Antieosinophilic Activity of Simendans

Hannu Kankaanranta, Xianzhi Zhang, Ritva Tumelius, Minna Ruotsalainen, Heimo Haikala, Erkki Nissinen, and Eeva Moilanen

The Immunopharmacology Research Group, Medical School, University of Tampere (H.K., X.Z., E.M.) and Department of Respiratory Medicine (H.K.) and Research Unit (E.M.), Tampere University Hospital, Tampere, Finland; Seinajoki Central Hospital, Seinajoki, Finland (H.K.); Orion Corporation, Espoo, Finland (R.T., M.R., H.H., E.N.); and Center for Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China (X.Z.)

Received April 5, 2007; accepted July 6, 2007

ABSTRACT

Simendans are novel agents used in the treatment of decompensated heart failure. They sensitize troponin C to calcium and open ATP-sensitive potassium channels and have been shown to reduce cardiac myocyte apoptosis. The aim of the present study was to evaluate whether simendans reduce pulmonary eosinophilia and regulate eosinophil apoptosis. Bronchoalveolar lavage (BAL) eosinophilia was evaluated in ovalbumin-sensitized mice. Effects of simendans on apoptosis in isolated human eosinophils were assessed by relative DNA fragmentation assay, annexin V-binding, and morphological analysis. Dextrosimendan [(+)-][4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono[propanedinitrile] reduced ovalbumin-induced BAL-eosinophilia in sensitized mice. Levosimendan [(−)-][4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl]phenyl]hydrazono[propanedinitrile] and dextrosimendan reversed interleukin (IL)-5-afforded survival of human eosinophils by inducing apoptosis in vitro. Even high concentrations of IL-5 were not able to overcome the effect of dextrosimendan. Dextrosimendan further enhanced spontaneous apoptosis as well as that induced by CD95 ligation, without inducing primary necrosis. Dextrosimendan-induced DNA fragmentation was shown to be dependent on caspase and c-Jun NH2-terminal kinase activation, whereas extracellular signal-regulated kinase, p38 mitogen-activated kinase, and ATP-sensitive potassium channels seemed to play no role in its actions. Taken together, our results show that simendans possess antieosinophilic activity and may be useful for the treatment of eosinophilic inflammation.

Eosinophils are thought to play a critical role in allergic diseases, such as allergic rhinitis, atopic dermatitis, and asthma (Gleich, 2000). In asthmatic patients, activation of eosinophils in the airways is thought to cause epithelial tissue injury, contraction of airway smooth muscle, and increased bronchial responsiveness. Apoptosis or programmed cell death may be an important feature in the resolution of asthmatic inflammation (Kankaanranta et al., 2005). Apoptosis is characterized by specific biochemical and morphological changes, including cell shrinkage, surface blebbing, DNA fragmentation, and loss of nucleoli (Kankaanranta et al., 2005), so that the apoptotic cell is phagocytosed intact without release of its contents. Eosinophil apoptosis is inhibited by cytokines IL-3, IL-5, and granulocyte macrophage-colony-stimulating factor (Kankaanranta et al., 2005), whereas eosinophil apoptosis is up-regulated by Fas (CD95/APO-1), a 45-kDa transmembrane protein belonging to the tumor necrosis factor receptor family (Kankaanranta et al., 2005). We and others have previously shown that eosinophil apoptosis is delayed in patients with asthma or inhalant allergy (Wedi et al., 1997; Kankaanranta et al., 2000). Furthermore, the number of eosinophils in asthmatic lung is elevated and is inversely correlated with the number of apoptotic eosinophils (Vignola et al., 1999).

Levosimendan is a novel agent with a dual mechanism of action developed and marketed for the treatment of decompensated heart failure. This agent sensitizes troponin C to DNA fragmentation, and loss of nucleoli (Kankaanranta et al., 2005), so that the apoptotic cell is phagocytosed intact without release of its contents. Eosinophil apoptosis is inhibited by cytokines IL-3, IL-5, and granulocyte macrophage-colony-stimulating factor (Kankaanranta et al., 2005), whereas eosinophil apoptosis is up-regulated by Fas (CD95/APO-1), a 45-kDa transmembrane protein belonging to the tumor necrosis factor receptor family (Kankaanranta et al., 2005). We and others have previously shown that eosinophil apoptosis is delayed in patients with asthma or inhalant allergy (Wedi et al., 1997; Kankaanranta et al., 2000). Furthermore, the number of eosinophils in asthmatic lung is elevated and is inversely correlated with the number of apoptotic eosinophils (Vignola et al., 1999).

Levosimendan is a novel agent with a dual mechanism of action developed and marketed for the treatment of decompensated heart failure. This agent sensitizes troponin C to

ABBREVIATIONS: IL, interleukin; levsimendan, (−)[4-(4,1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono[propanedinitrile]; dextrosimendan, (+)[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono[propanedinitrile]; BAL, bronchoalveolar lavage; JNK, c-Jun NH2-terminal kinase; DMSO, dimethyl sulfoxide; L-JNKI1, GRKKRRQRRR-PP-RPKRPTTLNLFVQRSD-amide; L-TAT, RKKRRQRRR-amide, negative control for L-JNKI1; fmk, fluoromethyl ketone; Z-Asp-CH2-DCC, benzoylloxycarbonyl-Asp-CH2CC-dichlorobenzene; PDB088059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole; PI, propidium iodide; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; KATP, ATP-sensitive potassium channel.
calcium in a manner dependent on calcium concentration, thereby increasing the effects of calcium on cardiac myofilaments during systole and improving contraction at low energy cost (Pollesello et al., 1994; Haikala et al., 1995). Levosimendan causes also vasodilatation through the opening of ATP-sensitive potassium channels (Yokoshiki et al., 1997). In addition to its beneficial effects in decompen sed heart failure (Follath et al., 2002), levosimendan has been shown to have beneficial effects in left ventricular failure complicating acute myocardial infarction by lowering the risk of death (Moiseyev et al., 2002). This suggests that levosimendan may affect the inflammatory events associated with myocardial infarction in addition to its beneficial effects on hemodynamics. In fact, supporting the possible anti-inflammatory effects, levosimendan has been shown to have beneficial effects in carrageenan-induced paw edema in rats (Haikala et al., 2004) and in experimentally induced septic shock in pigs (Oldner et al., 2001) to affect the inflammatory changes associated with decompensated advanced heart failure in humans (Parissis et al., 2004) and to reduce cardiac myocyte apoptosis in rats (Louhelainen et al., 2007).

Given the possible role of Ca2+ and K+ in eosinophil apoptosis (Beauvais et al., 1995, 1998; Bankers-Fulbright et al., 1998), our aim was to test the effects of simendans (Fig. 1) on pulmonary eosinophilia and on human eosinophil apoptosis. The present study describes the ability of dextrosimendan to reduce pulmonary eosinophilia in ovalbumin-sensitized mice and that simendans are able to induce apoptosis in human eosinophils and to reverse IL-5-afforded eosinophil survival as well as evaluates their possible mechanisms of action. We describe dextrosimendan as a novel anti-inflammatory drug candidate with anti eosinophilic properties.

Materials and Methods

Animals. BALB/c mice 6 to 8 weeks old (20–25 g) were purchased from M&B A/S (Ry, Denmark). The mice were housed in the animal facility of OrionPharma Ltd. (Espoo, Finland) in a thermostatically controlled room at 22 ± 2°C at a relative humidity of 40 to 70% with artificial illumination from 6:00 AM to 8:00 PM. Mice were fed commercial pellets (SDS rat and mouse no. 1 maintenance; Special Diets Services, Witham, UK) and given water ad libitum under specific pathogen-free conditions according to standard guidelines. All animal experiments were conducted with the permission of the Animal Ethics Committee of OrionPharma, in accordance with Finnish law and government regulations complying with the European Community guidelines for the use of experimental animals.

Allergen Sensitization and Exposure. The mice were sensitized by initial i.p. injection of ovalbumin [20 μg in 2 mg Al(OH)3/0.2 ml 0.9% NaCl] on day 0. After 1 week, these mice were further sensitized with the same reagent. On days 21, 22, and 23, the mice were exposed to aerosols of allergen (1% ovalbumin in 0.9% NaCl) for 20 min on each day. The aerosol challenge was performed in a sealed plastic chamber by using a jet nebulizer (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) at the pressure 1.5 bar.

Animal Treatments and Bronchoalveolar Lavage Analysis. Sensitized and challenged animals were treated with vehicle (2.5 ml/kg of 0.1% Tween 20 in 0.9% NaCl), dextrosimendan (0.1 and 1.0 mg/kg), or budesonide (1.0 mg/kg) intranasally 1 h before and 6 h after challenge at days 21 to 23. Twenty-four hours after the last challenge, animals were euthanized with an overdose of pentobarbital sodium (180 mg/kg i.p.). Bronchoalveolar lavage (BAL) was collected by washing lungs twice with 0.5 ml of phosphate-buffered saline (+37°C). Each wash included gentle flush for three times. Volume of BAL fluid was measured and filled into 1 ml, and the number of white blood cells was determined by a hemocytometer Sysmex Microcell Counter (TOA Medical Electronics Co. Ltd., Kobe, Japan). Differential cell counts were calculated from May-Grünewald-Giemsa-stained cytopsin slides (white blood cell number, 2 × 107/ml).

Human Eosinophil Purification. Blood (50–100 ml) was obtained from eosinophilic individuals. Eosinophils were isolated to >99% purity under sterile conditions as reported previously (Kankaanranta et al., 1999, 2000; Zhang et al., 2000). The cells were resuspended at 106 cells/ml and cultured in RPMI 1640 medium, 10% fetal calf serum, and antibiotics. Subjects gave informed consent to a study protocol approved by the ethics committee of Tampere University Hospital (Tampere, Finland).

Determination of Eosinophil Apoptosis. Unless otherwise stated, eosinophil apoptosis was determined by propidium iodide staining of DNA fragmentation and flow cytometry as described previously (Kankaanranta et al., 1999, 2000; Zhang et al., 2000). The cells showing decreased relative DNA content were considered to be apoptotic. Annexin V binding and morphological analysis was performed as previously reported (Zhang et al., 2002). Oligonucleosomal DNA fragmentation in eosinophils was analyzed by agarose gel DNA electrophoresis as described previously (Kankaanranta et al., 1999, 2000; Zhang et al., 2003). Eosinophil apoptosis is expressed as apoptotic index (number of apoptotic cells/total number of cells).

Immunoblot Analysis. Eosinophils were suspended at 106 cells/ml and cultured at 37°C. At the indicated time points, cells were centrifuged at 12,000g for 10 min. The cell pellet was lysed by boiling for 5 min in 30 μl of Laemmli buffer (×6), centrifuged at 12,000g, and debris was carefully removed. Samples were then stored at −20°C until analyzed. For immunoblot analysis, each protein sample was loaded on 8 to 12% SDS-polyacrylamide gel electrophoresis gel and electrophoresed for 2 h at 100 V. The separated proteins were transferred to nitrocellulose membrane (Hybond ECL) with semidry blotting pressure 1.5 bar.

Fig. 1. The chemical structures of levo- and dextrosimendans.
JNK. Control time curve with the solvent (0.5% DMSO) was prepared to see the change in JNK activation in similar conditions in the absence of dextrosimendan. The increase in activation of JNK by dextrosimendan is expressed as the phospho-JNK activity in dextrosimendan-treated cells as compared with the simultaneously prepared solvent control cells.

**Materials.** Levosimendan and dextrosimendan were obtained from OrionPharma Ltd. The synthesis of racemic simendans and isolation of levo- and dextrosimendan has been described earlier (Haikala et al., 1990; Nore et al., 1992). Reagents were obtained as follows: l-JNKI1 and l-TAT control peptide (Alexis Corp., Läufelfingen, Switzerland); Z-Asp-CH$_2$-DCB (Peptide Institute, Inc., Osaka, Japan); phosphospecific JNK monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA); horseradish peroxide-linked anti-rabbit IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK); and budesonide, diazoxide, glibenclamide, 5-hydroxydecanoate, and ovalbumin (Sigma Chemical Co, St. Louis, MO). Unless otherwise stated, the reagents were obtained as described previously (Kankaanranta et al., 1999, 2000, 2002, 2006; Zhang et al., 2000, 2002, 2003). The incubation time was 40 h unless otherwise stated. l-JNKI, l-TAT, PD98059, SB203580, and Z-Asp-CH$_2$-DCB were added 20 min before dextrosimendan. Stock solutions of levo-/dextrosimendan, PD98059, SB203580, and Z-Asp-CH$_2$-DCB were prepared in DMSO. Budesonide was dissolved in ethanol. The final concentration of DMSO in the culture was 0.5% and that of ethanol 0.2%. A similar concentration of the solvent was added to the control cultures.

**Statistics.** Results are expressed as means ± S.E.M. Statistical significance was calculated by Welch t test or analysis of variance for repeated measures supported by Dunnett or Student-Newman-Keuls test. Differences were considered significant if \( P < 0.05 \).

**Results**

**Effect of Dextrosimendan on Ovalbumin-Induced Lung Eosinophilia in Sensitized Mice.** In nonsensitized animals, the total number of eosinophils in BAL fluid after challenge was very low (0.001 ± 0.001 × 10$^5$ eosinophils accounting to 0.06 ± 0.04% of all cells in BAL; \( n = 17 \)). Sensitization with ovalbumin dramatically increased the number of eosinophils in BAL fluid after challenge (2.87 ± 0.65 × 10$^5$ eosinophils) accounting to 44 ± 3% of all cells in BAL. Treatment of animals with dextrosimendan (0.1 or 1 mg/kg) reduced the number of eosinophils in BAL as compared with vehicle-treated animals in a dose-dependent manner (Fig. 2). For comparison, treatment of animals with budesonide (1 mg/kg) almost completely prevented eosinophilia in BAL (2 ± 0.5% eosinophils in BAL; \( n = 20 \); \( P < 0.001 \) as compared with the sensitized and challenged control animals).

**Effects of Levo- and Dextrosimendan on IL-5-Afforded Eosinophil Survival.** During culture for 40 h, IL-5 induced human eosinophil apoptosis in a concentration-dependent manner. Maximal inhibition of apoptosis was obtained at 10 pM concentration of IL-5 (apoptotic indexes, 0.57 ± 0.09 and 0.07 ± 0.02 in the absence and presence of IL-5, respectively, \( n = 5 \), \( P < 0.001 \)). Levosimendan increased the number of apoptotic eosinophils in the presence of 10 pM IL-5 (i.e., reversed the effect of IL-5), with an EC$_{50}$ value of 6.5 ± 0.7 \( \mu \)M (Fig. 3A). This increase in the number of apoptotic cells was confirmed by showing increased phosphatidylserine expression on the outer leaflet of cell membrane of IL-5-treated cells, i.e., the percentage of annexin V-positive cells in the absence and presence of levosimendan (10 \( \mu \)M) was 7 ± 1 and 86 ± 4%, respectively. Dextrosimendan reversed IL-5-afforded human eosinophil survival in a concentration-dependent manner by inducing apoptosis (EC$_{50}$, 2.9 ± 0.4 \( \mu \)M; Fig. 3B). The ability of dextrosimendan to induce apoptosis in IL-5-treated human eosinophils was confirmed by showing the increase in the percentage of annexin V-positive eosinophils (5 ± 1 and 95 ± 1% in the absence and presence of 40 \( \mu \)M dextrosimendan, respectively, \( n = 6 \), \( P < 0.001 \)). Furthermore, an increase in the number of eosinophils showing the typical morphological features of apoptosis such as nuclear coalescence, chromatin condensation, and cell shrinkage was found with dextrosimendan (apoptosis index, 0.02 ± 0.01 and 0.98 ± 0.01 in the absence and presence of 40 \( \mu \)M dextrosimendan, respectively; Fig. 3, C and D). To further confirm the ability of dextrosimendan to induce eosinophil apoptosis, DNA breakdown, the typical hallmark of apoptosis was analyzed. IL-5 inhibited DNA breakdown, dextrosimendan (40 \( \mu \)M) reversed the IL-5-afforded inhibition of DNA breakdown, and a typical “ladder” pattern was found, indicating the occurrence of apoptotic cell death (Fig. 3E). Because dextrosimendan was found to be even more potent in inducing eosinophil apoptosis than levosimendan, dextrosimendan was used in further studies to evaluate the mechanisms of simendan-induced apoptosis in eosinophils.

Glucocorticoids are known to partially reverse the survival-prolonging action of IL-5 on eosinophils. However, this effect of glucocorticoids is abolished when IL-5 is used at higher concentrations (Zhang et al., 2000, 2002; Druilhe et al., 2003; Kankaanranta et al., 2005). Budesonide (1 \( \mu \)M) partly reversed cytokine-afforded survival in the presence of low (0.1–1 pM) but not in the presence of higher (10–100 pM) concentrations of IL-5 (Fig. 4A). To evaluate whether the effect of dextrosimendan is similar to glucocorticoids, its effects were studied in the presence of different concentrations of IL-5. Interestingly, increasing concentrations of IL-5 only partially reversed the effect of low concentration (3 \( \mu \)M) of dextrosimendan. In contrast, the effect of a higher concentration (10 \( \mu \)M) of dextrosimendan was not reversed even by high concentrations of IL-5 (Fig. 4B).
Effect of Dextrosimendan on Fas-Induced Eosinophil Apoptosis. There are only few compounds that are able to reverse the effect of IL-5 on eosinophil survival (Kankaanranta et al., 2005). One of those is nitric oxide, which has been shown to reverse the effect of IL-5 by inducing apoptosis (Zhang et al., 2003). However, nitric oxide can also reverse the apoptosis-inducing effect of Fas in eosinophils (Hebestreit et al., 1998). This prompted us to evaluate whether dextrosimendan has detrimental effects on Fas-induced apoptosis. Dextrosimendan (40 μM) further enhanced the apoptosis-inducing effect of Fas-ligation in human eosinophils as assessed by using the relative DNA fragmentation assay (apoptotic index, 0.71 ± 0.07 and 0.96 ± 0.01 in the absence and presence of dextrosimendan, n = 6, P < 0.05) and annexin V-binding analysis (59 ± 4 and 96 ± 1% annexin V-positive cells in the absence and presence of dextrosimendan, n = 6, P < 0.001).

Effect of Dextrosimendan on Spontaneous Eosinophil Apoptosis. Dextrosimendan enhanced apoptosis of cytokine-deprived eosinophils in a concentration-dependent manner with an EC50 value of 2.3 ± 0.5 μM (Fig. 5). This was confirmed by morphological analysis of eosinophils cultured for 22 h in the absence and presence of dextrosimendan (40 μM) (apoptotic indexes, 0.21 ± 0.05 and 0.99 ± 0.01, respectively, n = 6, P < 0.001). The increased exposure of phosphatidylserine on the outer-leaflet of the cell-membrane was further confirmed by using annexin V binding assay, where the corresponding percentages of annexin V-positive cells were 37 ± 5 and 96 ± 1% in the absence and presence of 40 μM dextrosimendan (n = 6, P < 0.001).

Effect of Dextrosimendan on Primary Eosinophil Necrosis. An important feature for a drug possessing anti-eosinophilic activity is that it should not induce primary necrosis that could lead to the release of eosinophil contents to the surrounding tissue. To evaluate this possibility, the effects of dextrosimendan on primary eosinophil necrosis were evaluated by using counterstaining with annexin V and propidium iodide, where positive staining with propidium iodide indicates a rupture of the plasma membrane, and the absence of staining with annexin V indicates that the cell has not undergone apoptosis. Thus, cells showing positive staining with propidium iodide (PI) but not with annexin V can be considered to show the typical feature of primary necrosis, i.e., the plasma membrane breakdown. In the absence of IL-5, the percentages of PI+/annexin V− cells were 4 ± 1 and 1 ± 1% in the absence and presence of 40 μM dextrosimendan, respectively (n = 6, P > 0.05). In the presence of IL-5 (10 pM), the corresponding percentages were 3 ± 1 and 2 ± 1% (n = 6, P > 0.05). These results show that dextrosimendan does not induce primary necrosis in eosinophils.

Effect of Caspase Inhibition on Dextrosimendan-Induced Apoptosis. A pan-caspase inhibitor, Z-Asp-CH2-DCB (20–200 μM), significantly reversed dextrosimendan (10 μM)-induced apoptosis in IL-5-treated eosinophils during 40-h incubation (Table 1).

Role of Mitogen-Activated Protein Kinases in Dextrosimendan-Induced Apoptosis in Eosinophils. When IL-5-treated eosinophils were incubated at 37°C in the presence of dextrosimendan (10 μM), a time-dependent increase in the activity of JNK was detected using Western blotting with an anti-pJNK antibody that recognizes the dual phosphorylated (i.e., activated) JNK (Fig. 6, A and B). To evaluate the functional role of JNK activation in dextrosimendan-induced apoptosis in IL-5-treated cells, a novel cell-permeable inhibitor peptide specific for JNK, 1-JNKI1 (Bonny et al., 2001), was employed. 1-JNKI1 (10 μM), but not the negative control peptide L-TAT, almost completely reversed dextrosimendan (10 μM)-induced apoptosis in IL-5-treated eosinophils (Figs. 6C and 7, A and B). To see whether JNK activation is central to the dextrosimendan-induced apoptosis, the effect of 1-JNKI1 (10 μM) on dextrosimendan-induced apoptosis was analyzed by using the morphological analysis...
and measurement of phosphatidylserine appearance on the outer cell membrane using annexin V binding assay. Interestingly, l-JNKI1 did not reduce the number of cells showing the typical early signs of apoptosis such as apoptotic morphology or phosphatidylserine expression on the outer cell membrane. By using morphological criteria for apoptosis, in dextrosimendan (10 μM)- and IL-5 (10 pM)-treated cells, the apoptotic indexes were 0.76 ± 0.03 and 0.70 ± 0.03 in the presence of 10 μM l-TAT and 10 μM l-JNKI1, respectively, after culture for 20 h (Fig. 7, E and F). Likewise, the percentage of annexin V-positive cells were not reduced by l-JNKI1
as compared with cells treated with L-TAT (76 ± 3 and 71 ± 4% in the presence of 10 μM L-TAT and 10 μM L-JNK11, respectively; Fig. 7, C and D).

ERK and p38 MAPK have been proposed to be involved in the regulation of eosinophil apoptosis (Kankaanranta et al., 1999; Hall et al., 2001). Thus, to evaluate the role of these kinases in dextrosimendan-induced eosinophil apoptosis, we used a pharmacological approach to inhibit the activity of ERKs by using a mitogen-activated protein kinase inhibitor PD098059 and p38 MAPK by SB203580. However, neither PD098059 (1–10 μM) nor SB203580 (1–10 μM) affected dextrosimendan (10 μM)-induced apoptosis in IL-5-treated human eosinophils (Table 2).

Effect of KATP Channel-Modulating Compounds on Eosinophil Apoptosis. A report (Yokoshiki et al., 1997) suggested that levosimendan is a KATP channel opener. To evaluate whether modulation of the KATP channel-opening state could affect eosinophil apoptosis, the effects of chemically unrelated KATP channel-modulating compounds were studied. Diazoxide (5–100 μM; a KATP channel opener) only slightly increased the number of apoptotic eosinophils in the presence of 10 pM IL-5 (apoptotic indexes, 0.09 ± 0.01 and 0.17 ± 0.02 in the absence and presence of 100 μM diazoxide, respectively, n = 5, P < 0.01). Likewise, KATP channel inhibitors glibenclamide (0.03–10 μM) and 5-hydroxydecanoic acid (5–50 μM) were not able to modify IL-5-afforded eosinophil survival to a significant level. The apoptotic indexes in IL-5 (10 pM)-treated cells were 0.08 ± 0.01 and 0.07 ± 0.01 in the absence and presence of 10 μM glibenclamide, respectively (n = 5, P > 0.05), and 0.07 ± 0.01 and 0.09 ± 0.01 in the absence and presence of 50 μM 5-hydroxydecanoic acid (n = 5, P < 0.01).

### TABLE 2

The effect of MAP kinase inhibitors PD098059 and SB203580 on dextrosimendan (10 μM)-induced apoptosis in human eosinophils

<table>
<thead>
<tr>
<th>Apoptotic Index</th>
<th>Mean ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD098059</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.94 ± 0.01</td>
</tr>
<tr>
<td>1 μM</td>
<td>0.95 ± 0.01</td>
</tr>
<tr>
<td>10 μM</td>
<td>0.96 ± 0.01</td>
</tr>
<tr>
<td>SB203580</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.91 ± 0.03</td>
</tr>
<tr>
<td>1 μM</td>
<td>0.94 ± 0.01</td>
</tr>
<tr>
<td>200 μM</td>
<td>0.95 ± 0.02</td>
</tr>
</tbody>
</table>

Discussion

In the present study, we have shown that the Ca^{2+}-sensitizing and KATP channel-opening simendans reduce pulmonary eosinophilia in mice and induce apoptosis in IL-5-treated human eosinophils. Furthermore, simendans are able to enhance spontaneous eosinophil apoptosis without inducing primary necrotic cell death. Their mechanism of action seems to involve caspase activation as well as JNK-mediated DNA breakdown.

In the treatment of asthma, glucocorticoids reduce the number of eosinophils and suppress the eosinophilic inflammation. This was evidenced by the reduction of the number of eosinophils in the BAL fluid of sensitized and challenged mice by budesonide. In addition to their anti-inflammatory effects, induction of eosinophil apoptosis is currently considered as one of the mechanisms of how glucocorticoids reduce eosinophilic inflammation in asthma (Druilhe et al., 2003; Walker et al., 2003; Walsh et al., 2003). Clinically, the net effect of glucocorticoids is a combination of direct anteosinophilic effects and suppression of production of those cytokines that drive the eosinophilic inflammation. Glucocorticoids are able to enhance spontaneous eosinophil apoptosis at clinically relevant drug concentrations (Zhang et al., 2000, 2002). In addition, glucocorticoids partly reverse IL-5-afforded survival, but the effect of steroids falls off as the concentration of IL-5 increases (Zhang et al., 2000, 2002; Druilhe et al., 2003; Kankaanranta et al., 2005). Likewise, in the present study, the apoptosis-inducing effect of budesonide was abolished by high concentrations of IL-5. In contrast to glucocorticoids, the effect of dextrosimendan was not reduced by higher concen-
trations of IL-5, thus suggesting that the mechanism of action of dextrosimendan is different from that of glucocorticoids. This suggests that the effect of simendans might be complementary to that of glucocorticoids. However, there exists the possibility that, in addition to the induction of eosinophil apoptosis, simendans might also have other anti-inflammatory effects such as inhibition of synthesis or release of cytokines or chemokines.

The basic mechanism of action of simendans in the treatment of cardiac failure is that they bind to the cardiac troponin C to increase its sensitivity to calcium without an increase in the free intracellular calcium concentration (Haikala et al., 1995). Although activation of several, mainly G-protein-coupled, receptors is known to lead to an increase in the intracellular calcium in eosinophils, to our knowledge, there is no published literature on the existence of any of the calcium-binding proteins or troponins in eosinophils. Furthermore, to our knowledge, the only report evaluating the role of free intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) in the regulation of eosinophil apoptosis is that by Murray et al. (2003) showing that a temporary increase in the [Ca\(^{2+}\)]\(_i\), by leukotrienes B\(_4\) or D\(_4\) is not a sufficient signal to affect apoptosis in eosinophils. Simendans are known not to increase the level of [Ca\(^{2+}\)]\(_i\), but to sensitize the effects of cardiac troponin C to calcium. Levosimendan is approximately 10 times more potent in binding to cardiac troponin C than dextrosimendan (Sorsa et al., 2004) but is 2-fold less potent in inducing apoptosis in IL-5-treated eosinophils. This suggests that the apoptosis-inducing mechanism of action of simendans does not depend on binding to cardiac troponin C. Whether there exist different types of calcium-binding proteins in eosinophils that are more sensitive to dextrosimendans does not depend on binding to cardiac type troponin C. However, the presence of caspases 3, 6, 7, 8, and 9 during apoptosis in eosinophils has recently gained attention (Kankaanranta et al., 1999; Hall et al., 2001; Gardai et al., 2003; Zhang et al., 2003). There exists some controversy whether extracellular regulated kinase pathway is involved in the survival-prolonging action of cytokines or not (Kankaanranta et al., 1999, 2005; Hall et al., 2001), whereas p38 MAPK seems to be involved in spontaneous eosinophil survival (Kankaanranta et al., 1999). By using pharmacological inhibitors for mitogen-activated protein kinase kinase and p38 MAPK, we were able to exclude ERK and p38 MAPK as targets of dextrosimendan. Recently, JNK has been proposed to be involved in dexamethasone- (Gardai et al., 2003) and nitric oxide-induced (Zhang et al., 2003) and in spontaneous (Hasala et al., 2007) eosinophil apoptosis. Dextrosimendan induced activation of JNK as evidenced by Western blot analysis showing an increase in the amount of phosphorylated JNK. Inhibition of JNK activity by a specific inhibitor, 1-JNKI1, reversed the effect of dextrosimendan when apoptosis was measured using the relative DNA fragmentation assay, suggesting that JNK mediates dextrosimendan-induced apoptosis. However, when the effects of 1-JNKI1 on dextrosimendan-induced apoptosis were analyzed using morphological features of apoptosis and the expression of phosphatidylserine on the outer leaflet of the cell membrane (annexin V binding assay), 1-JNKI1 was not able to reverse the effect of dextrosimendan. Taken together, these results suggest that JNK is activated in human eosinophils in response to dextrosimendan and mediates dextrosimendan-induced DNA breakdown, but JNK activation is not involved in the early signaling of dextrosimendan-induced apoptosis. The role of JNK in the regulation of apoptosis in other cell types, mainly of malignant nature, has been widely studied, and it has been found to have both pro- and antiapoptotic effects. It may mediate intrinsic apoptosis pathway by inducing cytochrome c release from mitochondria, leading to caspase activation and cell death (Lin and Dibling, 2002; Manning and Davis, 2003). In addition, JNK has been shown to mediate oxidative stress-induced DNA fragmentation in cardiac smooth muscle cells (Turner et al., 1998). The exact relationship between JNK activation and DNA fragmentation in eosinophils remains to be established.

In the present study, dextrosimendan, used at a dose of 1 mg/kg as nasal inhalation, produced a significant reduction in the number of eosinophils in BAL in sensitized and challenged mouse. Thus, the concentrations of dextrosimendan administered locally into the lung reached levels that have remarkable antieosinophilic activity. This suggests that the results obtained are likely to have clinical importance. Interestingly, levosimendan, at doses similar (0.3–3 mg/kg orally) to those used in the present study, was recently reported to reduce the high-salt diet-associated increase in cardiac myocyte apoptosis in hypertensive Dahl/Rapp rats (Louhelainen et al., 2007). Likewise, levosimendan has been shown to reduce apoptosis in cardiac myocytes isolated from rat ventricles, probably because of the activation of mitochondrial K\(_{\text{ATP}}\) channels (Maytin and Colucci, 2005). Furthermore, treatment with levosimendan has been shown to reduce the circulating apoptosis mediators in humans (Parissis et al., 2004). This suggests that simendans do not induce apoptosis in all cell types. However, the reason why simendans induce apoptosis in eosinophils but protect cardiac myocytes remains unknown at the present. Taken together, our results suggest that compounds with simendan structure have re-
Kankaanranta et al.

markable antieosinophilic activity and thus are potent candidates for the treatment of eosinophil inflammatory conditions in addition to their well-known effect on cardiovascular performance.

Acknowledgments

We thank Tanja Kuusela for skillful technical assistance and Heli Maatta for secretarial help.

References


Kankaanranta H, Ilmarinen P, Zhang X, Nissinen E, and Moilanen E (2006) Antieosinophilic activity and thus are potent can-

Kankaanranta H, Ilmarinen P, Zhang X, Nissinen E, and Moilanen E (2005) Antieosinophilic activity and thus are potent can-