

Recovery of neutrophil apoptosis by ectoine: a new strategy against lung inflammation

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Abstract

The life span of neutrophilic granulocytes has determining impact on the intensity and duration of neutrophil driven lung inflammation. Based on the compatible solute ectoine, we aimed to prevent anti-apoptotic reactions in neutrophils triggered by the inflammatory microenvironment in the lung.

Neutrophils from COPD patients and control individuals were exposed to inflammatory mediators and xenobiotics in the presence or absence of ectoine. The in vivo relevance of this approach was tested in xenobiotic-induced lung inflammation in rats.

The reduction of apoptosis rates of ex vivo exposed neutrophils from persons of all study groups was significantly restored in the presence of ectoine. However, natural apoptosis rates not altered by inflammatory stimuli were not changed by ectoine. Mechanistic analyses demonstrated the preventive effect of ectoine on the induction of anti-apoptotic signalling. Neutrophilic lung inflammation induced by single or multiple exposition of animals to environmental particles was reduced after the therapeutic intervention with ectoine. Analyses of neutrophils from bronchoalveolar lavage indicate that the in vivo effect is due to the restoration of neutrophil apoptosis. Ectoine, a compound of the highly compliant group of compatible solutes, demonstrates a reproducible and robust effect on the resolution of lung inflammation.

Key words: carbon black, chronic obstructive pulmonary disease, granulocyte-macrophage colony-stimulating factor, emphysema, leukotriene B₄, phosphatidylinositol 3-kinase

Introduction:

Neutrophilic inflammation of the lung is an important component of the innate immune response against viral and bacterial pathogens (1). Neutrophilic granulocytes are recruited from the circulation into the airways by immune complexes containing the chemokine CXCL8 (interleukine-8; IL-8) which is secreted from inflammatory or epithelial cells (2). Pathogens are then affected by the release of reactive oxygen species and pathogen-destroying enzymes like myeloperoxidase, elastase, and matrix metalloproteinases. These reactions, however, have also adverse effects on the lung tissue and neutrophilic inflammation is therefore strictly regulated not to persist after successful pathogen defence. Accordingly, upon entry of neutrophils into the lung, intracellular signalling cascades are being triggered which lead to neutrophil apoptosis and, ultimately, to their removal by macrophage-mediated phagocytosis (3).

Apart from biogenic pathogens, lung inflammation can also be induced by occupational or environmental exposure to xenobiotics or by tobacco smoking (4). Ongoing exposure may then result in chronic lung inflammation and eventually severe lung diseases such as chronic obstructive pulmonary disease (COPD). For this reason, some therapeutic approaches in COPD focus on the resolution of the neutrophilic lung inflammation (5). Unfortunately, corticosteroids as the most important group of anti-inflammatory drugs, which in other types of inflammation reliably reduce inflammatory cell numbers, are not effective in the therapy of COPD (6). Studies on the molecular mechanisms of glucocorticoid resistance of neutrophils suggest that oxidative stress from xenobiotic exposure and/or the inflammatory cells themselves have impact on glucocorticoid receptor activity regulated by histone deacetylases (7).

An alternative approach to reduce the number of neutrophils in chronic lung inflammation therefore builds on the concept that neutrophil life span may be reduced if anti-apoptotic reactions triggered by the inflammatory microenvironment are prevented (8). In a pro-inflammatory situation, inflammatory mediators like granulocyte-macrophage colony-stimulating factor (GM-CSF) or leukotriene B₄ (LTB₄) counteract natural apoptosis (9,10). The activation of PI3-K (phosphatidylinositol 3-kinase) and Akt (protein kinase B) signalling reduces the proteolytic turn-over of Mcl-1, the predominant anti-apoptotic protein in neutrophils, and thereby delays naturally scheduled apoptosis leading to an increase of the local inflammatory reaction (11). Accordingly, pharmaceutical strategies have been developed, which target anti-apoptotic signalling via Akt (12). This signalling cascade, however, has pleiotropic effects in different cell types and pharmacological intervention at this level may have severe side effects (12).

Compatible solutes are being used by many cells from bacteria up to higher vertebrates in order to counteract extreme situations like osmotic stress, heat, or desiccation (13). These zwitter-ionic substances are known *not* to disturb physiological processes in a broad concentration range. By interaction with the hydration layer of macromolecules, the presence of these substances appears to promote thermodynamically stable conformations. Based on the biophysical principle of 'preferential exclusion' (14) these substances have stabilizing effects on macromolecules and are therefore used in heterologous systems including biotechnical use but also for skin care products and dermatological applications with beneficial effects (15). We have previously shown that, ectoine, a compatible solute from *Halomonas elongata* is well tolerated when it is instilled in the lungs of rats (16). In these studies, ectoine reduced mitogen activated protein kinase (MAPK) activation and IL-8 expression in lung epithelial cells in vitro, as well as in animals which were

intratracheally treated with carbon nanoparticles (CNP; 17). The elicitation of these endpoints by environmentally relevant model particles proved to be specifically mediated by PI3-K and Akt signalling events (18), indicating that compatible solutes, and ectoine in particular, prevent molecular stress responses mediated by this mechanism.

In the present study we asked whether the compatible solute ectoine is able to prevent stress-induced signalling pathways responsible for the regulation of neutrophil apoptosis which is also known to be modulated by Akt-dependent signalling (19). As ectoine is well tolerated when directly applied to the lung of animals, it could be useful for the treatment of neutrophilic lung inflammation and, in particular, of COPD. We therefore assessed the influence of ectoine on the prevention of apoptosis in neutrophils isolated from healthy volunteers, COPD patients, and clinical controls. In a next set of experiments, the effects of ectoine on lung neutrophil apoptosis and the persistence of lung inflammation was studied in vivo in animals treated with environmental model particles.

Methods:

Volunteers and patients. The group of young healthy volunteers consisted of six male and four female individuals (age 29.9 +/- 4.2 years). Male patients with a stable COPD were compared to male control individuals. Demographic informations as well as health status (including COPD criteria) of patients and controls are given in table 1. Both groups were recruited from a cohort of patients with symptoms of coronary artery disease. All subjects were screened by clinical history, physical examination, electrocardiogram at rest, and routine chemical analyses. Medication was discontinued on the day of the investigations. Patients with severe chronic heart failure, renal insufficiency (glomerular filtration rate < 30 ml/min), a malignant

disease, an inflammatory disease as indicated by an increased CRP > 5 mg/l, vasculitis or Raynaud's syndrome were excluded. The study was approved by the local ethics committee on human research of the Heinrich-Heine-University Düsseldorf and written informed consent was obtained from all study subjects before enrolment.

Isolation and treatment of human neutrophils. Neutrophil isolation and apoptosis measurement were performed as described (19). Prior (2 h) to the treatment with particles or inflammatory mediators, ectoine (in PBS) or PBS (sham control) were added to the cultures. Cells were then treated with 33 µg/ml CNP in PBS, 300 nM LTB₄ (Calbiochem/Merk, Nottingham, UK) in 0,1 % EtOH, or 20 ng/ml GM-CSF (Cell Signaling Technology) in PBS. Cells were harvested after 6 h (for the analyses of signalling proteins) and 16 h (for measurement of apoptosis), from the same samples.

Quantification of apoptotic cells. Blood neutrophils (4×10^5) were suspended in 300 µl hypotonic solution containing propidium iodide (PI). The red fluorescence of PI was measured flow cytometrically (FACScan cytometer, BD Biosciences). A minimum of 10^4 events were counted per sample. Western blots and the respective protein preparations were performed as described (17,19).

Particle suspensions and compound solutions. CNP, 14 nm diameter (Carbon Black, Printex 90, Degussa, Frankfurt, Germany) and ectoine solution ((S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid, LPS-free, ultrapure 99%, bitop AG, Witten, Germany) were characterized and prepared as described (17).

Animal experiments. Female Fisher 344 rats (8 weeks old, Charles River Laboratories, Germany) were instilled intratracheally with a volume of 0.4 ml particle suspension, ectoine solution (doses indicated in the figures), or PBS, respectively, under inhalation anesthesia (isoflurane, 5 %, 2 min). Animals were sacrificed by

exsanguination under pentobarbital anesthesia, after the indicated periods. Lung lavages were performed using 4 x 5 ml PBS. Differential cell counts were performed after Giemsa/May-Grünwald staining of lavage cells. Cell free lavage fluids were used for cytokine assays. Lung tissues were minced, shock frozen and stored at -80° C until further use. All animal experiments were performed after relevant permission according to German animal protection laws. Myeloperoxidase was measured by ELISA using a rat myeloperoxidase kit (Cell Sciences, Canton, MA). For apoptosis measurements, lavage cells from each animal were suspended in 1 ml PBS and subjected to Percoll (Biochrom, Berlin, Germany) centrifugation. Cell pellets were washed once with PBS and resuspended in 300 µl hypotonic solution (0.1% sodium citrate, 0.1% Triton X 100) containing 50 µg/ml PI and subsequently subjected to fluorescence measurements.

Statistics. Due to the lognormal distribution of data, statistical analysis was performed after logarithmic transformation. Analysis of variance was used throughout (proc mixed, SAS 9.2 SAS Institute Inc. Cary, NC, USA). If appropriate, repeated measures analysis of variance was used (proc glm, SAS 9.2). Main effects and interactions were tested. Changes were estimated relative to the respective controls and are given with their 95% confidence interval (CI:95%). A result was considered as statistically significant, when the error probability p was less than 0.05. Multiple testing was avoided. The main hypothesis per experiment was tested. In the rare cases of two tested hypotheses per experiment, Bonferroni correction was applied ($p=0.025$). Each experiment is graphically represented by one figure. Data are depicted as mean values and standard deviations.

Results:

Ectoine restores spontaneous apoptosis in isolated human neutrophils.

Peripheral blood neutrophils were isolated from young healthy volunteers and subsequently subjected to treatments which mimic the inflammatory situation in the lung (figure 1A). Besides the inflammatory mediators GM-CSF (20 ng/ml) and LTB₄ (300 nM), carbon nanoparticles (CNP, 33 µg/ml) which are accepted as representative for ultrafine particulate air pollution (20), were used for cell treatment. All these pro-inflammatory stimulants significantly reduced the percentage of apoptotic neutrophils by a factor of 0.61 (95%CI: 0.53 - 0.69), 16 hours after exposure (figure 1A). This effect was equal for the three stimulants used. The pre-treatment of neutrophils with 1 mM ectoine 2 h prior to inflammatory mediators or particles nearly completely restored apoptosis rates (0.82, 95%CI: 0.82 - 0.99), independent from the anti-apoptotic agent and highly significant (repeated measures analysis of variance, F-test $p < 0.0001$).

Neutrophils in general do not respond well to anti-inflammatory therapies like corticosteroid treatment (6) and, in the case of COPD, may even become resistant to this kind of therapy (7). We therefore repeated the experiments with blood neutrophils from patients with COPD as well as from clinical control individuals (figure 1B). Although higher background apoptosis rates were observed in these groups, all three inflammatory stimulants reduced the number of apoptotic cells both from clinical controls and from patients with COPD (0.49, 95%CI: 0.42 - 0.59 and 0.58, 95%CI: 0.50 - 0.67, respectively). This reduction again was much smaller after ectoine pre-treatment in cells from both groups (0.87, 95%CI: 0.80 - 0.95 for patients with COPD, and 0.84, 95%CI: 0.79 - 0.90 for controls). The effects proved to be significant ($p < 0.0001$) and independent of the inflammatory stimulant and from the COPD status (repeated measures analysis of variance, F-test).

Ectoine acts preventive at the level of membrane-coupled signalling. The cellular and molecular mechanisms underlying the effect of ectoine on apoptosis was further investigated in neutrophils from healthy volunteers treated with carbon particles (33 µg/ml). Increasing concentrations of ectoine gradually restored apoptosis rates (figure 2A). The ectoine effect was significant (repeated measures analysis of variance, F-test, $p < 0.001$). To investigate whether ectoine itself has any pro-apoptotic activity, human neutrophils from 3 male and 2 female human donors were exposed to a broad dose range (0.01 mM – 10 mM) of ectoine (figure 2B). Compared to sham treated controls, no ectoine effect ($p = 0.3024$) was detected.

As a next step, the effects of ectoine on anti-apoptotic signalling was investigated. For this purpose, neutrophils isolated from healthy volunteers were analyzed for Akt phosphorylation at Ser⁴⁷³ as a specific downstream event of PI3-K signalling. Phosphorylation levels in comparison to total Akt amounts in Western blot analyses showed a significant (repeated measures analysis of variance, F-test, $p < 0.0001$ after Bonferroni correction) activation of this pathway by CNP, GM-CSF, and LTB₄ (figure 2C), indicating that all anti-apoptotic stimuli trigger the same anti-apoptotic pathway. Importantly, in all three cases, pre-incubation of human neutrophils with ectoine prevented the activation of this pathway. In these cells, phosphorylation levels were not significantly different from those before application of the anti-apoptotic stimuli. The ectoine effect was significant (repeated measures analysis of variance, F-test, $p = 0.012$ after Bonferroni correction). As a consequence of Akt activation, protein amounts of anti-apoptotic Mcl-1 were increased after the treatment with CNP, GM-CSF, and LTB₄ (3.0 times, 95%CI: 2.0 - 4.4). Pretreatment with ectoine largely prevented this key anti-apoptotic event (1.6 times, 95%CI: 1.0 - 2.4; figure 2D). The ectoine effect again was significant (repeated measures analysis of variance, F-test, $p = 0.0173$).

Ectoine restores apoptosis rates in vivo and reduces pre-existing neutrophilic inflammation. We next asked whether ectoine has also preventive effects in vivo on neutrophilic inflammation which was induced in lungs of Fischer 344 rats by intra-tracheal instillation of xenobiotic model particles. As depicted in figure 3A, inflammation was first induced by a single instillation of 2.5 mg/kg CNP followed by two consecutive ectoine applications given at day 1 and day 2. At day 3, bronchoalveolar lavage (BAL) was performed and specimens were analysed for neutrophil apoptosis as well as inflammatory parameters. As figure 3B shows, CNP exposure alone significantly reduced the percentage of apoptotic cells (0.59, 95%CI: 0.45 - 0.78) indicating that anti-apoptotic mechanisms had been elicited by CNP and / or CNP-triggered inflammatory mediators. In CNP-exposed animals, which subsequently were treated twice with 0.1 mM or 1 mM ectoine, an improvement of neutrophil apoptosis was observed (0.64, 95%CI: 0.48 - 0.84, or 0.82, 95%CI: 0.62 - 1.07, respectively). The ectoine effect proved to be statistically significant ($p=0.0215$, analysis of variance, F-test).

Importantly, advanced neutrophil apoptosis correlated with the significant reduction of lung inflammation measured as percentage of neutrophils and amount of cinc-1 (neutrophil recruiting IL-8 homologue) in BAL (figure 3C). After CNP exposure 4.4 times (95%CI: 3.2 - 5.6) as many neutrophils and 3.1 times (95%CI: 2.4 - 4.0) higher concentrations of cinc-1 as without treatment could be observed in BAL. After additional application of 1 mM ectoine, the elevation was reduced to 3.1 times (95%CI: 2.2 - 4.4) for the number of neutrophils and 2.1 times (95%CI: 1.6 - 2.6) for the concentration of cinc-1. The ectoine effect was significant for both outcomes (analysis of variance, F-test, $p=0.0005$). This therapeutic effect was confirmed at the level of neutrophil activation by measuring myeloperoxidase (figure 3D). MPO levels were increased 19.6 times (95%CI: 11.6 - 41.8). This increase was reduced to 10.3

times (95%CI: 5.3 - 60.8) after additional treatment with ectoine (analysis of variance, F-test, $p=0.0633$). We had previously reported that CNP activates the MAP-K Erk1/2 in lung epithelial cells (21). Assessment of Erk1/2 phosphorylation in lung homogenates of animals revealed, that ectoine also significantly ($p=0.0166$, analysis of variance, analysis of variance, F-test) diminished this tissue response (figure 3D). In aggregate, these observations strongly indicate that ectoine can reduce a pre-existing neutrophilic lung inflammation.

Ectoine effects during repetitive xenobiotic exposure in vivo. In a real life scenario, however, chronic inflammation would result from continuous rather than from a single exposure to xenobiotics. In order to mimic this situation, we administered ectoine (1 mM) twice after one, two, or three instillations of CNP, respectively (figure 4A). In these experiments, neutrophil apoptosis was significantly reduced by each CNP application to 66.0% (95%CI: 53.9% - 80.9%), and was restored each time (85.8%, 95%CI: 69.6% - 105.9%) when this treatment was followed by two instillations of 1 mM ectoine. The restoration was equally pronounced in all three exposure scenarios, no significant interaction between exposure scenario and ectoine effect was detected. The ectoin effect was significant (analysis of variance, F-test, $p=0.0091$; figure 4B).

The preventive effect of ectoine therapy was also observed at the level of inflammation. Under all experimental conditions, ectoine significantly reduced neutrophil numbers and cinc-1 levels in BAL of CNP-treated rats (figure 4C). These effects were significant ($p<0.0001$, analysis of variance, F-test) for both endpoints and the dimension of the effect was equally pronounced in all three exposure scenarios. The therapeutic effect of ectoine was also demonstrated by comparative measurement of inflammatory cytokines in BAL from animals with pre-existing inflammation using membrane-coupled cytokine arrays. Figure 4D demonstrates that

in addition to cinc-1, several other pro-inflammatory factors (TNF-alpha, GM-CSF, IL-1alpha, IL1 beta, IL-4, IL-6, and cinc-2) which were elevated due to the xenobiotic particle treatment were significantly reduced upon ectoine treatment to 41.2% (95%CI: 31.4% - 54.1%, repeated measures analysis of variance, F-test $p < 0.0001$) of the value without ectoine, while IFN-gamma and anti-inflammatory IL-10 only changed to 81% (95%CI: 48.0% - 137%) of the value before treatment, which was not significant.

Persistent reduction of neutrophil numbers in rat lungs by ectoine. We next studied whether the preventive effect of ectoine persists during the course of inflammation. For that purpose, 2.5 mg/kg CNP were injected once, either with or without ectoine, in the lungs of animals. Animals were sacrificed at different time points from 12 hours up to one week (168 h) after exposure and BAL was taken. Interestingly, the effect of ectoine significantly changed over time (analysis of variance, interaction time*ectoin effect, F-test $p < 0.0001$). 12 h and 24 h after the application of CNP, ectoine did not significantly reduce the number of lung neutrophils in particle treated rats (figure 5A). In marked contrast, from 48 h up to 168 h the reduction of neutrophils by ectoine was significant ($p = 0.002$). The number of neutrophils without ectoine treatment was 1.2 times (95%CI: 1.1 - 1.4) higher than that with treatment with no further changes over time. Similarly, from 48 h on, cinc-1 concentrations in BAL were 2.5 (95%CI: 2.0 - 3.08) times higher after CNP treatment than after additional ectoine treatment ($p < 0.0001$; figure 5B).

Discussion:

Ectoine treatment prevents anti-apoptotic reactions in human neutrophils.

Chronic exposure to inhalable xenobiotics is able to cause neutrophilic lung inflammation leading to emphysema and COPD (22). In this case, xenobiotics trigger

the release of pro-inflammatory cytokines and chemokines, which lead to an influx and activation of neutrophils (23). Under these circumstances and regardless of the inducing agent, chronic neutrophilic inflammation is the key pathogenic mechanism in a vicious cycle of necrotic tissue damage and increased recruitment and activation of neutrophils (24). The neutrophilic inflammation remains, or due to ongoing tissue destruction, is aggravated, even when the inducing agent is removed, e.g. after cessation of smoking (4). In this inflammatory scenario neutrophils come into contact with inflammatory mediators but also with the xenobiotics present during ongoing exposure. Neutrophil life span may be extended not only by cellular factors but also by the xenobiotics themselves.

In line with this concept, the presented data clearly demonstrate that spontaneous apoptosis of human neutrophils is counteracted not only by GM-CSF and LTB₄ but also by ultrafine carbon particles which are a main constituent of particulate air pollution (20). CNP in several cells are able to induce oxidative stress which triggers membrane signalling pathways (25). Oxidative stress has been described as trigger of anti-apoptotic pathways in neutrophils (26). Our data demonstrate a direct anti-apoptotic effect of environmentally relevant inhalable particles on human neutrophils which may contribute to the persistence of the inflammation during ongoing exposure.

The present study, moreover, provides compelling evidence that the compatible solute ectoine, is able to prevent anti-apoptotic reactions of neutrophils induced by inflammatory stimulants. This conclusion is based on the following observations: (i) Anti-apoptotic reactions of neutrophils isolated from young healthy volunteers either induced by CNP as environmentally relevant model particles or by inflammatory mediators are completely prevented. (ii) Investigating neutrophils from male COPD patients as well as from clinical control individuals, a striking prevention of anti-

apoptotic reactions by ectoine was observed. (iii) The activation of anti-apoptotic membrane-coupled signalling pathways by inflammatory stimulants in neutrophils is prevented in the presence of ectoine.

Ectoine acts preventive rather than pro-apoptotic. As ectoine did not alter apoptosis rates of neutrophils which were *not* stimulated by CNP or inflammatory factors, we conclude that ectoine does *not* act pro-apoptotic. Instead, our data are consistent with the assumption that ectoine has the capacity to prevent anti-apoptotic mechanisms which are known to be activated within the inflammatory microenvironment.

The cell signalling cascade involving PI3-K and Akt has been described as the key mechanism for the anti-apoptotic effects of LTB₄ and GM-CSF (9,10). The same pathway was identified to be triggered by CNP-induced oxidative stress (25,27). Interestingly, ectoine has been shown to prevent stress-induced membrane-dependent signalling cascades in some systems. In human keratinocytes, e.g., the UVA stress response can be reduced by ectoine pre-treatment through mechanisms which involve the prevention of second messenger ceramide from cell membrane lipid rafts (28). Also, the compatible solutes ectoine and hydroxyectoine have been recently found to act on lipid monolayers and bilayers and to affect fluidity of membranes (29). The reduction of Akt signals to background resulting in diminished Mcl-1 levels indicates that this membrane-coupled pathway is the target of the preventive effect of ectoine in neutrophils as well as in epithelial cells.

Ectoine as a therapeutic strategy to reduce ongoing neutrophilic lung inflammation. The key question for the therapeutic usefulness of ectoine in lung inflammation is whether the prevention of delayed neutrophil apoptosis has the potential to reduce the neutrophilic infiltrate during the course of an ongoing inflammation. In this regard we have clearly shown that in the *in vivo* model of carbon

particle-induced lung inflammation, ectoine restored neutrophil apoptosis rates and at the same time reduced lung inflammation.

In humans, however, the situation might be more complex. Therapeutic or preventive strategies have to cope with the fact that such interventions may be counteracted by self-perpetuating mechanisms of the inflammation itself and/or by continuous exposure from air pollution. In the present study, ectoine was found to reproducibly prevent delayed apoptosis and to reduce inflammation in animals which had been repetitively exposed to CNP. We therefore believe, that ectoine can reduce neutrophilic lung inflammation under conditions which may be representative for the real life situation in humans.

We also show that the effect of ectoine persists over the whole period of lung inflammation which was elicited by a single particle exposure. Interestingly, the general pattern of the release of neutrophil-recruiting cinc-1 in BAL is consistent with a biphasic response. This kinetic may be best explained by different cell types known to produce this chemokine. During the early recruiting phase, cinc-1 is likely to be mainly produced by epithelial cells and macrophages, while at later time points the gradually growing and ultimately dominating number of neutrophils may be responsible for cinc-1 release. This would imply that ectoine affects not only lung epithelial cells, as described earlier (17), but in particular neutrophils. Irrespective of the cinc-1 expression pattern, these in vivo data demonstrate a highly reproducible, robust, and persistent effect of ectoine on neutrophil numbers and cinc-1 levels, resulting in an accelerated resolution of lung inflammation.

Neutrophil recruitment appears to be not directly affected by ectoine. At early time points after the induction of inflammation (12 h and 24 h), when neutrophil numbers are dominated by chemokine mediated influx, no ectoine effects can be observed in exposed animals. Later however, when differences in apoptosis rates are considered

to influence neutrophilic lung inflammation, the effect of ectoine becomes obvious. These data corroborate findings of earlier studies in which we observed that rapid neutrophil recruitment within 4 h after the application of lipopolysaccharide is not influenced by ectoine (17). Both results demonstrate that ectoine does not suppress neutrophil influx which is necessary for the defence of pathogens.

The CNP exposure scenario chosen for the recent experiments was primarily designed to rapidly induce severe inflammation. It employed high doses of CNP which in the human lung would most likely result from a cumulative process requiring years of environmental exposure. We therefore believe that the exposure regimen used in the present study is over- rather than underestimating human exposure scenarios. It is therefore not unlikely that patients who are continuously exposed to environmental air pollution may also benefit from a regular application of compatible solutes which have been described as a very compliant group of natural substances with so far no known side effects in humans (30). So far inhalation studies with humans inhaling ectoine have not been published. As a first approach for the treatment of chronic neutrophilic lung inflammation, feasibility as well as efficacy studies have to be performed.

In conclusion, the data presented here demonstrate that instillation of the compatible solute ectoine in the lungs of animals suffering from neutrophilic inflammation induced by environmental model particles can exert significant therapeutic effects. Ectoine appears to act by preventing anti-apoptotic reactions and reducing the life span of lung infiltrating neutrophils. The ectoine effects were observed regardless (i) whether neutrophil apoptosis was delayed by xenobiotics or by pro-inflammatory factors, and (ii) whether isolated human blood neutrophils (from patients, controls, or volunteers) or lung neutrophils recruited after exposition to carbon particles were

studied. We therefore propose that compatible solutes such as ectoine may be effectively used in clinical settings for the treatment of neutrophilic lung inflammation.

Acknowledgements:

The work was supported by project funding from the Ministerium für Umwelt Naturschutz und Reaktorsicherheit (BMU), Deutsche Forschungsgemeinschaft (GRK 1427 and SFB 728), Zukunftswettbewerb NRW, and bitop AG. The excellent technical assistance of Ragnhild Wirth and Winfried Brock as well as the professional help with animal keeping of Siegrid Martin and Petra Groß are gratefully acknowledged.

References:

1. Chaudhuri N, Sabroe I. Basic science of the innate immune system and the lung. *Paediatr Respir Rev* 2008; 9: 236-242.
2. Bergion DA, Reeves EP, Meleady P, Henry M, McElvaney OJ, Carroll TP, Condrón C, Chotirmall SH, Clynes M, O'Neill SJ, McElvaney NG. α -1 Antitrypsin regulates human neutrophil chemotaxis induced by soluble immune complexes and IL-8. *J Clin Invest* 2010; 120: 4236-4250.
3. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. *Nat Immunol* 2005; 6: 1191-1197.
4. MacNee W. Pathogenesis of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005; 2: 258-266.
5. Beeh KM, Beier J. Handle with care: targeting neutrophils in chronic obstructive pulmonary disease and severe asthma? *Clin Exper Allergy* 2006; 36: 142-157.

6. Barnes PJ, Adcock IM. Glucocorticoid resistance in inflammatory diseases. *Lancet* 2009; 73: 1905-1917.
7. Ito K, Yamamura S, Essilfie-Quaye S, Cosio B, Ito M, Barnes PJ, Adcock IM. Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF-kappaB suppression. *J Exp Med* 2006; 203: 7-13.
8. Duffin R, Leitch AE, Fox S, Haslett C, Rossi AG. Targeting granulocyte apoptosis: mechanisms, models, and therapies. *Immunol Rev* 2010; 236: 28-40.
9. Klein JB, Rane MJ, Scherzer JA, Coxon PY, Kettritz R, Mathiesen JM, Biridi A, McLeish KR. Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. *J Immunol* 2000; 164: 4286-4291.
10. Petrin D, Turcotte S, Gilbert A-K, Rola-Pleszynski M, Stankova J. The anti-apoptotic effect of leukotriene B₄ in neutrophils: A role for phosphatidylinositol 3-kinase, extracellular signal-regulated kinase and Mcl-1. *Cell Signal* 2005; 18: 479-487.
11. Dzhagalov I, St. John A, He Y-W. The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages. *Blood* 2007;109: 1620-1626.
12. Ghigo A, Damilano F, Braccini L, Hirsch E. PI3K inhibition in inflammation: toward tailored therapies for specific diseases. *BioEssays* 2010; 32: 185-196.
13. Yancey PH. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J Exp Biol* 2005; 208: 2819-2830.
14. Arakawa T, Timasheff SN. The stabilization of proteins by osmolytes. *Biophys J* 1985; 47: 411-414.

15. Lentzen G, Schwarz T. Extremolytes: natural compounds from extremophiles for versatile applications. *Appl Microbiol Biotechnol* 2006; 72: 623-634.
16. Galinski EA, Pfeiffer H-P, Trüper HG. 1,4,5,6-Tetrahydro-2methyl-4-pyrimidinecarboxylic acid: a novel cyclic amino acid from halophilic bacteria of the genus *Ectothiorhodospira*. *Eur J Biochem* 1985; 149: 135-139.
17. Sydlik U, Gallitz I, Albrecht C, Abel J, Krutmann J, Unfried K. The compatible solute ectoine protects against nanoparticle-induced neutrophilic lung inflammation. *Am J Respir Crit Care Med* 2009; 180: 29-35.
18. Unfried K, Sydlik U, Bierhals K, Weissenberg A, Abel J. Carbon nanoparticle-induced lung epithelial cell proliferation is mediated by receptor-dependent Akt activation. *Am J Physiol Lung Cell Mol Physiol* 2008; 291: 725-733.
19. Paunel-Görgülü A, Zörnig M, Lötgers T, Altrichter J, Rabenhorst U, Cinatl J, Windolf J, Scholz M. Mcl-1-mediated impairment of the intrinsic apoptosis pathway in circulating neutrophils from critically ill patients can be overcome by Fas. *J Immunol* 2009; 183: 6189-6206.
20. Donaldson K, Tran L, Jimenez LA, Duffin R, Newby DE, Mills N, MacNee W, Stone V. Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. *Part Fibre Toxicol* 2005; 2: 10.
21. Sydlik U, Bierhals K, Soufi M, Abel J, Schins RP, Unfried K. Ultrafine carbon particles induce apoptosis and proliferation in rat lung epithelial cells via specific signaling pathways both using EGF-R. *Am J Physiol Lung Cell Mol Physiol* 2006; 291: L725-L733.

22. Schikowski T, Sugiri D, Ranft U, Gehring U, Heinrich J, Wichmann H-E, Krämer U. Long-term air pollution exposure and living close to busy roads are associated with COPD in women. *Respir Res* 2005; 5: 152.
23. Luo HR, Loison F. Constitutive neutrophil apoptosis: Mechanisms and regulation. *Am J Hematol* 2007; 83: 288-295.
24. Quint JK, Wedzicha JA. The neutrophil in chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 2007; 119: 1065-1071.
25. Weissenberg A, Sydlik U, Peuschel H, Schroeder P, Schneider M, Schins RP, Abel J, Unfried K. Reactive oxygen species as mediators of membrane-dependent signaling induced by ultrafine particles. *Free Rad Biol Med* 2010; 49: 597-605.
26. Melley DD, Evans TW, Quinlan GJ. Redox regulation of neutrophilic apoptosis and the systemic inflammatory response syndrome. *Clin Science* 2005;108:413-424.
27. Peuschel H, Sydlik U, Haendeler J, Büchner N, Stöckmann D, Kroker M, Wirth R, Brock W, Unfried K. c-Src-mediated activation of Erk1/2 is a reaction of epithelial cells to carbon nanoparticle treatment and may be a target for a molecular preventive strategy. *Biol Chem* 2010; 391: 1327-1332.
28. Grether-Beck S, Timmer A, Felsner I, Brenden H, Brammertz D, Krutmann J. Ultraviolet A-induced signaling involves a ceramide-mediated autocrine loop leading to ceramide de novo synthesis. *J Invest Dermatol* 2005; 125: 545-553.
29. Harishchandra RK, Wulff S, Lentzen G, Neuhaus T, Galla HJ. The effect of compatible solute ectoines on the structural organization of lipid monolayer and bilayer membranes. *Biophys Chem* 2010; 150: 37-46.

30. Kanapathipillai M, Lentzen G, Sierks M, Park CB. Ectoine and hydroxyectoine inhibit aggregation and neurotoxicity of Alzheimer's β -amyloid. *FEBS Lett* 2005; 579: 4775-4780.

Tables:

Table 1 Demographic details and health status of patients with COPD and control subjects

	COPD	control subjects	p value
n	10	10	
demographic information			
Age [years]	66.8 ± 7.5	64.7 ± 12.4	
Sex [%male]	100	100	
Hight [m]	1.76 ± 0.05	1.77 ± 0.08	n.s. ⁽¹⁾
Weight [kg]	85.4 ± 12.4	90.1 ± 21.2	n.s.
Body mass index [kg/m ²]	27.6 ± 3.2	28.3 ± 4.6	n.s.
Current smoker [%]	10	0	
Former smoker [%]	90	90	
Packyears	46.5 ± 18.3	23.9 ± 12.2	0.006
COPD GOLD classification			
I [%]	20	0	
II [%]	50	0	
III [%]	30	0	
IV [%]	0	0	
lung function⁽²⁾			
FVC ⁽³⁾ [% pred]	77 ± 18	86 ± 9	n.s.
FEV ₁ ⁽⁴⁾ [% pred]	62 ± 20	86 ± 8	0.008
FEV ₁ /FVC [% pred]	60 ± 9	79 ± 9	0.001
Total lung capacity [% pred]	100 ± 13	90 ± 11	n.s.
Residual volume [% pred]	151 ± 29	106 ± 17	0.003
blood parameters			
C-reactive protein [mg/dL]	0.46 ± 0.31	0.50 ± 0.28	n.s.
White blood cell count [/L]	8.14 ± 3.34	7.22 ± 2.70	n.s.
coronary artery disease			
1-vessel disease [%]	30	10	
2-vessel disease [%]	0	10	
3-vessel disease [%]	70	80	

¹ Not significant² Lung function values are given as percentage of predicted values³ Forced vital capacity⁴ Forced expiratory volume in one second

Figure legends:**Figure 1. Ectoine restores apoptosis rates in isolated human neutrophils.**

A: Apoptotic cells (%) from neutrophils isolated from 10 young healthy volunteers and representative histogram plots of sub-G1 measurements. Cells were 2 h pre-treated with 1 mM ectoine followed by a 16 h treatment with 33 μ g/ml CNP, 300 nM LTB₄, 20 ng/ml GM-CSF, or PBS. B: Apoptotic cells (%) from neutrophils isolated from patients with COPD or clinical control individuals (each n=10) treated as described in A.

Figure 1

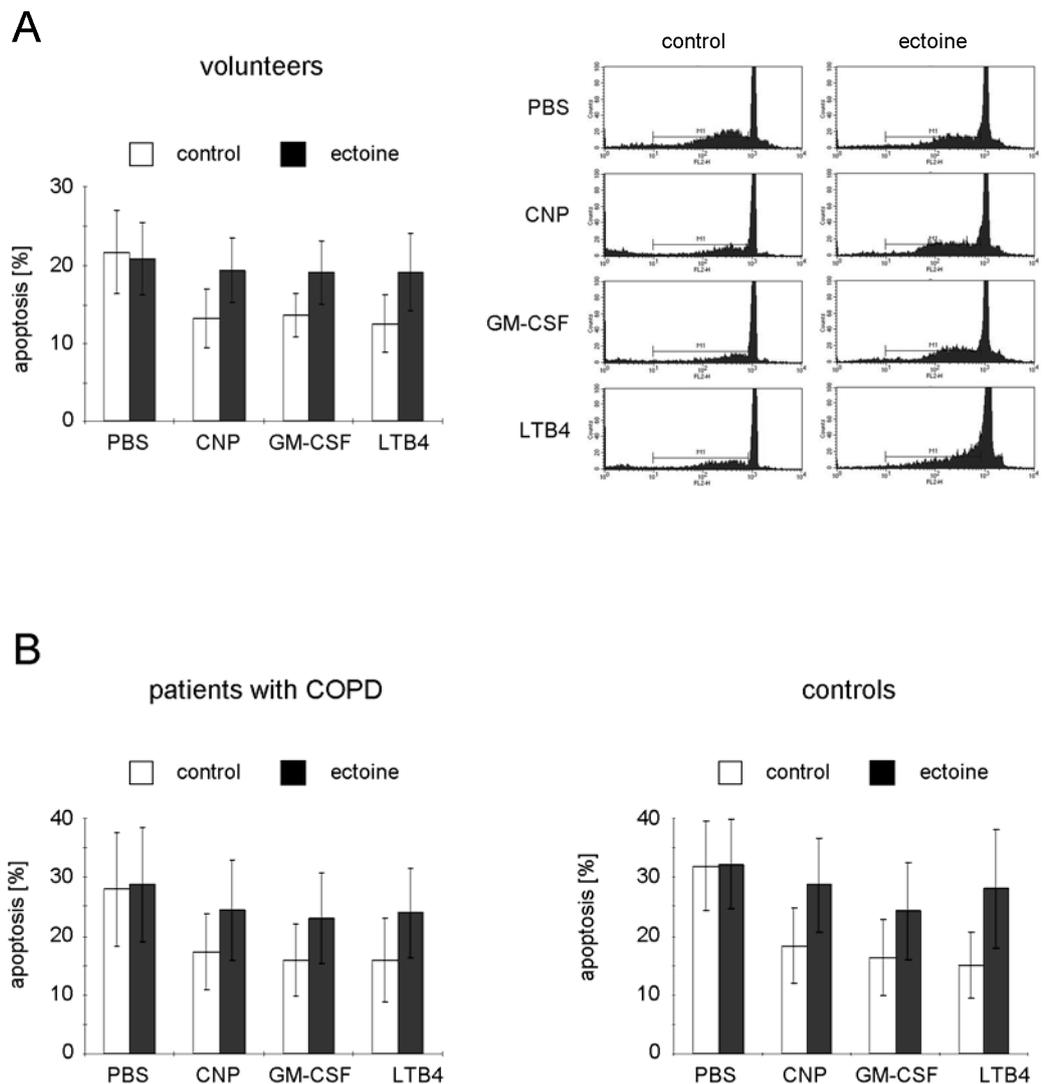


Figure 2. Ectoine prevents anti-apoptotic reactions via Akt-mediated signalling.

A: Apoptotic cells (%) from neutrophils isolated from 5 young healthy volunteers. Cells first treated (2 h) with the indicated doses [mM] of ectoine then treated 16 h with 33 μ g/ml CNP or PBS. B: Percentage of apoptotic cells from neutrophils isolated from 5 volunteers treated 18 h with the indicated doses (mM) of ectoine alone. C: Quantification and representative immunoblots of Akt and D: Mcl-1 in neutrophils 6 h after treatments with PBS, 33 μ g/ml CNP, 20 ng/ml GM-CSF, or 300 nM LTB₄. Ectoine pre-treatment was 1 mM for 2 h.

Figure 2

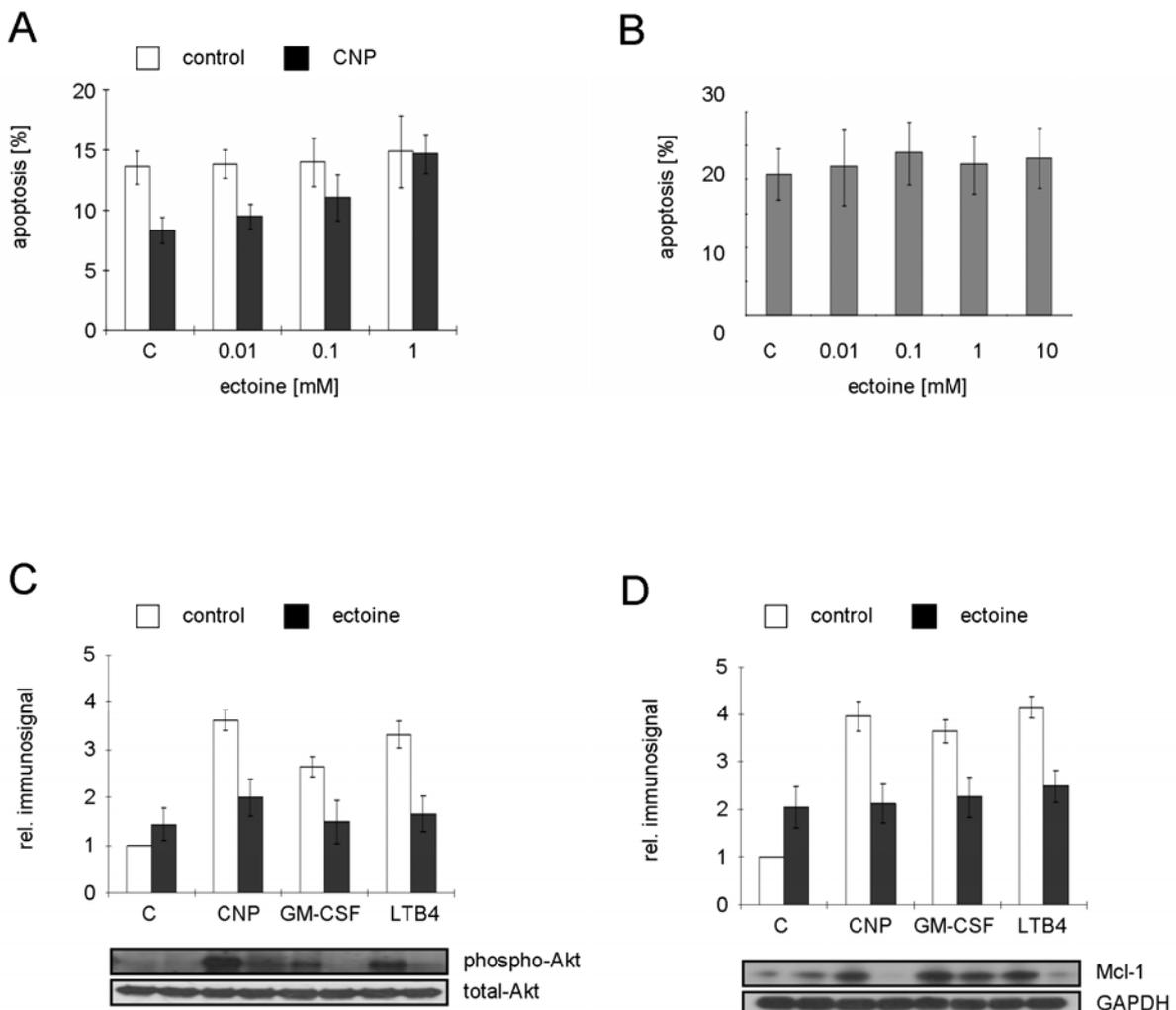


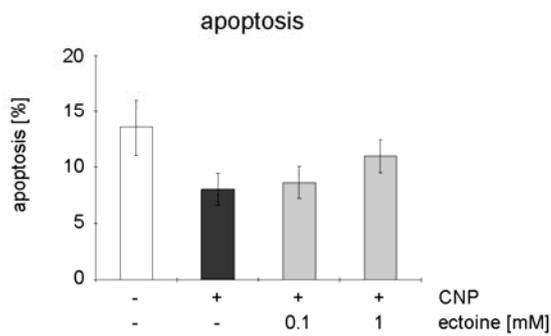
Figure 3. Ectoine reduces inflammation by shortening neutrophilic life span in lung inflammation in vivo. A: Setup of animal experiment with Fischer 344 rats. CNP (2.5 mg/kg bodyweight) and ectoine (E) were intra-tracheally instilled (PBS served as sham for both treatments). B: Apoptosis rats of neutrophils isolated from BAL of 4 animals treated according to figure 3A. C: BAL parameters (% neutrophils and cinc-1) of animals treated according to figure 3A (n = 7). D: Myeloperoxidase in BAL and phosphorylation of Erk1/2 in lung tissue of animals (n = 5) exposed according to the scenario depicted as d2 in figure 4.

Figure 3

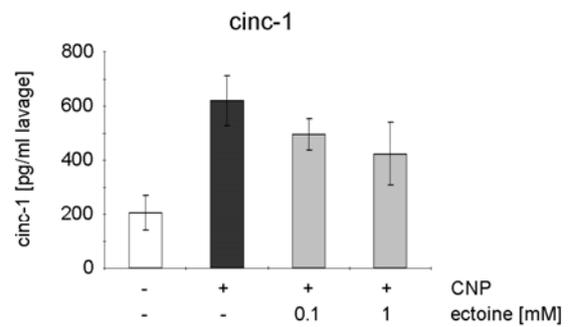
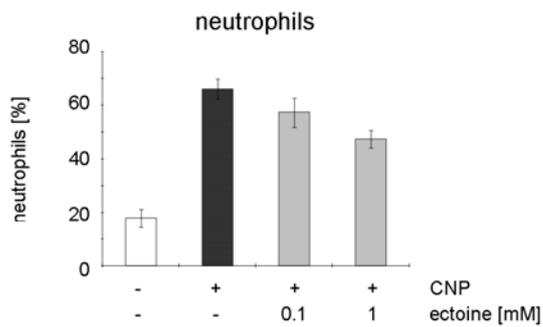
A



B



C



D

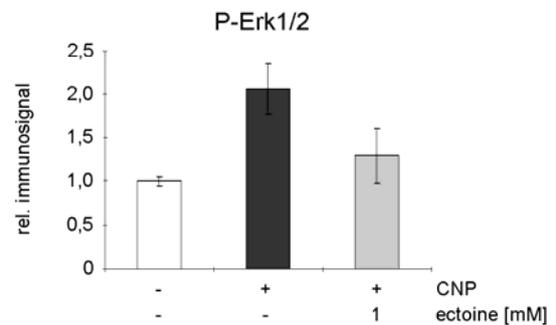
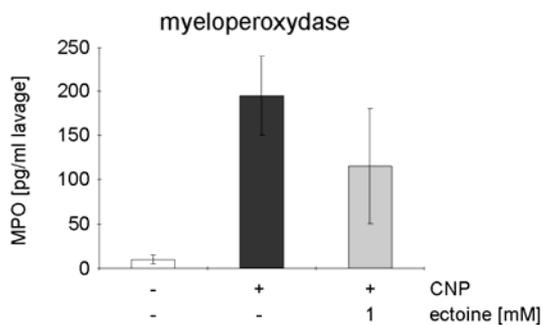


Figure 4. Enhanced apoptosis rates induced by repetitive exposure to CNP are reduced by ectoine. A: Different designs of animal experiments d 1 – d 3, Fischer 344 rats were instilled once, twice, or three times with CNP (2.5 mg/kg bodyweight)

and subsequently treated twice after each CNP instillation with ectoine (E or PBS as sham control for both treatment). B: Relative apoptosis rates of neutrophils isolated from animals treated according to d 1 (n = 4), d 2 (n = 3 for control animals; n = 5 for CNP + PBS; n = 5 for CNP + ectoine), and d 3 (n = 3 for control animals; n = 6 for CNP + PBS; n = 3 for CNP + ectoine). C: Parameters of BAL (% neutrophils and cinc-1) from animals repeatedly treated with CNP and 1 mM ectoine according to designs d 1 (n = 5), d 2 (n = 5), and d 3 (n = 6 for CNP + PBS; n = 3 for CNP + ectoine). D: Cytokine pattern of BAL from control animals (n = 3), animals treated according to d 1 with CNP (n = 4), or treated according to d 1 with CNP and ectoine (n = 5).

Figure 4

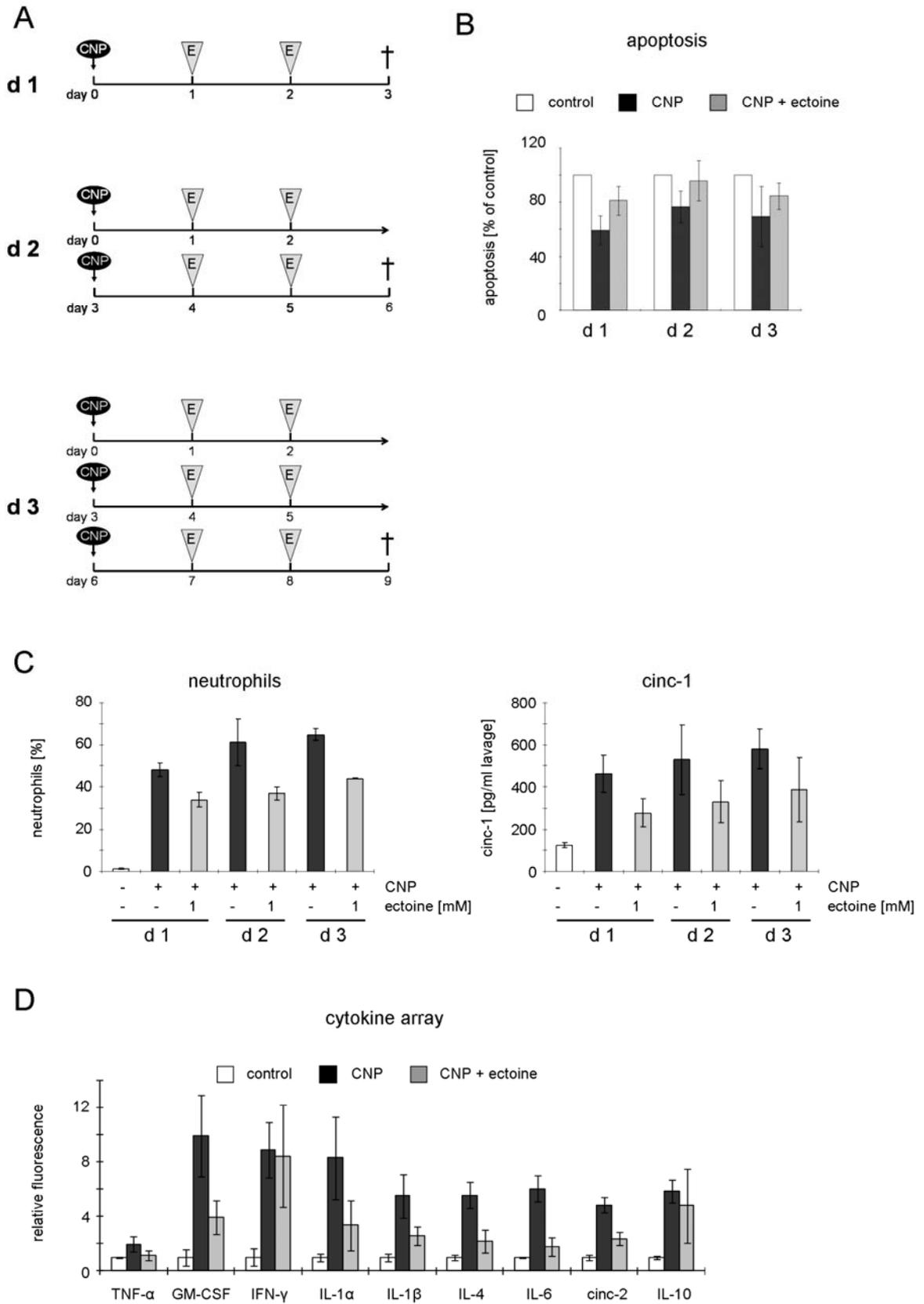


Figure 5. Ectoine effects on the resolution of CNP-induced lung inflammation.

BAL parameters (A, % neutrophils and B, cinc-1) from Fischer 344 rats intratracheally instilled with 2.5 mg/kg body weight in the presence or absence of 1 mM ectoine. Control animals were treated with 0.4 ml PBS. Animals (3 - 5 per group per time point) were sacrificed and lavaged at the indicated time intervals after instillation.

Figure 5

