubation of eosinophils with pertussis toxin had no effect on isoproterenol-mediated eosinophil survival (data not shown).

**PI3K is involved in the antiapoptotic effect of isoproterenol.** PI3K (13) and MEK (26) have been shown to mediate cell survival in many cell types. To determine whether the PI3K pathway or the MEK pathway is involved eosinophil survival, we studied the effects of the PI3K inhibitors wortmannin and LY-294002 and the MEK inhibitor PD-98059 on the antiapoptotic effect of isoproterenol. Preincubation of eosinophils with either 10 nM wortmannin or 1 µM LY-294002 for 30 min blocked the antiapoptotic effect of isoproterenol (Fig. 3A), whereas preincubation of eosinophils with 50 nM PD-98059 had no effect on isoproterenol-mediated eosinophil survival. In the absence of isoproterenol, the inhibitors had no effect on eosinophil survival. Pretreatment with wortmannin also blocked the antiapoptotic effect of 8-Br-cAMP.

To explore whether isoproterenol exerts its antiapoptotic action through activation of Akt, we tested the effect of 5 mM 1,2-diacyl-sn-glycerol-3-phosphate (DAG) and the PI3K inhibitor LY-294002 for 30 min and then treated with isoproterenol (10−8 M) for 10 or 30 min. A: levels of Akt phosphorylated at Ser473 (phospho-Akt) and total Akt protein levels were measured by Western blotting with anti-phospho-Akt (Ser473) antibody and anti-Akt antibody, respectively. B: eosinophils were lysed and analyzed by Western blotting with anti-phospho-FKHR (Ser256) antibody (top) and anti-FKHR antibody (bottom). Anti-phospho-FKHR (Ser256) antibody cross-reacts with phosphorylated AFX.

**DISCUSSION**

In this study, we demonstrated that incubation of airway eosinophils with isoproterenol or the long-acting β2-agonist formoterol in vitro inhibits both spontaneous apoptosis of eosinophils and apoptosis induced by Fas receptor activation. Incubation with cAMP or the direct adenylate cyclase activator forskolin inhibited eosinophil apoptosis. We also showed that the PI3K inhibitors and an Akt inhibitor completely blocked isoproterenol- and cAMP-mediated eosinophil survival. This was not observed for MEK inhibitor PD-98059. Isoproterenol rapidly induced phosphorylation of Akt, a downstream effector of PI3K, in a PI3K-dependent manner. Isoproterenol also PI3K dependently induced phosphorylation-inactivation of FKHR. These findings indicate that the inhibition of airway eosinophil apoptosis by β-agonists is mediated by the PI3K-Akt-FKHR pathway.

Although the PI3K-Akt pathway is an important regulator of cell survival in various cell types, little is known regarding its role in eosinophil apoptosis. GM-CSF and IL-5 inhibit apop-
tosis (21, 24, 51, 56) and activate PI3K in eosinophils (11), but PI3K is reported not to be involved in antiapoptotic signaling of GM-CSF in human peripheral blood eosinophils (38). We confirmed that PI3K pathway was not involved in the antiapoptotic effect of GM-CSF on murine airway eosinophils. The present study shows that isoproterenol rapidly induces Akt phosphorylation and that PI3K inhibitors block the survival effect of isoproterenol on eosinophils, indicating that activation of the PI3K-Akt pathway plays a central role in the survival programming properties of β-adrenoceptor agonists in lung eosinophils. The present findings and those of a previous study showing PI3K-independent signaling after GM-CSF stimulation suggest that regulation of eosinophil survival by the PI3K-Akt pathway is likely dependent on the survival stimulus.

Most of the actions of the β2-adrenergic receptor are mediated through Gs proteins and cAMP (23, 50). In addition to coupling to Gs protein, β2-adrenoceptors can also couple to Gi protein (14). Although it has been reported that stimulation of β2-adrenoceptors inhibits apoptosis of cardiac myocyte via a Gi-coupled pathway (12), pertussis toxin had no effect on isoproterenol-mediated eosinophil survival. cAMP and the adenylyl cyclase activator forskolin inhibited eosinophil apoptosis; in contrast, an inhibitor of adenylate cyclase DDA blocked the antiapoptotic effect of isoproterenol. These data suggest that β-agonists increase intracellular cAMP by activating adenylyl cyclase via a Gs-protein-coupled receptor in eosinophils. The role of cAMP in regulating apoptosis is thought to be cell type specific. It delays apoptosis in neutrophils (35, 41, 43) but induces pro-apoptotic signals in thymocytes (37). We have confirmed earlier reports of an antiapoptotic effect of cAMP on eosinophils (8, 22, 42, 57). It has recently been reported that there is cross-talk between the cAMP-dependent signaling pathway and the PI3K pathway, but the effects of cAMP on PI3K-Akt activity are quite varied. cAMP activates Akt through a PI3K-dependent mechanism in thyroid cells (7, 9), whereas inhibition of the PI3K-Akt pathway by cAMP has been reported in fibroblasts (30, 54). Furthermore, it has been reported that there are survival stimuli activating Akt via PI3K-independent mechanisms (19, 44). The present study suggests that PI3K-dependent Akt activation is an important facet of β-agonist/cAMP-regulated antiapoptotic signals in eosinophils.

Several substrates of Akt have been identified, including components of the cell death machinery, caspase-9 and a Bcl-2 family member Bad (5). Phosphorylation of Bad and caspase-9 by Akt suppresses their pro-apoptotic functions, accounting at least in part for the potent survival function of Akt. However, we were unable to detect phosphorylation of Bad in eosinophils (data not shown). Recent studies have shown that the effect of Akt on cell-survival responses is mediated by the regulation of the Foxo subfamily of forkhead transcription factors (2, 6). We demonstrated that isoproterenol rapidly induced phosphorylation/inactivation of FKHR in a PI3K-dependent manner. These downstream targets involved in Akt-mediated eosinophil survival require further investigation. Clarifying the point in the signaling cascade at which PI3K-Akt inhibits eosinophil apoptosis would permit development of new specific inhibitors, and this could have important therapeutic implications for asthma.

Regarding eosinophils in the airways of patients with asthma, local expansion of eosinophils from CD34+/IL-5R+ precursor cells is thought to contribute to tissue eosinophilia in addition to recruitment of mature eosinophils that differentiates in the bone marrow (18, 49). Furthermore, different apoptotic responses have been reported between peripheral blood eosinophils and lung eosinophils (45). In the present study, airway eosinophils were obtained by bronchoalveolar lavage after aerosol allergen provocation of immunized mice, and the survival of eosinophils was assessed. Comparison of the role of PI3K-Akt pathway in survival between local eosinophils and peripheral blood eosinophils will be important, and further studies are needed to evaluate whether the present findings represent the in vivo situation.

In summary, we have shown that β-agonists and cAMP inhibit spontaneous apoptosis of airway eosinophils. PI3K inhibitors and an Akt inhibitor blocked isoproterenol-mediated eosinophil survival, and isoproterenol rapidly induced phosphorylation of Akt and phosphorylation/inactivation of FKHR in eosinophils in a PI3K-dependent manner. These findings indicate that the PI3K-Akt-FKHR pathway provides a critical survival signal induced by β-agonists in airway eosinophils. Identifying the pivotal event in signaling for eosinophil survival might provide novel therapeutic strategies aimed at controlling eosinophilic inflammation.

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