Mechanisms of TNF-α stimulation of amiloride-sensitive sodium transport across alveolar epithelium

NORIMASA FUKUDA, CHRISTIAN JAYR, AHMED LAZRAK, YIBING WANG, RUDOLF LUCAS, SADIS MATALON, and MICHAEL A. MATTHAY

1Cardiovascular Research Institute, University of California, San Francisco, California 94143-0130; 2Department of Anesthesiology, Physiology, and Biophysics, University of Alabama, Birmingham, Alabama 35223; and 3Department of Biological Chemistry, Weizmann Institute of Science, 76100 Rehovot, Israel

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Fukuda, Norimasa, Christian Jayr, Ahmed Lazrak, Yibing Wang, Rudolf Lucas, Sadis Matalon, and Michael A. Matthay. Mechanisms of TNF-α stimulation of amiloride-sensitive sodium transport across alveolar epithelium. Am J Physiol Lung Cell Mol Physiol 280:L1258–L1265, 2001.—Because tumor necrosis factor (TNF)-α can upregulate alveolar fluid clearance (AFC) in pneumonia or septic peritonitis, the mechanisms responsible for the TNF-α-mediated increase in epithelial fluid transport were studied. In rats, 5 μg of TNF-α in the alveolar instillate increased AFC by 67%. This increase was inhibited by amiloride but not by propranolol. We also tested a triple-mutant TNF-α that is deficient in the lectinlike tip portion of the molecule responsible for its membrane conductance effect; the mutant also has decreased binding affinity to both TNF-α receptors. The triple-mutant TNF-α did not increase AFC. Perfusion of human A549 cells, patched in the whole cell mode, with TNF-α (120 ng/ml) resulted in a sustained increase in Na⁺ currents from 82 ± 9 to 549 ± 146 pA (P < 0.005; n = 6). The TNF-α-elicited Na⁺ current was inhibited by amiloride, and there was no change when A549 cells were perfused with the triple-mutant TNF-α or after preincubation with blocking antibodies to the two TNF-α receptors before perfusion with TNF-α. In conclusion, although TNF-α can initiate acute inflammation and edema formation in the lung, TNF-α can also increase AFC by an amiloride-sensitive, cAMP-independent mechanism that enhances the resolution of alveolar edema in physiological conditions by either binding to its receptors or activating Na⁺ channels by means of its lectinlike domain.

To investigate the mechanisms by which TNF-α upregulates AFC, we carried out both in vivo and isolated cell studies. First, using intact rats, we determined whether intratracheal instillation of TNF-α increased AFC by amiloride-sensitive mechanisms in rats and whether the TNF-α effect in rats was inhibited by a β-antagonist or accelerated by a β-agonist. Also, to gain insight into how TNF-α may upregulate AFC, we carried out experiments in rats with a triple-mutant TNF-α that lacks the lectinlike region of the molecule that is responsible for the membrane conductance-activating effect of the molecule (26, 33); the triple mutant also has a modest decrease in binding to the two known TNF-α receptors TNFRI and TNFRII (32). To elucidate the mechanisms of TNF-α action, we measured Na⁺ currents across A549 cells, a human alveolar epithelial cell line that possesses many characteristics of type II cells, including Na⁺-selective amiloride-sensitive channels (31), patched in the whole mode. These measurements were performed before and after perfusion of A549 cells with TNF-α, TNF-α plus amiloride, or the triple-mutant TNF-α and across A549 cells preincubated with blocking antibodies to TNFRI and TNFRII before perfusion with TNF-α.

Address for reprint requests and other correspondence: M. A. Matthay, Cardiovascular Research Institute, Univ. of California, 505 Parnassus Ave., HSW-825, San Francisco, CA 94143-0130 (E-mail: mmat@itsa.ucsf.edu).

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MATERIALS AND METHODS

Male Sprague-Dawley rats (n = 46; 250–350 g) were used for all animal experiments. The rats were housed in air-filtered, temperature-controlled units with food and water. All procedures were approved by the University of California, San Francisco Committee on Animal Research.

Surgical Preparation for AFC Measurements

Rats were anesthetized with pentobarbital sodium (50–100 mg/kg ip). A tracheostomy was done, and a 0.2-mm-diameter endotracheal tube (PE-240, Clay Adams, Becton Dickinson, Parsippany, NJ) was inserted. The rats were maintained in the right decubitus position and ventilated (Harvard Apparatus, Millis, MA) with 100% O2, peak airway pressure of 12–15 cmH2O, and positive end-expiratory pressure of 3 cmH2O. The respiratory rate was adjusted to maintain arterial Pco2 between 35 and 45 mmHg. A catheter was inserted into the right carotid artery to monitor systemic blood pressure and obtain blood samples. Body temperature was always kept constant at 38°C by placing the animals on a thermostatically controlled pad. This protocol was according to previous studies by Jayr et al. (27) and Rezaiguia et al. (44).

Preparation of the Instillate

A 5% isosmolar bovine serum albumin (BSA; Sigma, St. Louis, MO) solution with Ringer lactate was prepared according to previous reports (24, 30, 49). We added 1 mg of anhydrous Evans blue dye and 0.5 μCi of 125I-labeled human albumin (Merck-Frosst, Montreal, PQ) to the instillate. In some studies, 10% serum albumin (Merck-Frosst, Montreal, PQ) was used to calculate the flux of plasma protein into the vascular tracer 15 min before instillation. The vascular tracer was obtained for measurement of total protein, 125I radioactivity, and arterial blood gas determinations. We instilled 6 ml/kg of isosmolar fluid into both lungs. At the end of studies (60 min), the rats were exsanguinated and the lungs were removed through a midline sternotomy. An alveolar fluid sample (0.1–0.2 ml) was aspirated with a 3-ml syringe and Silastic tubing that was passed into a wedged position in both lungs. Total protein and radioactivity of the alveolar fluid sample were measured. The lungs were homogenized for extravascular lung water measurements and radioactivity counts.

Specific Protocols: Rat Studies

Basal alveolar fluid clearance was determined in group 1 (n = 4 rats). The effects of TNF-α (5 μg/rat; n = 7 rats) and the triple-mutant TNF-α (5 μg/rat; n = 5 rats) on AFC were studied in group 2. The effect of amiloride (10–3 M; n = 5 rats) on basal AFC and the combined effect of amiloride (10–3 M) and wild-type TNF-α (5 μg/rat; n = 6 rats) on AFC were studied in group 3. The effect of terbutaline (10–4 M; n = 5 rats) on basal AFC and the combined effect of terbutaline (10–4 M) and wild-type TNF-α (n = 5 rats) on AFC were studied in group 4. The effect of propranolol (10–3 M; n = 5 rats) on TNF-α-stimulated was studied in group 5.

Measurement of AFC

According to prior studies by our laboratory (7, 18, 27, 37, 41, 44), AFC was estimated by measuring the increase in the final concentration of the alveolar protein tracer compared with the initial instilled tracer protein concentrations. We subtracted the dry weight of the added protein in the lung water calculation from the final alveolar sample.

Patch Clamp

Cell line and culture methods. A549 cells were purchased from American Type Culture Collection (Manassas, VA) in the 76th passage. They were suspended in DMEM-F12 medium (Cellgro) supplemented with 1% penicillin-streptomycin and 10% fetal calf serum, plated on plastic tissue culture flasks, rinsed with 100% humidity. All experiments were carried out on cells between the 76th and the 97th passages.

Electrophysiology, patch-clamp recording, and analysis. Macroscopic currents were recorded from A549 cells in the whole cell recording mode of the patch-clamp technique (23).

Between 24 and 36 h before any electrophysiological measurements, the A549 cells were lifted from the tissue plates with 2.5% trypsin-EDTA (Sigma) for 3–5 min at 37°C and then seeded on 12-mm-diameter glass coverslips in DMEM-F12 medium. The coverslip was rinsed with standard external solution (SES; 310–320 mosM) just before the onset of the measurements. The composition of SES was 145 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 2.0 mM MgCl2, 5.5 mM glucose, and 10 mM HEPES, pH 7.4 (with NaOH). The cells were then transferred to the recording chamber that was mounted on the stage of an inverted microscope (IMT-2, Olympus, Tokyo, Japan) for patch-clamp recordings.

The pipettes were made from LG16-type capillary glass (Dagan, Minneapolis, MN) with a vertical puller (model PB-7, Narishige, Japan). The intrapipette solution consisted of 135 mM potassium methylsulfonic acid, 10 mM KCl, 6 mM NaCl, 1.0 mM MgATP, 2.0 mM Na2ATP, 10.0 mM HEPES, and 0.5 mM EGTA, pH 7.2 (with 1 N KOH), at 22°C (standard internal solution; 300 mosM). The pipette resistance varied from 3 to 5 MΩ when back-filled with standard internal solution. The pipette offset potential was corrected just before gigaseal formation. Series resistance and capacitance transients were compensated for with the patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA).
The junction potentials between the pipette solution and the bath solution at the tip of the pipette and between the perfusing solution and the agar bridge were corrected for as described in a prior report (5). The preparation was grounded with a Ag-AgCl electrode connected to the bath via an agar bridge (2%). The solution in the recording chamber (400 µl) was changed with a gravity-driven perfusion system.

The cell membrane potential was held at −40 mV during all whole cell recordings. Inward currents were elicited by altering the membrane potential from the holding value (−40 mV) by −100 mV for 450 ms every 5 s with the Clampex program (pCLAMP, Axon Instruments). The currents were digitized at 5 kHz with a digital-to-analog, analog-to-digital converter (DigiData 1200A, Axon Instruments), filtered through an internal four-pole Bessel filter at 2 kHz, and stored on the hard disk of a computer.

After baseline currents were recorded, A549 cells, while still patched in the whole cell mode, were perfused with SES containing either 10 or 120 ng of wild-type or triple-mutant TNF-α for 2–3 min, which resulted in a steady-state increase in inward Na+ currents. The cells were then perfused with either SES alone or SES containing 10 µM amiloride for 5–8 min. Inward Na+ currents were measured continuously throughout this period. To further investigate the potential mechanisms by which TNF-α increased Na+ currents, A549 cells, patched in the whole cell mode, were incubated with SES containing anti-human soluble (s) TNFRI and sTNFRII antibodies (5 ng each; R&D Systems) or an equivalent amount of nonspecific mouse IgG for 3 min. The specificity of the antibodies has been demonstrated (38). They were then perfused with the SES containing sTNFRI, sTNFRII, and 120 ng/ml of TNF-α. Inward Na+ currents were measured as above.

Statistics

All data are means ± SD unless otherwise noted. AFC data were analyzed by one-way ANOVA followed by Mann-Whitney U-test post hoc. Time-dependent effects were analyzed by repeated-measures ANOVA followed by Student-Newman-Keuls test post hoc. Significance was defined as P < 0.05.

RESULTS

Effect of Wild-Type TNF-α and Amiloride on AFC in Rats

Wild-type TNF-α (5 µg/rat) increased AFC by 67% (P < 0.05) over 1 h in the ventilated rats. Amiloride decreased basal AFC by 41% (P < 0.05) and prevented the TNF-α-induced upregulation of AFC (P < 0.05; Fig. 1). A lower dose of TNF-α (1 µg/rat) did not increase AFC (data not shown).

decreased basal AFC by 41% (P < 0.05) and prevented the TNF-α-induced upregulation of AFC (P < 0.05; Fig. 1). A lower dose of TNF-α (1 µg/rat) did not increase AFC (data not shown).

Effect of β-Adrenergic Stimulation and Blockade on AFC During TNF-α Instillation in Rats

Terbutaline alone increased AFC by 65% in rats (P < 0.05), similar to the effect of wild-type TNF-α. However, the combination of terbutaline and TNF-α had no additional effect on AFC (Fig. 2). Propranolol, a β-adrenergic receptor antagonist, did not decrease the TNF-α-stimulated AFC in rats (Fig. 2).

Effect of Wild-Type TNF-α and Triple-Mutant TNF-α on AFC in Rats

Wild-type TNF-α significantly increased AFC by 67% in ventilated rats (P < 0.05), but the triple-mutant TNF-α did not upregulate AFC (Fig. 3).

Patch-Clamp Experiments on A549 Cells With Wild-Type and Triple-Mutant TNF-α

Whole cell inward current was elicited by the application of −100 mV to cells patched in the whole cell...
mode and held at \(-40\) mV. Perfusion of A549 cells with 10 ng/ml of TNF-α did not increase the baseline current (data not shown). However, perfusion with 120 ng/ml of TNF-α \((n = 6\) cells\) resulted in a large increase in the inward Na\(^+\) current, starting within 30 s from the start of perfusion (Fig. 4A). This increase was sustained when the cell was reperfused with SES alone (Fig. 4A) but was completely abolished when amiloride \((10 \mu M)\) was added into the perfusion medium (Fig. 4B). On the other hand, perfusion with the triple-mutant TNF-α \((120\) ng/ml\) did not increase the inward currents (Fig. 5). Perfusion with TNF-α in the presence of two blocking antibodies to TNFRI and TNFRII failed to increase the inward current (Fig. 6). In contrast, when equiva-

![Fig. 4. A: effect of wild-type TNF-α on Na\(^+\) current in a A549 cell patched in the whole cell mode. The pipette was filled with standard internal solution (see MATERIALS AND METHODS for details). The cell was held at \(-40\) mV, and an inward current (negative current) was elicited by an application of a \(-100\)-mV pulse. The cell was perfused with either standard external solution (SES) or SES containing TNF-α \((120\) ng/ml) as indicated. B: typical experiment that was repeated 6 times with the same experimental conditions as in A except that the A549 cell was perfused with SES containing 10 \(\mu M\) amiloride after perfusion with TNF-α.

![Fig. 5. Effect of triple-mutant TNF-α on Na\(^+\) current in an A549 cell patched in the whole cell mode. The experimental conditions are described in Fig. 4A except that this A549 cell was perfused with SES containing the triple-mutant TNF-α \((120\) ng/ml\). In sharp contrast to the result in Fig. 4A, the inward Na\(^+\) current was not altered during perfusion. Results represent a typical experiment, which was repeated 6 times.](http://ajplung.physiology.org/)

The major findings of these studies can be summarized as follows. I) Wild-type TNF-α upregulated AFC in rats, a finding consistent with the prior study by Rezaiguia et al. (44) in rats with pneumonia as well as
with the recent study of sepsis (10). 2) Propranolol, a β-adrenergic antagonist, did not decrease TNF-α-stimulated AFC in rats. 3) No additional upregulation occurred with the combination of TNF-α and a β-adrenergic agonist. 4) Amiloride blocked the TNF-α-stimulated AFC in rats. 5) The triple-mutant TNF-α did not stimulate AFC in rats. 6) In patch-clamp studies of the human alveolar epithelial A549 cell line, TNF-α stimulated Na⁺ influx in A549 cells, an effect that was inhibited by amiloride. The triple-mutant TNF-α did not induce Na⁺ current in the A549 cells, and the TNFR blocking antibodies abolished the effect of TNF-α.

Because the effect of TNF-α occurred within 30 s from the onset of perfusion in the A549 cells and within 1 h from its instillation in the distal airspaces of the rat, it is apparent that the primary mechanism does not depend on a transcriptional effect of TNF-α. Because the TNF-α effect was inhibitable by amiloride in both the intact rat lung studies and the isolated human A549 epithelial cells, the primary pathway for augmented fluid transport in lung epithelium depends on Na⁺ transport. We then tried to determine whether the TNF-α effect could be mediated by an effect on membrane conductance by a receptor-independent mechanism. In a prior study (26), peritoneal macrophages from TNFR double-knockout mice still showed an increase in ion channel activity in the presence of wild-type TNF-α, and this activity was amiloride sensitive. Also, a peptide mimicking the lectinlike domain, with no binding to the two TNFRs, can still trigger increases in membrane conductance and also be inhibited by amiloride (33). Because the triple-mutant TNF-α did not stimulate AFC in the rat studies, it seemed plausible that the effect of TNF-α was mediated by receptor-independent mechanisms in the lung.

But this interpretation could be incomplete, because the TNF-α mutant retains some receptor affinity, although the affinity is decreased 5-fold for TNFR1 and 10-fold for TNFRII (32). Thus the results of the in vivo studies could be consistent with a receptor-dependent effect, a receptor-independent effect, or both.

Interestingly, the patch-clamp studies in the A549 cells also demonstrated a rapid amiloride-inhibitable uptake of Na⁺ in the presence of wild-type TNF-α, consistent with the data in the intact rat lung studies. Also, the triple-mutant TNF-α did not increase Na⁺

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**Fig. 6.** A: perfusion of A549 cells with wild-type TNF-α in the presence of blocking antibodies to its receptors did not increase Na⁺ currents. An A549 cell was patched in the whole cell mode as described in Fig. 4A and was perfused with SES containing anti-human soluble TNF receptor types I and II (sTNFRI and sTNFRII, respectively) antibodies (5 ng each) for 10 min. When a stable value of the inward Na⁺ current was obtained, the solution was switched to one containing sTNFRI, sTNFRII, and 120 ng/ml of TNF-α for ~5 min. No change in the inward current was observed. In contrast, when equivalent amounts of nonimmune IgG were substituted for the primary antibodies (B), TNF-α rapidly increased the whole cell current. At the end of this period, the cell was perfused with SES containing 10 μM amiloride, which blocked the existing Na⁺ current. Results represent a typical experiment, which was repeated 6 times.

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**Fig. 7.** Mean values of inward Na⁺ currents elicited after perfusion with wild-type TNF-α (120 ng/ml), TNF-α (120 ng/ml) in the presence of blocking antibodies to TNFRI and TNFRII (5 ng/ml each), or triple-mutant TNF-α (120 ng/ml). Values are means ± SE; n = 6 cells. *P < 0.005 compared with other groups.
influx in the A549 cells, also consistent with the results of the rat studies. Therefore, we took advantage of the availability of specific human TNFRI and TNFRII blocking antibodies to determine whether the effect of TNF-α in the A549 cells was receptor mediated. The results provided direct evidence in these cells for a receptor-dependent effect. Several studies (39, 46–48, 50) have shown that TNFRI is expressed in the lung in airway epithelium and alveolar epithelium and that both receptors exist in A549 cells.

However, the mechanism by which TNF-α increases Na⁺-dependent AFC in vivo may be considerably more complicated and may involve multiple pathways. TNF-α could stimulate the release of other mediators in vivo, such as transforming growth factor-α (9, 52), which has been shown to rapidly upregulate AFC in rats (17). Furthermore, it is certainly plausible that receptor-independent effects may occur in vivo in the lung and that some of the TNF-α effects on enhancing alveolar fluid reabsorption across the alveolar epithelium could be secondary to direct effects on the cell membrane.

The combination of TNF-α and a β-adrenergic agonist, terbutaline, did not have an additive effect on increasing AFC in the TNF-α-instilled rats. Also, propranolol, a β-adrenergic antagonist, did not inhibit the TNF-α-induced upregulation of alveolar epithelial fluid transport in rats. These results are similar to the findings after instillation of either endotoxin (21) or bacteria (44) into rat lungs. Taken together, the data indicate that TNF-α-induced alveolar epithelial fluid transport is not mediated by an endogenous release of epinephrine, a finding that is in agreement with the results of a recent peritonitis study in rats (10).

Which signaling mechanism mediated the TNF-α-induced Na⁺ uptake? TNF-α is known to decrease intracellular cAMP (13, 40), and the recent study by Börjesson et al. (10) showed that TNF-α upregulation of AFC in rats with peritonitis was associated with no change in cAMP in the lung. Therefore, enhanced Na⁺ and fluid transport from TNF-α probably does not depend on a cAMP-mediated process. Interestingly, G proteins can mediate several effects of TNF-α (25), and a G protein has been shown to contribute to Na⁺ transport in fetal alveolar type II cells (19, 20, 30, 34). Also, pertussis toxin can inhibit transepithelial Na⁺ transport (2). However, there is no clear evidence for the association of G protein with an immunopurified alveolar type II cell Na⁺ transport at this time (6). Thus further investigation will be needed to elucidate the mechanisms of TNF-α signaling in alveolar epithelial cells.

TNF-α has been shown in several studies (11, 49) to increase lung and systemic vascular permeability. Also, in vitro studies indicated that TNF-α can alter short-circuit current across cultured alveolar type II cells (56), and TNF-α decreases surfactant protein B expression (43). Thus TNF-α can have a deleterious effect on the lung endothelium and the alveolar epithelium, although the data in this study and other studies (10, 44) documented a beneficial effect of TNF-α on lung fluid balance by upregulating alveolar epithelial fluid transport.

In summary, TNF-α upregulates alveolar epithelial Na⁺ and fluid transport by an amiloride-sensitive, catecholamine-independent mechanism as demonstrated by studies in both intact rats and isolated human A549 alveolar epithelial cells. In the isolated cell studies, the effect was mediated by a TNFR-dependent process, although the intact rat studies do not clearly distinguish between receptor-dependent and -independent effects of TNF-α. Overall, these results provide further evidence for a beneficial effect of TNF-α on lung fluid balance that may be germane to clinically important pathological conditions such as pneumonia (44) or peritonitis (10).

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REFERENCES


