Impairment of macrophage survival by NaCl: implications for early pulmonary inflammation in cystic fibrosis

GWENDOLYN S. KERBY,1,2 VINCENT COTTIN,1 FRANK J. ACCURSO,2 FUKUN HOFFMANN,1 EDWARD D. CHAN,3 VALERIE A. FADOK,4 AND DAVID W. H. RICHES1,3–5
1Program in Cell Biology, Department of Pediatrics, National Jewish Medical and Research Center, Denver 80206; and Departments of 2Immunology, 3Pediatrics, 4Medicine, and 5Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262

Received 16 July 2001; accepted in final form 24 February 2002

Kerby, Gwendolyn S., Vincent Cottin, Frank J. Accurso, Fukun Hoffmann, Edward D. Chan, Valerie A. Fadok, and David W. H. Riches. Impairment of macrophage survival by NaCl: implications for early pulmonary inflammation in cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 283: L188–L197, 2002—Inflammation, characterized by the presence of proinflammatory chemokines and neutrophils, is a hallmark of early airway disease in infants with cystic fibrosis (CF), although the underlying mechanisms remain poorly defined. In this study, we evaluated the role of NaCl and the ensuing hyperosmolar effect on tumor necrosis factor (TNF)-α signaling and apoptosis in macrophages. Incubation of mouse macrophages with NaCl activated p38mapk and the p46jnk and p54jnk c-jun NH2-terminal kinase isoforms, but not p42mapk/erk2 or Akt. Similar results were obtained with sorbitol, suggesting a general response to osmolarity. Strikingly, the activation of p42mapk/erk2 and Akt by TNF-α was also inhibited in the presence of NaCl. Because the activation of p42mapk/erk2 and Akt has been associated with survival responses, we investigated the effect of NaCl on macrophage apoptosis. The results indicated a synergistic increase in apoptosis when macrophages were exposed to TNF-α in the presence of NaCl compared with stimulation with TNF-α alone or NaCl alone. Furthermore, pharmacological inhibition of p42mapk/erk2 and Akt mimicked the effect of NaCl. Collectively, these findings indicate that modest elevations in NaCl differentially regulate the activation of mitogen-activated protein kinases and Akt and potentiate macrophage apoptosis. We speculate that augmentation of macrophage apoptosis in CF airways may result in decreased clearance of neutrophils and interleukin (IL)-8 in their airways in the absence of detectable airway infection. The CF gene encodes a defective cystic fibrosis transmembrane conductance regulator (CFTR) protein that leads to abnormal electrolyte transport in epithelial cells. Although much research has been dedicated to elucidating exactly how the CF electrolyte transport defect leads to persistent lung infection and inflammation, the mechanism is not fully resolved.

The electrolyte composition of airway surface liquid (ASL) in healthy individuals and CF subjects has been difficult to determine and has been a source of recent controversy (16). Initial studies reported that bronchial chloride concentrations were increased in CF subjects compared with subjects with chronic bronchitis (170 ± 79 mM and 85 ± 54 mM, respectively) (14). Similar findings were reported by Joris and colleagues (21) in their study of ASL [Cl]− in CF patients (129 ± 5 mM) and normal subjects (84 ± 9 mM). A similar increase in [Na+] was found in CF subjects (121 ± 4 mM) compared with control subjects (82 ± 6 mM). In addition, in a bronchial epithelial cell xenograft model in nu/nu mice, Goldman and colleagues (15) found that xenografts of CF bronchial epithelial cells produced abnormally high [Na+] and [Cl]− levels ([Na+] = 172 ± 9 mM; [Cl−] = 178 ± 9 mM) compared with normal grafts ([Na+] = 83 ± 3 mM; [Cl−] = 83 ± 3 mM). However, other studies have failed to detect significant differences in electrolyte concentrations of ASL obtained from normal, CF, and chronic bronchitis subjects (20, 23).

Given the findings suggesting that electrolyte concentrations in the ASL of patients with CF are hypertonic with respect to serum concentration as well as with respect to the ASL of normal subjects, we were intrigued by the possible role that differences in Na+ and Cl− concentrations may play in the early, exuberant inflammatory response in CF and, in particular, their effects on macrophage functions. Previous studies...
have shown that different cell types respond to elevated levels of NaCl and the ensuing hyperosmolar effect by activating members of the mitogen-activated protein kinase (MAPK) family, including p38mapk, p46nk (c-jun NH2-terminal kinase), and p54nk isoforms (13). Activation of these serine/threonine kinases has been shown to contribute to protective stress responses as well as to induce apoptosis in cells stressed by exposure to hyperosmosality (12, 25, 39). In addition, Shapiro and Dinarello (32) have shown that macrophages respond to hyperosmosality by expressing and secreting increased levels of proinflammatory cytokines, including tumor necrosis factor (TNF)-α and IL-8. These findings thus provide important clues that elevated NaCl or hyperosmosality in general may contribute to changes in macrophage function in the airways of patients with CF. To address this question, we investigated the effect of hyperosmosality on the activation of MAPKs and Akt and on the induction of macrophage apoptosis in primary cultures of mouse macrophages and in the human monocyte-like cell line THP-1. As we will show, hyperosmolality enhanced the activation of p38mapk p46nk and p54nk isoforms induced by the proinflammatory cytokine TNF-α but inhibited the activation of p42mapk/erk2 (extracellular signal-related kinase) and Akt. In addition, these conditions potentiated macrophage apoptosis.

MATERIALS AND METHODS

Materials. C3H/HeJ mice were bred at the National Jewish Center Biological Resource Center (Denver, CO) and were used throughout the study to avoid the possibility of stimulation by trace amounts of endotoxin contaminants (28). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Whittaker Bioproducts (Walkersville, MD), and fetal bovine serum (FBS) was obtained from Irvine Scientific (Santa Ana, CA). The human monocyte-like cell line THP-1 was obtained from American Type Culture Collection (Rockville, MD). Recombinant c-Jun1 (C-20), and anti-p42mapk/erk2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or Upstate Biotechnology (Lake Placid, NY). Donkey anti-rabbit and sheep anti-mouse IgG F(ab’)2 horseradish peroxidase-conjugated antibodies, [γ-32P]ATP, and enhanced chemiluminescence kits were purchased from Amersham Life Science (Arlington Heights, IL). Recombinant mouse TNF-α and apoptosis detection kits were obtained from R&D Systems (Minneapolis, MN), and the in situ cell death detection kit was obtained from Roche Molecular Biochemicals (Indianapolis, IN).

Macrophage isolation and culture. Bone marrow-derived macrophages were cultured from femoral and tibial bone marrow, as previously described (30). The growth medium was DMEM containing 100 U/ml penicillin, 100 µg/ml streptomycin, 10% (vol/vol) heat-inactivated FBS, and 10% (vol/vol) L929 cell-conditioned medium (as a source of colony stimulating factor-1). Bone marrow cells were cultured at a density of 2.4 × 105 cells/cm2 at 37°C in a 10% CO2 atmosphere for 5–6 days. One hour before stimulation, one-half of the medium was removed and centrifuged at 1,000 rpm for 10 min to remove any nonadherent cells. NaCl, sorbitol, and/or TNF-α were added at twice the concentrations indicated. The conditioned media were then added back to the macrophage monolayers at the time of stimulation. This method of stimulation was used to avoid the stimulation of MAPKs that occurs upon transfer to fresh medium. THP-1 cells were cultured in RPMI 1640 containing 10% (vol/vol) heat-inactivated fetal calf serum and 1% (wt/vol) glucose, 100 U/ml penicillin, and 100 µg/ml streptomycin and were maintained at densities between 0.2–1 × 106 cells/ml. The cells were split every 3–4 days.

In vitro kinase assays. JNK activity was measured in lysates of mouse macrophages as previously described (8). GST-c-Jun1 (C-20) was bound to sepharose beads as the affinity matrix and substrate for phosphorylation in the presence of [γ-32P]ATP. Kinase activities of p38mapk, p42mapk/erk2, and p44mapk/erk1 were measured by immunoprecipitation with the appropriate antibody followed by in vitro kinase assays using recombinant activating transcription factor-2 as substrate in the presence of [γ-32P]ATP as previously described (37). All reactions were terminated by the addition of 2× Laemmli sample buffer containing 20 mM dithiothreitol (DTT) and were boiled for 5 min. The proteins were then separated by SDS-PAGE on 12% gels and transferred to nitrocellulose membranes. The 32P-labeled substrates were detected by autoradiography.

Western blot analysis. Macrophage monolayers were lysed on ice with 300 µl of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 5 µg/ml aprotinin, and 1 mM Na3VO4) at 4°C, the insoluble material was removed by centrifugation at 14,000 rpm for 10 min, and protein was equalized in each sample using the bicinchoninic acid method (29). To each sample, 5× Laemmli sample buffer containing 20 mM dithiothreitol and were boiled for 5 min. Samples were separated by SDS-PAGE on 12% gels and were transferred onto nitrocellulose membranes. The blots were washed in Tris-Tween-buffered saline [20 mM Tris, pH 7.6, containing 137 mM NaCl and 0.05% (vol/vol) Tween] and blocked with 5% (wt/vol) dry milk dissolved in Tris-Tween-buffered saline for 1 to 2 h. The membranes were probed overnight at 4°C with phosphospecific 44mapk/erk1, 42mapk/erk2, and 38mapk, or JNK antibodies, or nonphosphospecific 42mapk/erk2, 38mapk, or JNK1 antibodies in 5% (wt/vol) bovine serum albumin and 0.02% (wt/vol) sodium azide as described (36). Phosphorylation of Akt at Ser473 was detected by immunoprecipitation of total Akt from lysates of ~2 × 107 bone marrow macrophages or THP-1 cells as described (37), followed by Western blotting with anti-phospho-Ser473 Akt antibody. In some experiments, Ser473 phosphorylated Akt was detected directly by SDS-PAGE of macrophage whole cell lysates, followed by Western blotting as described above. Bound antibody was detected with antirabbit or anti-mouse IgG F(ab’)2 horseradish peroxidase-conjugated antibody as the secondary antibody for 1 h. The enhanced chemiluminescence method was used to detect bound conjugated secondary antibody.

Quantification of apoptosis. Macrophage monolayers were grown on bacteriological grade petri dishes and stimulated as described in RESULTS for 5 h. Cells were then removed from the culture plates using a gentle jet technique so that cells would not be damaged or unnecessarily disrupted. In all experiments, an unstimulated negative control and an ani-
sonycin-positive control for apoptosis were used. Cells were treated as described in the apoptosis detection kit and as previously reported by Bratton et al. (6). Briefly, the cells were centrifuged at 1,000 rpm for 10 min at 4°C, washed twice with 5 ml of cold 1× Dulbecco’s PBS, and suspended in 1× binding buffer at a concentration of 1 × 10^6 cells/ml. One hundred microliters of cells were transferred to 5-ml culture tubes and stained with 100 ng of fluorescein-conjugated annexin V and 500 ng of propidium iodide reagent. The reactions were gently vortexed and allowed to incubate for 15 min at room temperature. Each sample was given 400 μl of binding buffer, and samples were analyzed by flow cytometry. Analysis was done on a Becton Dickinson (San Jose, CA) FACScalibur flow cytometer, and the results were analyzed with PC Lysis software (Becton Dickinson). Annexin-positive cells were determined as described in the apoptosis detection kit by setting quadrants to separate viable cells from propidium iodide-permeant cells and nonapoptotic cells from the staining highly for the FITC-labeled annexin V probe. Percent apoptosis was determined from the cells staining greater than the control population threshold.

To confirm the apoptosis seen above, cells were also studied using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay to detect fragmented DNA. Bone marrow-derived macrophages grown on glass coverslips in 12-well plates were stimulated as described in DNA. Bone marrow-derived macrophages grown on glass coverslips in 12-well plates were stimulated as described in the results for 24 h. Macrophages were washed with PBS, fixed for 15 min at room temperature in a solution containing 3% (wt/vol) paraformaldehyde and 3% (wt/vol) sucrose in PBS (pH 7.5), washed again, and permeabilized with 0.2% (vol/vol) Triton X-100 for 10 min. Cells were then incubated with terminal transferase reaction solution containing fluorescein-conjugated dUTP for 1 h at 37°C as recommended by the manufacturer (Roche Diagnostics, Indianapolis, IN). The cells were washed three times with 0.03 M sodium citrate, pH 7.4, containing 0.3 M sodium chloride to remove unbound nucleotides and were then washed with PBS. DNA was stained with Hoechst-33342 at 10 μg/ml. After being washed with PBS, the coverslips were incubated overnight in PBS supplemented with 0.02% sodium azide and mounted in a solution containing 90% glycerol, 10% Tris-HCl, pH 8.5, and 20 mg/ml o-phenylenediamine as an antifading agent. Cells were observed with a Leica DMRXA confocal immunofluorescence microscope using a ×100 plan objective. The percentage of TUNEL-positive cells was determined by counting three times at least 200 cells with a confocal microscope.

Osmolality and electrolyte measurement. Osmolality was measured by freezing-point depression using an Advanced Micro-Osmometer (model 3MO; Advanced Instruments, Norwood, MA). Sodium and chloride concentrations were quantitated by ion-selective electrodes using a Beckman CX3 automated chemistry analyzer (Brea, CA). The osmolality, sodium, and chloride measurements of the media with NaCl or sorbitol added are shown in Table 1.

Statistical analysis. The apoptosis data are shown as means ± SE. Results were analyzed using a nonparametric repeated measures analysis of variance to compare different conditions. Statistics were calculated using SAS version 6.12 (Cary, NC). P < 0.05 was considered significant.

RESULTS

Hyperosmolar activation of JNK and p38<sub>mapk</sub>, but not ERK, in mouse macrophages. To investigate the effect of the addition of NaCl on MAPK activation, we used in vitro kinase assays to quantify the activity of JNK, p38<sub>mapk</sub>, p42<sub>mapk</sub>/erk<sub>2</sub>, and p44<sub>mapk</sub>/erk<sub>1</sub>. Macrophage monolayers either were left unstimulated or were stimulated with media containing increasing concentrations of NaCl (0–200 mM) for 10 min and were then lysed as described in MATERIALS AND METHODS. We also conducted experiments in which cell lysates from unstimulated and NaCl-exposed macrophages were analyzed by Western blotting using phosphospecific antibodies for JNK, p38<sub>mapk</sub> or ERK. The same blots were then probed with the respective nonphosphospecific antibodies to control for equal loading. Preliminary experiments established that 10 min was the optimal time point of MAPK activation. As can be seen in Fig. 1A, after stimulation with NaCl, JNK activity was increased as detected using both the in vitro kinase assay and Western blot analysis. JNK activity was stimulated in response to increasing concentrations of NaCl and peaked at 200 mM. Figure 1B illustrates the results of the in vitro kinase assay and Western blot analyses for p38<sub>mapk</sub>, which also showed increased kinase activity first seen at 50 mM NaCl and peaking at 200 mM NaCl. In the corresponding Western blot using phosphospecific p38<sub>mapk</sub> antibody, maximal stimulation was seen at 200 mM NaCl. The effect of NaCl on p42<sub>mapk</sub>/erk<sub>2</sub> activity is illustrated in Fig. 1C. In contrast to the effects of NaCl on JNK and p38<sub>mapk</sub>, there was no activation of p42<sub>mapk</sub>/erk<sub>2</sub> or p44<sub>mapk</sub>/erk<sub>1</sub> above baseline in response to NaCl in either the kinase assay or Western blots using phosphospecific anti-p42/ p44<sub>mapk</sub> antibody. Thus increasing concentrations of NaCl stimulate JNK and p38<sub>mapk</sub> but do not stimulate p42<sub>mapk</sub>/erk<sub>2</sub> or p44<sub>mapk</sub>/erk<sub>1</sub> activity.

Based on the results shown above, we questioned whether the observed pattern of MAPK activation was due to a hyperosmolar effect of NaCl. Accordingly, macrophages were exposed to increasing concentrations of sorbitol (0–400 mM), yielding osmolalities of 320–780 mosmol/kg H2O, for 10 min. MAPK activity was quantitated with in vitro kinase assays and Western blot analysis using phosphospecific antibodies for each MAPK. As shown in Fig. 2A, JNK activity, as detected by in vitro kinase assay and Western blot analysis, was stimulated with increasing concentrations of sorbitol beginning at 200 mM and reaching a maximum at 400 mM. The effect of sorbitol on p38<sub>mapk</sub> activation, as detected by in vitro kinase assay and Western blot analysis, is illustrated in Fig. 2B and demonstrates increased p38<sub>mapk</sub> kinase activity with increasing concentrations of sorbitol beginning at 100 mM and reach-

<table>
<thead>
<tr>
<th>Media + Sorbitol</th>
<th>Media + NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbitol (mM)</td>
<td>Final Media mosmol/kg H2O</td>
</tr>
<tr>
<td>0</td>
<td>319.8</td>
</tr>
<tr>
<td>25</td>
<td>349.5</td>
</tr>
<tr>
<td>50</td>
<td>352.5</td>
</tr>
<tr>
<td>100</td>
<td>417.0</td>
</tr>
<tr>
<td>200</td>
<td>541.8</td>
</tr>
<tr>
<td>400</td>
<td>780.5</td>
</tr>
</tbody>
</table>

Table 1. Osmolality and sodium chloride measurements of media
The activation of p38 mapk by sorbitol is also reflected in the Western blot using phosphospecific p38 mapk antibody. The effect of sorbitol on ERK activity is shown in Fig. 2C. As seen with NaCl, there was no activation of p42 mapk or p44 mapk above baseline at any concentration of sorbitol tested using either the kinase assay or Western blot analysis. Thus increasing sorbitol concentrations activated p38mapk and JNK and had no effect on p42mapk or p44mapk activation, suggesting a more general response of the cells to hyperosmolarity, as opposed to a specific response to NaCl.

**MAPK activation by hyperosmolarity and TNF-α.** In addition to the possibility of exposure to ASL containing increased amounts of NaCl in CF compared with normal ASL, macrophages and other cell types are also exposed to a multitude of inflammatory and other stimuli, including TNF-α, in the airways of patients with CF. We therefore questioned whether costimulation of macrophages with TNF-α under hyperosmolar conditions would affect MAPK signaling. Macrophage monolayers were stimulated with a fixed concentration of TNF-α (1 ng/ml) in media containing increasing concentrations of NaCl (0–200 mM) for 10 min. MAPK activity was quantified by in vitro kinase assays and Western blot analysis using phosphospecific antibodies for each MAPK. As shown in Fig. 3A and as previously reported (8, 27), TNF-α alone activated JNK, whereas the addition of NaCl increased phosphorylation of JNK beginning at 100 mM and peaking at 200 mM. Figure 3B shows that the TNF-α-induced p38mapk activation
was also modestly increased by the addition of NaCl. p42mapk/erk2 activity is shown in Fig. 3C under the same conditions as described above. TNF-α alone activated p42mapk/erk2; however, unlike the potentiation of JNK and p38mapk activation, the addition of NaCl markedly diminished the activity of p42mapk/erk2. p42mapk/erk2 activity was decreased with as little as 50 mM NaCl in the in vitro kinase assay and continued to decrease to baseline with 200 mM NaCl (Fig. 3C). This was demonstrated by both in vitro kinase assay and Western blot using phosphospecific p42/p44mapk antibody. Thus costimulation with TNF-α and increasing concentrations of NaCl resulted in a modest increase in the activation of JNK and p38mapk and a significant inhibition of p42mapk/erk2 and p44mapk/erk2 activation.

To determine whether the changes in MAPK activity in the presence of TNF-α and NaCl were also due to an effect of hyperosmolarity, we stimulated macrophage monolayers with TNF-α (1 ng/ml) and increasing concentrations of sorbitol (0–400 mM) for 10 min. MAPK activity was quantified using in vitro kinase assays and confirmed with Western blot analysis using phosphospecific antibodies for the appropriate MAPK. As shown in Fig. 4A, JNK was activated with TNF-α alone, and activation increased with the addition of increasing concentrations of sorbitol. Figure 4B illustrates the activity of p38mapk when stimulated with TNF-α plus increasing concentrations of sorbitol. Again, TNF-α alone activated p38mapk, and the activity

Fig. 3. Costimulation with NaCl (0–200 mM) and tumor necrosis factor-α (TNF-α; 1 ng/ml) added to media on macrophage MAPK activity. MAPK activation included JNK (A), p38mapk (B), and ERK (C). Activity was assessed with kinase assays and Western blot analysis using phosphospecific and nonphosphospecific antibodies as described in MATERIALS AND METHODS. Examples shown are representative of at least 3 independent experiments with different macrophage samples.

Fig. 4. Costimulation of macrophages with sorbitol (0–400 mM) and TNF-α (1 ng/ml) added to media on MAPK activity including JNK (A), p38mapk (B), and ERK (C). Activity was assessed with kinase assays and Western blot analysis using phosphospecific and nonphosphospecific antibodies as described in MATERIALS AND METHODS. Examples shown are representative of at least 3 independent experiments with different macrophage samples.
was modestly increased with increasing concentrations of sorbitol. The effect of sorbitol on p42mapk/erk2 activity in response to TNF-α is shown in Fig. 4C. The kinase assay and Western blot demonstrate that TNF-α alone activated p42mapk/erk2, and the addition of increasing concentrations of sorbitol decreased ERK activity. The inhibition of p42mapk/erk2 activation began at 100 mM sorbitol and declined to baseline at 400 mM. Thus costimulation of macrophages with TNF-α and increasing concentrations of sorbitol resulted in the activation of JNK and p38mapk while inhibiting the activation of p42mapk/erk2, suggesting that the changes in MAPK activation are a response to hyperosmolarity.

**Hyperosmolarity inhibits the activation of Akt.** An important function of p42mapk/erk2 is in the control of cell proliferation and in cell survival responses. In view of the striking inhibitory effect of hyperosmolarity on the activation of p42mapk/erk2, we next questioned whether the inhibitory effect of hyperosmolarity also extends to another prosurvival protein kinase signaling pathway, namely the activation of Akt (9). Macrophage monolayers were stimulated with TNF-α (10 ng/ml) in either the presence or absence of 100 mM NaCl for 10 min before lysis and analysis of Akt activation by Western blotting with a phosphospecific antibody directed at Ser473. As can be seen in Fig. 5A, Akt was basally phosphorylated at low levels in mouse macrophages, consistent with the presence of growth factors in the culture medium, whereas stimulation with TNF-α markedly increased phosphorylation of Akt at Ser473. However, under hyperosmolar conditions induced by 100 mM NaCl, the phosphorylation of Akt was reduced to basal levels (Fig. 5A). Figure 5B shows a control experiment confirming the inhibitory effect of hyperosmolarity on the phosphorylation of p42mapk/erk2. We also observed an inhibition of Akt phosphorylation in the human embryonic epithelial cell line HEK-293 (data not shown), suggesting that epithelial cells may behave similarly to macrophages in this respect. Thus hyperosmolarity inhibits the activation of the survival pathways involving both p42mapk/erk2 and Akt while potentiating the activation of the stress-responsive pathways involving JNK and p38mapk.

**Effect of hyperosmolarity and TNF-α on macrophage apoptosis.** Based on previous studies associating the activation of JNK and p38mapk and the inhibition of ERK with apoptosis (39), we investigated the effect of TNF-α and hyperosmolarity induced by NaCl on the induction of macrophage apoptosis. Mouse macrophage monolayers were exposed to 1 ng/ml of TNF-α and 100 mM NaCl, either alone or combined, for 5 h. The level of apoptosis was then quantified by flow cytometry after staining with propidium iodide and annexin V. Figure 6A illustrates the effect of various conditions on apoptosis. Cells exposed to normal media had a baseline rate of apoptosis of 12.6 ± 1.2%, and when exposed to anisomycin, a positive control, apoptosis was induced in 50.6 ± 2.4% of cells (P < 0.001). The addition of TNF-α alone or hyperosmolarity alone did not significantly increase the degree of apoptosis compared with normal media (TNF-α 12.8 ± 1.7%, P = 0.88; NaCl 18.3 ± 2.3%, P = 0.08). However, the combination of TNF-α and hyperosmolarity induced apoptosis in 23.1 ± 3.3% of macrophages (P = 0.007). These studies were reproduced using the same conditions with mouse bone marrow-derived macrophages exposed to 1 ng/ml of TNF-α and hyperosmolarity induced with 100 mM NaCl alone or together for 24 h when apoptosis was detected using a TUNEL assay. Figure 6B demonstrates the effects of these conditions on apoptosis using this method. In cells exposed to normal media, apoptosis was undetectable, but after exposure to anisomycin, apoptosis was detected in 67.5 ± 16.4% of the cells (P < 0.001). Stimulation with TNF-α alone did not result in any significant increase in apoptosis above unstimulated levels (2.4 ± 1.1%, P = 0.13). However, hyperosmolarity alone resulted in a significant apoptotic response (8.4 ± 2.7%, P = 0.003), although the macrophages had an even greater rate of apoptosis when exposed to TNF-α under hyperosmolar conditions (23.9 ± 4.7%, P = 0.003). Thus exposure of macrophages to TNF-α under hyperosmolar conditions potentiates apoptosis by both methods of detection.

**Involvement of both p42mapk/erk2 and Akt in protection against apoptosis.** In view of these findings, we next addressed the role of p42mapk/erk2 and Akt in the protection against apoptosis. Because both kinase cascades promote distinct survival responses in different cell systems, we considered it possible that either or
was detected by staining with annexin V and propidium iodide (see MATERIALS AND METHODS). Columns represent percent apoptosis; n = 3. Values represent means ± SE.

![Graph](image.png)

**Fig. 6.** Macrophage apoptosis after exposure to normal media, 1 ng/ml of TNF-α, 100 mM NaCl, 1 ng/ml of TNF-α and 100 mM NaCl, or anisomycin added to media. A: macrophage apoptosis was increased after exposure to TNF-α and NaCl. Macrophage apoptosis was detected by staining with annexin V and propidium iodide (see MATERIALS AND METHODS). Columns represent percent apoptosis; n = 11. B: macrophage apoptosis was increased after exposure to TNF-α and/or NaCl. Macrophage apoptosis was detected by TdT-mediated dUTP nick end labeling (TUNEL) assay (see MATERIALS AND METHODS). Columns represent percent apoptosis; n = 3. Values represent means ± SE.

both kinase cascades may be involved in the protection against apoptosis after stimulation with TNF-α. Thus we reasoned that pharmacological inhibition of either pathway alone or both pathways together would mimic the effects of hyperosmolarity on macrophage apoptosis. Mouse bone marrow-derived macrophages were incubated with PD-98059, a MEK1 inhibitor, LY-294002, a phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor, or both inhibitors to block the activation of ERK, Akt, or both pathways, respectively. The cells were then left unstimulated or were stimulated with TNF-α (10 ng/ml) in the presence and absence of the inhibitors for 18 h before quantifying the degree of apoptosis by TUNEL assay. As shown in Fig. 7A, in the absence of TNF-α, incubation in PD-98059 or LY-294002 each modestly augmented the basal level of apoptosis, whereas the effect of coincubation with both inhibitors resulted in an additive increase in the basal level of apoptosis. These findings suggest that both pathways may exert modest prosurvival effects, likely as a result of the low level of activation of these pathways seen in unstimulated conditions (Fig. 5). Incubation with PD-98059 or LY-294002 in the presence of TNF-α led to an augmentation in apoptosis compared with the level seen in the absence of either inhibitor. However, coincubation with both PD-98059 and LY-294002 in the presence of TNF-α led to a synergistic increase in the degree of apoptosis to a level that was comparable with that in cells incubated with TNF-α under hyperosmolar conditions (Fig. 7A), indicating that both ERK and Akt combine to exert a protective effect against TNF-α-induced apoptosis.

We also determined whether pharmacological inhibition of ERK and Akt activation likewise mimicked the effect of hyperosmolarity by potentiating TNF-α-induced apoptosis in the human monocyte-like cell line THP-1. THP-1 cells were incubated with medium alone or TNF-α (10 ng/ml) in the presence and absence of PD-98059, LY-294002, or the combination of both inhibitors for 18 h before flow cytometric quantification of the degree of apoptosis by annexin V and propidium iodide staining. As shown in Fig. 7B, incubation with PD-98059 alone had no effect on the baseline level of apoptosis. Incubation in LY-294002 alone modestly potentiated baseline apoptosis consistent with the constitutive activation of Akt observed in THP-1 cells (Fig. 7C). Combined incubation with PD-98059 and LY-294002 induced a further increment in apoptosis. As also shown in Fig. 7B, the level of apoptosis seen when THP-1 cells were stimulated with TNF-α in the presence of PD-98059 or LY-294002 alone was similar to that seen in the absence of TNF-α. However, the degree of apoptosis was markedly augmented when the THP-1 cells were stimulated with TNF-α in the presence of both PD-98059 or LY-294002, and, like the bone marrow-derived macrophages, was similar to the level of apoptosis seen when these cells were incubated with TNF-α under hyperosmolar conditions. The inhibitory effects of NaCl on the phosphorylation of p42mapk/erk2 and Akt in THP-1 cells are shown in Fig. 7C. Collectively, these findings suggest that both p42mapk/erk2 and Akt contribute to the protection against apoptosis induced by TNF-α.

**DISCUSSION**

CF is the most common inherited fatal disease of Caucasians in the United States, with >90% of patients dying from progressive and unrelenting cycles of airway inflammation, infection, mucus plugging, and bronchiectasis. However, while much has been learned about the pathogenesis of pulmonary inflammation and infection, the precise mechanism(s) that connects the genetic mutations in the CFTR to fulminant lung disease is not well understood (11, 19). Until 1995, conventional dogma supported the view that the in-
tense neutrophil recruitment and accumulation in the distal airways in CF arose primarily in response to colonization with common CF pathogens, especially *Pseudomonas aeruginosa* (24). However, previous studies from this and other laboratories have shown that pulmonary inflammation, characterized by increased levels of IL-8, neutrophils, and neutrophil secretory products, is established early in life and can be detected in bronchoalveolar lavage fluid from infants with CF as young as 4 wk of age (4, 22). These findings have recently been substantiated in a pathogen-free, fetal tracheal xenograft model in *scid* mice. Tirouvanziam et al. (35) found that approximately eightfold higher levels of IL-8 accumulated in the luminal fluid of xenografts from fetuses bearing mutations in CFTR compared with non-CF fetal tracheal xenografts. In addition, murine neutrophils were found to accumulate in higher numbers in the lamina propria of CF tracheal xenografts compared with non-CF xenografts. Collectively, these studies suggest that abnormalities in the function of CFTR are involved in early airway inflammation in CF and that inflammation can arise in the apparent absence of detectable infection. These observations thus raise the question of how the inflammatory response is both initiated and perpetuated in CF.

Characterization of the composition of ASL has led to two theories regarding the pathogenesis of lung disease in CF. One hypothesis, originating from Michael Welsh’s group in Iowa, proposes that the ASL in CF has an increased NaCl content compared with normal ASL and has been referred to as the “high-salt hypothesis” (33). The other hypothesis, proposed by Richard Boucher’s group in North Carolina, proposes that CF ASL has a reduced volume compared with normal ASL and has often been referred to as the “low-volume hypothesis” (23, 26). In the present study, we investigated the effect of hyperosmolarity induced by NaCl on both the signaling responses of macrophages to the proinflammatory cytokine TNF-α and on macrophage survival in the presence of TNF-α. The principal findings of this study are that 1) the pattern of MAPK and Akt phosphorylation and activation, induced by TNF-α, is altered by hyperosmolarity and that 2) the changes in MAPK and Akt activation are accompanied by an increase in macrophage apoptosis. Previous studies have shown that p42mapk/erk2, p38mapk, p46 jnk, and p54jnk isoforms are rapidly and simultaneously activated in mouse macrophages in response to TNF-α (8, 37, 38). The most striking change in MAPK and Akt activation observed in the present study was the hyperosmolarity-dependent inhibition of the phosphorylation and activation of both kinases. These changes in kinase activation were also seen when macrophages were stimulated in the presence of sorbitol, suggesting that the alteration in activation was not a response to NaCl per se but represented a more generalized response to hyperosmolarity. Importantly, while the activation of p38mapk, p46 jnk, and p54 jnk was induced only at relatively high concentrations of NaCl, the inhibition of p42mapk/erk2 and Akt activation was seen at

---

**Fig. 7.** Pharmacological inhibition of the activation of p42mapk/erk2 and Akt with PD-98059 and LY-294002, respectively, augments apoptosis of mouse macrophages and the monocyte-like cell line THP-1 in the presence of TNF-α. A: mouse macrophages were stimulated as indicated for 18 h before quantification of the level of apoptosis by TUNEL assay. Data represent means ± SE of 3 independent experiments. B: THP-1 cells were stimulated as indicated for 18 h, and the level of apoptosis was determined by flow cytometry after staining with annexin V and propidium iodide. C: effect of NaCl on the constitutive and TNF-α-dependent phosphorylation of p42mapk/erk and Akt in THP-1 cells as determined by Western blotting with phosphospecific p42mapk/erk2 and Akt antibodies. Data in B and C are representative of at least 3 independent experiments.
concentrations of NaCl similar to those that have been reported in the ASL of patients with CF.

A growing body of literature supports the conclusion that hyperosmolarity exerts significant effects on cell function and on the activity of innate host defense systems. Studies reported by Smith and colleagues (33) provided the first demonstration that increases in the concentration of NaCl inhibited the ability of a β-defensin activity to kill P. aeruginosa when exposed both in vitro and in cultures of airway epithelial cells. Goldman et al. (15) subsequently cloned human β-defensin-1 and confirmed its exquisite sensitivity to NaCl. In addition, using human bronchial epithelial cell xenografts in nu/nu mice, they were able to demonstrate impaired killing of P. aeruginosa in cells isolated from patients with CF. Other studies have also shown that cultured human bronchial gland epithelial cells isolated from a CF patient bearing the ΔF508 mutation secrete increased amounts of IL-8 compared with non-CF cells in response to NaCl (34). The results from in vitro studies have also provided insights into how hyperosmolar conditions are sensed. Based on earlier work on the yeast-osmosensing gene HOG1, Han et al. (17) cloned the mammalian homolog, p38\textsuperscript{mapk} and showed it to be activated in response to exposure of macrophages to hyperosmotic stress. Similarly, the p46\textsuperscript{jnk} and p54\textsuperscript{jnk} isoforms have been shown to be activated in response to hyperosmotic stress in a variety of cell types (13). An additional study also suggested that the activation of JNK after exposure to hyperosmolarity was associated with clustering and internalization of the cell surface receptors for epidermal growth factor, TNF, and IL-1 (31). Last, pharmacological inhibitor studies conducted by Hashimoto et al. (18) and Shapiro and Dinarello (32) have shown that the induction of IL-8 production in response to hyperosmotic stress is dependent on the activation of p38\textsuperscript{mapk}. Thus increased concentrations of NaCl in the ASL of patients with CF compared with non-CF subjects might be expected to have significant effects on the innate inflammatory response in both the presence and absence of colonization with common CF pathogens.

The results of the present study have also provided insight into a potential mechanism that may also contribute to the high numbers of neutrophils that are seen in CF pathogen-colonized adolescent patients and adults with CF as well as in infants, namely decreased neutrophil clearance as a consequence of the induction of macrophage apoptosis in the presence of a proinflammatory stimulus (TNF-α) and increased levels of NaCl resulting in conditions of hyperosmolarity. Hyperosmolarity alone was not sufficient to induce macrophage apoptosis as has previously been reported in neutrophils (2, 12). Similarly, exposure to TNF-α alone was found to be ineffective at inducing macrophage apoptosis. The results of the present study clearly implicate the downregulation in ERK and Akt activation as contributing to the increase in apoptosis seen when macrophages interact with TNF-α under hyperosmolar conditions. Previous studies have shown that growth factor withdrawal from rat PC-12 pheochromocytoma cells is accompanied by a sustained activation of the JNK and p38\textsuperscript{mapk} pathways and a concurrent inhibition of p42\textsuperscript{mapk}/erk2 activation (39). This pattern of divergence in MAPK activation pathways, which is analogous to the results of the present study, also led to the induction of apoptosis of PC-12 cells (39). Similar results have also been found in human Jurkat T lymphocytes in which activation of JNK and p38\textsuperscript{mapk} in the absence of p42\textsuperscript{mapk}/erk2 activation also resulted in apoptosis (25). Likewise, inhibition of Akt activation either by pharmacological inhibition of PI 3-kinase or with dominant inhibitory Akt mutants has been shown to block growth factor-mediated survival responses via the phosphorylation-dependent inhibition of the proapoptotic activity of Bad (10) and by blocking Fas ligand expression via the phosphorylation of the forkhead transcription family member FKHR1 (7). Similarly, Berra and colleagues (5) have shown that combined inhibition of both p42\textsuperscript{mapk}/erk2 and Akt promotes apoptosis in HeLa cells through effects on the activation of p38\textsuperscript{mapk}. Thus since both TNF-α and NaCl are believed to be present at elevated concentrations in the ASL of the CF airway, it is tempting to speculate that the excessive burden of neutrophils may be partially due to apoptosis of airway macrophages, thereby depriving neutrophils of a major clearance pathway.

In summary, the present study demonstrates that exposure of mouse macrophages to TNF-α in the presence of increasing NaCl leads to an activation of p46\textsuperscript{jnk}, p54\textsuperscript{jnk}, and p38\textsuperscript{mapk} isoforms and a concomitant inhibition of the activation of p42\textsuperscript{mapk}/erk2 and Akt. Under these conditions, macrophages were also induced to undergo apoptosis via the inhibitory effects of hyperosmolality on the activation of p42\textsuperscript{mapk}/erk2 and Akt. We speculate that this may partially explain the persistence of inflammation in CF through impairment of an important mechanism for the clearance of neutrophils.

The authors thank Linda Remigio and Cheryl Leu for excellent technical assistance and Marci Sontag for statistical support. This work was supported by a Cystic Fibrosis Foundation Pilot and Feasibility Project grant within the University of Colorado Research and Development Program in Cystic Fibrosis and by National Heart, Lung, and Blood Institute Public Health Service Grant HL-65326. G. S. Kerby was supported by a Harry Shwachman Cystic Fibrosis Clinical Investigator Award from the Cystic Fibrosis Foundation. V. Cottin was supported, in part, by a traveling fellowship from the Société de Pneumologie de Langue Française, the Association pour la Recherche contre le Cancer Fondation Alain Philippe, Fondation Lavoisier du Ministère des Affaires Etrangères, and a Michael and Eleanor Stobin 1999 Pediatric Fellowship from National Jewish Medical and Research Center, Denver, CO. This work was also supported by National Center for Research Resources Grant MO1-RR00069 and the Mike McMorris Cystic Fibrosis Center.

REFERENCES

2. Aoshiba K, Yasui S, Hayashi M, Tamaoki J, and Nagai A. Role of p38 mitogen-activated protein kinase in spontaneous