FGF-10 prevents mechanical stretch-induced alveolar epithelial cell DNA damage via MAPK activation

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Submitted 22 May 2002; accepted in final form 9 October 2002

Upadhyay, Daya, Eduardo Correa-Meyer, Jacob I. Sznaider, and David W. Kamp. FGF-10 prevents mechanical stretch-induced alveolar epithelial cell DNA damage via MAPK activation. Am J Physiol Lung Cell Mol Physiol 284: L350–L359, 2003; 10.1152/ajplung.00161.2002.—Cyclic stretch of alveolar epithelial cells (AEC) can alter normal lung barrier function. Fibroblast growth factor-10 (FGF-10), an alveolar type II cell mitogen that is critical for lung development, may have a role in promoting AEC repair. We studied whether cyclic stretch induces AEC DNA damage and whether FGF-10 would be protective. Cyclic stretch (30 min of 30% strain amplitude and 30 cycles/min) caused AEC DNA strand break formation, as assessed by alkaline unwinding technique and DNA nucleosomal fragmentation. Pretreatment of AEC with FGF-10 (10 ng/ml) blocked stretch-induced DNA strand break formation and DNA fragmentation. FGF-10 activated AEC mitogen-activated protein kinase (MAPK), and MAPK inhibitors prevented FGF-10-induced AEC MAPK activation and abolished the protective effects of FGF-10 against stretch-induced DNA damage. In addition, a Grb2-SOS inhibitor (SH3b-p peptide), a RAS inhibitor (farnesyl transferase inhibitor 277), and a RAF-1 inhibitor (forskolin) each prevented FGF-10-induced extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in AEC. Moreover, N17-A549 cells that express a RAS dominant/negative protein prevented the FGF-10-induced ERK1/2 phosphorylation and RAS activation in AEC. We conclude that cyclic stretch causes AEC DNA damage and that FGF-10 attenuates these effects by mechanisms involving MAPK activation via the Grb2-SOS/Ras/RAF-1/ERK1/2 pathway.

alveolar epithelial cells; fibroblast growth factor-10; mitogen-activated protein kinase; deoxyribonuclease

MECHANICAL VENTILATION with high tidal volumes over-distends and disrupts alveolar epithelial cells (AEC), resulting in alveolar edema and inflammation (8, 11, 41). Normal tidal breathing imposes an estimated ~1% strain on AEC, whereas high tidal volume ventilation can significantly increase the strain (7). Although the mechanisms by which cyclic stretch causes lung injury are not fully established, cyclical stretch induces an oxidative stress, cytokine release, and apoptosis (12, 13, 34, 37).

Restoration of normal alveolar epithelial barrier function is crucial for resolving pulmonary edema and improving outcomes in patients with respiratory failure (31, 38). Accumulating evidence suggests that growth factors, particularly keratinocyte growth factor [KGF; also known as fibroblast growth factor (FGF)-7], are important in preventing lung injury from various causes, including oxidative stress and overdistension by mechanical ventilation (42, 43). The mechanisms implicated in mediating the protective effects of KGF include stimulating AEC proliferation (35), augmenting AEC expression of surfactant apoproteins (28), limiting oxidant-induced increases in lung epithelial cell permeability (40), and promoting DNA repair (43). FGF-10 is a recently described 19.3-kDa heparin-binding protein that is structurally similar to KGF (14). FGF-10 is a potent alveolar type II cell (AT2 cell) mitogen that is predominantly expressed by lung mesenchymal cells and is required for lung development (20, 38). FGF-10 promotes epithelial cell motility, differentiation, migration, and wound healing (16).

There is no information on the signaling pathways induced by FGF-10. However, RAS/Raf-1 signaling typically occurs when FGFs bind their receptors, resulting in downstream activation of the mitogen-activated protein kinase (MAPK) family of proteins that include extracellular signal-regulated kinases (ERK) 1 and 2 (29). MAPKs have an important role in regulating cell growth, differentiation, apoptosis, and inflammation, and these effects are cell type and stimulus specific (8, 22). Cyclic stretch induces MAPK activation in various cells, including AEC (10, 19). However, the cellular consequences of MAPK activation are unclear.

DNA strand breaks (DNA-SB) are among the earliest cellular changes that occur after an oxidative stress (27). Recent studies show that DNA damage may also occur in mechanically stretched cells (2, 12). Although the underlying mechanisms are not known, Aikawa et al. (2) suggested an important role for reactive oxygen species (ROS) derived from Na(D)PH oxidase. In the

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present study, we reasoned that mechanical stretch might cause AEC injury in part by inducing DNA damage and that FGF-10 would attenuate these effects by mechanisms dependent on MAPK activation. We report here that cyclic stretch causes AEC DNA damage, as assessed by DNA-SB formation and by a DNA fragmentation assay. Furthermore, we show that FGF-10 blocks AEC DNA damage via mechanisms involving the Grb2-SOS/RAS/RAF-1/ERK1/2 pathways. Finally, we demonstrate that cyclic stretch-induced ROS, derived in part from NADPH oxidase activation, mediate AEC DNA damage.

METHODS

Materials

FGF-10 was purchased from R&D Systems (Minneapolis, MN). U-0126, PD-98059, and polyclonal anti-ERK1/2 antibodies p44/p42 were purchased from Promega (Madison, WI). Monoclonal anti-phosphorylated ERK1/2 antibodies were purchased from New England Biolabs (Beverly, MA). Farne-syl transferase inhibitor (FTI 277), hSOS n10 SHh3 Binding Peptide (SH3b-p), was from Calbiochem-Novabiochem (La Jolla, CA). Diphenyleniodine was purchased from Cayman (Ann Arbor, MI). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO).

Cell Culture

A549 cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in DMEM supplemented with 1-glutamine (0.5 μg/ml), nonessential amino acids, penicillin (100 U/ml), streptomycin (200 μg/ml), and 10% FBS (GIBCO, Grand Island, NY). For each experiment, we used a seeding density of 3.0 × 10^5 cells/well plated on collagen I-coated Silastic membranes in six-well plates (Costar, Cambridge, MA). The cells were grown to confluence over 24 h in a humidified 95% air-5% CO_2 incubator at 37°C.

Cyclic Stretch

AEC were subjected to mechanical stretch using the Flexercell Strain Unit (FX-3000; Flexercell), as previously described (10). Briefly, AEC on elastomer membranes were exposed to a cyclically applied vacuum (22.3 kPa) on the underside of the membranes, and a computer controlled the duration, amplitude, and frequency of the applied stretch. The vacuum produced a 30% elongation on the flexible bottom membranes at a frequency of 30 cycles/min, with a stretch/relaxation ratio of 1:1. We estimate that the level of strain in our model corresponds to AEC elongation that may occur during high-tidal volume mechanical ventilation that is associated with ventilator-induced lung injury (7). Controls consisted of cells plated on the same plates but not exposed to cyclic stretch. The vacuum of the Flexercell Strain Unit and six-well plates was maintained in a 37°C humidified incubator with 5% CO_2.

DNA-SB Assay

Alkaline unwinding and ethidium bromide fluorescence technique. The AEC were treated with FGF-10 (10 ng/ml) for 1 h and then exposed to mechanical stretch for 30 or 60 min. After mechanical stretch, the cells were washed with PBS and placed on ice, and DNA damage was assessed by alkaline unwinding and ethidium bromide fluorescence, as previously described (17). In some experiments, AEC were treated with selective MAPK inhibitors, U-0126 (10 μM) or PD-98059 (100 μM), for 15 min before FGF-10 (10 ng/ml) was added for 1 h. Because ethidium bromide preferentially binds to double-stranded DNA (ds-DNA) in alkali, the relative amounts of nonbroken ds-DNA and broken single-stranded DNA can be assessed. Fluorescence was determined with a model 450 Sequoia Turner fluorometer (Mountain View, CA) with excitation at 520 nm and emission at 585 nm. The results were expressed as previously described (17, 43) in which the percentage of total ds-DNA is defined as (F − F_{min})/(F_{max} − F_{min}) × 100, where F is the fluorescence in the experimental condition, F_{min} is the background ethidium bromide fluorescence determined after converting all the DNA into single-strand form, and F_{max} is the fluorescence determined from cells not exposed to alkaline unwinding conditions.

Comet Assay

The Comet assay was performed according to the manufacturer's instructions using a Comet assay kit (Trevigen). AEC were treated with FGF-10 (10 ng/ml) for 1 h and then exposed to mechanical stretch for 1 h. Cells were washed with Ca^{2+}- and Mg^{2+}-free PBS (Trevigen). The cell suspension was mixed with liquefied agarose at a 1:10 (vol/vol) ratio. A small aliquot of this mixture was immediately transferred to the slide provided. After cell lysis at 4°C, slides were treated with alkali solution (0.3 M NaOH and 1 mM EDTA) for 1 h and then exposed to mechanical stretch for 30 or 60 min, and then cell lysates were prepared for the ELISA. In some experiments, AEC were pretreated with FGF-10 (10 ng/ml) for 1 h and exposed to stretch for 30 or 60 min, and then cell lysates were prepared for the ELISA. In some experiments, AEC were pretreated with a highly selective MAPK inhibitor, as described above. The results are expressed as the percentage of control relative absorbance units.

DNA Fragmentation Assay

DNA fragmentation was assessed using a highly sensitive commercially available ELISA (Roche, Indianapolis, IN) according to the manufacturer's specifications. The assay involves a one-step sandwich immunoassay that detects nucleosomes consisting of histone-complexed DNA fragments (mono- and oligonucleosomes) characteristic of cells undergoing apoptosis. The AEC were pretreated with FGF-10 (10 ng/ml) for 1 h and exposed to cyclic stretch for 30 or 60 min, and then cell lysates were prepared for the ELISA. In some experiments, AEC were pretreated with a highly selective MAPK inhibitor, as described above. The results are expressed as the percentage of control relative absorbance units.

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine-5'-Triphosphate-Biotin Nick End-Labeling Assay

Cyclic stretch-induced apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated deoxyuridine-5'-triphosphate-biotin nick end-labeling (TUNEL) staining in AEC, as previously described (3).

Dichlorofluorescein Assay

ROS generation in AEC was assessed by 2',7'-dichlorodihydrofluorescein (DCFH). The diacetate ester form of DCFH, 2',7'-dichlorodihydrofluorescein diacetate, is taken up by the cells and deacetylated to form DCFH. DCFH then forms a highly fluorescent compound, dichlorofluorescein (DCF), in a reaction with the ROS. DCFH was added to AEC for 30 min at room temperature. AEC were treated with FGF-10 (10...
ng/ml) for 1 h and then exposed to mechanical stretch for 60 min. DCF fluorescence was measured using an excitation wavelength of 485 nm and an emission of 530 nm.

**MAPK Assay**

Preparation of lysates and total protein isolation. AECs were treated with FGF-10 (200 ng/ml) for the desired times (0, 3, 5, 10, 15, 30, and 60 min). In some of the ERK1/2 Western blot experiments, AEC were treated with U-0126 (10 μM) for 0.5 h before exposure to FGF-10 (200 ng/ml) for 10 min. The treated cells were washed with ice-cold PBS, lysed with 0.5 ml ice-cold cell lysis buffer containing phenylmethylsulfonyl fluoride (PMSF, 1 mM), incubated on ice for 5 min, sonicated four times for 5 s each, and microcentrifuged at 15,000 rpm (Beckman centrifuge) for 5 min, and then the supernatant was collected. Protein content was determined by the Bradford technique (6) using a Bio-Rad protein assay system (Bio-Rad, Hercules, CA).

Immunoprecipitation. Cell lysates (200 μg of total protein in 200 μl) were mixed with a 15-μl suspension of immobilized phospho-p44/42 MAPK monoclonal antibody and incubated for 4 h at 4°C with continuous gentle rocking. After microcentrifugation for 30 s at 4°C, the pellet was washed two times with 500 μl of 1x lysis buffer and kept on ice until use in the Western analysis.

Western blot analysis. Samples of protein (35 μg) were size fractionated by 1% SDS-12% PAGE and transferred to nitrocellulose membranes (Immobilon P, Schleicher & Shuell, Keene, NH) using a semidyry transfer apparatus (Bio-Rad). Incubation of blots with a monoclonal antibody that specifically recognizes the dually phosphorylated active form of ERK1 and ERK2 (New England Biolabs) was performed overnight at 4°C. Blots were developed with an enhanced chemiluminescence (ECL) detection kit (ECL+; Amersham, Buckinghamshire, UK) used as recommended by the manufacturer. The bands were quantified by densitometric scan (Eagle Eye II; Stratagene, La Jolla, CA).

**ERK assay.** The ERK activity was determined exactly as described in a commercially available p44/p42 antibody kit (New England Biolabs). Total protein (45 μg) from the lysates was immunoprecipitated with an immobilized phosphospecific p44/p42 MAPK antibody (Thy202/Pyr204) monoclonal antibody by overnight incubation at 4°C with gentle rocking. After the beads were washed two times with lysis buffer and then two times with kinase reaction buffer (KRB; 25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 1 mM NaCl, 1% Igepal CA-630, 10 mM MgCl2, 1 mM EDTA, 2% glycerol, 1 mM NaF, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PMSF. Fresh cell lysates were diluted to 1 μg/ml total cell protein with MLB and treated with glutathione. Cell lysates (500 μg) were then incubated with 15 μl Raf-1 RBD agarose conjugate/assay, and the mixture was rocked gently at 4°C for 30 min. The agarose beads were collected by pulsing, and the supernatant was drained off. The beads were washed three times with MLB, suspended in 100 μl 2x wash buffer (MLB) containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl2, 1 mM EDTA, 2% glycerol, 1 mM NaF, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PMSF.

**Activated Ras Affinity Precipitation Assay**

A functional assay for the activated Ras was done with an affinity precipitation assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer’s instructions and as described elsewhere (33). Serum-starved AEC were exposed to FGF-10 (200 ng/ml) for 10 min followed by cyclic stretch for 10 min. After treatment, cells were washed two times with cold PBS and lysed in 200 μl of Mg2+ lysis-wash buffer (MLB) containing 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl2, 1 mM EDTA, 2% glycerol, 1 mM NaF, 1 mM Na3VO4, 1 μg/ml leupeptin, and 1 mM PMSF. Fresh cell lysates were diluted to 1 μg/ml total cell protein with MLB and treated with glutathione. Cell lysates (500 μg) were then incubated with 15 μl Raf-1 RBD agarose conjugate/assay, and the mixture was rocked gently at 4°C for 30 min. The agarose beads were collected by pulsing, and the supernatant was drained off. The beads were washed three times with MLB, suspended in an appropriate amount of Laemmli sample buffer, and boiled for 5 min. Supernatants were collected and loaded on a 12% SDS-PAGE. The gel was transferred to a nitrocellulose membrane and probed with 1 μg/ml anti-Ras, clone Ras10 (Upstate Biotechnology), overnight at 4°C. A dilution of horseradish peroxidase conjugate antimouse antibody was used as the secondary antibody, and the enhanced chemiluminescence reagents were used for the final protein detection.

**Statistics**

All data are expressed as means ± SE. An unpaired Student's t-test was used to assess the difference between two groups. One-way ANOVA was performed when more than two groups were compared with a single control, and then differences between individual groups within the set were assessed by a multiple-comparison test (Tukey) when the F statistic was <0.05. A P value of <0.05 was considered significant.

**RESULTS**

**Cyclic Stretch Induces AEC DNA Damage**

To determine whether cyclic stretch caused AEC DNA-SB formation, AEC were exposed to cyclic stretch for various times (5, 15, 30, and 60 min) using a Flexercell apparatus, which generated cyclic stretch at a frequency of 30 cycles/min and 30% strain amplitude. As shown in Fig. 1, cyclic stretch induced AEC DNA-SB, as assessed by alkaline elution and ethidium bromide fluorescence. Cyclic stretch reduced ds-DNA levels to 40% of control levels after as little as 30 min of cyclic stretching, and DNA-SB persisted after 60 min. To determine whether cyclic stretch caused AEC DNA-SB formation by using single-cell gel electrophoresis (Comet) assay, we treated AEC with FGF-10 (10 ng/ml) for 1 h and then exposed them to cyclic stretch for 1 h using a Flexercell apparatus. DNA-SB formation was assessed by using a commercially available Comet assay kit (Trevigen), as described above. As shown in Fig. 2, cyclic stretch induced significant AEC DNA-SB formation compared with control. FGF-10 completely attenuated cyclic stretch-induced AEC DNA-SB formation at 60 min. To determine whether the DNA-SB observed was associated with DNA fragmentation, we used a sensitive DNA nucleosomol fragment assay with cyclic stretch. Cyclic stretch induced negligible levels of AEC DNA fragmentation after 30 min, but significant increases were noted by 60 min (Fig. 3). Notably, AEC DNA damage over these short time periods of cyclic stretch (30–60 min) was not associated with significant cell death, as assessed by lactate dehydrogenase (LDH) release (data not shown).
FGF-10 Attenuates Cyclic Stretch-Induced AEC DNA Damage

Given the critical role of FGF-10 in lung development and KGF, a closely related FGF family member in preventing AEC DNA damage (38, 43), we tested whether FGF-10 would prevent cyclic stretch-induced DNA damage. Compared with control, AEC treated with FGF-10 (10 ng/ml) for 1 h before cyclic stretch reduced stretch-induced AEC DNA-SB at 30 min by 50% (Fig. 4). FGF-10 also completely blocked stretch-induced AEC DNA fragmentation at 60 min (Fig. 3). MAPK-Dependent Pathways Mediate the Protective Effects of FGF-10

To determine whether MAPK-dependent pathways mediate the protective effects of FGF-10, we pretreated AEC with a specific MAPK inhibitor (U-0126, 10 μM) for 15 min followed by treatment with FGF-10 (10 ng/ml) for 1 h and cyclic stretch. U-0126 blocked the protective effect of FGF-10 against cyclic stretch-induced DNA fragmentation. FGF-10 Activates AEC MAPK

To confirm that FGF-10 activates AEC MAPK, we determined the time course of MAPK activation as assessed by Western analysis of Elk-1 in serum-starved AEC treated with FGF-10 (200 ng/ml). As shown in Fig. 5A, FGF-10-induced AEC MAPK activation was detected by 3 min, peaked at 10 min, and remained elevated at 60 min, the latest time point examined. As expected, the MAPK inhibitors U-0126...
Fig. 4. FGF-10 attenuated cyclic stretch-induced AEC DNA damage, and the protective effects of FGF-10 are mediated by MAPK-dependent signal transduction pathway. AEC pretreated with FGF-10 (10 ng/ml) for 1 h before cyclic stretch attenuated cyclic stretch-induced AEC DNA-SB formation at 30 min. AEC were pretreated with MAPK inhibitors [U-0126 (10 μM) or PD-98059 (100 μM)] for 15 min before treatment with FGF-10 (10 ng/ml), and then DNA-SB assay was performed. Both inhibitors blocked the protective effect of FGF-10 against stretch-induced DNA damage (mean ± SE, n = 3, *P < 0.05; †P < 0.005 stretch vs. FGF-10 + stretch; ‡P < 0.005 FGF-10 + stretch vs. U-0126 + FGFI0 + stretch). Both inhibitors by themselves did not cause any significant toxic effect.

(Fig. 5B) and PD-98059 (data not shown) completely prevented FGF-10-induced AEC MAPK activation.

**FGF-10, but not Cyclic Stretch, Induces AEC MAPK Activation via Activation of the Grb2-SOS/Ras/Raf-1 Pathway**

To determine the role of an adaptor protein, GRB2, and the guanine nucleotide exchange protein SOS, which are upstream signaling proteins for Ras/Raf-1 kinase, serum-starved AEC were incubated with the SH3 binding domain (SH3b-p), an agent that blocks Ras/Raf-1 signaling via Grb2-SOS. After AEC were treated with SH3b-p for 2 h, they were exposed to FGF-10 or cyclic stretch for 10 min. SH3b-p completely abolished FGF-10-induced MAPK activation (Fig. 6). Similar to a previous report (10) and the Ras/Raf-1 inhibitor studies described below, SH3b-p did not block stretch-induced MAPK activation.

To determine whether cyclic stretch or FGF-10 activates the Ras/Raf-1 pathway, AEC were treated with a Ras inhibitor, FTI 277 (10 μM), or a Raf-1 inhibitor, forskolin (50 μM), for 15 min. The cells were then exposed to FGF-10 (200 ng/ml) or cyclic stretch (30 cycles/min, 30% strain amplitude) for 10 min, and then MAPK activation was assessed by determining the levels of phosphorylated ERK1/2 by Western analysis.

Each inhibitor blocked FGF-10-induced AEC MAPK activation to the level seen with cyclic stretch alone (Fig. 6). A previous study also demonstrated that each of these inhibitors blocked cyclic stretch-induced MAPK activation (10). This suggests that FGF-10, but not cyclic stretch, causes AEC MAPK activation via the Grb2-SOS/Ras/Raf-1 pathway.

To corroborate that FGF-10, but not cyclic stretch, activates RAS, we used a functional Ras pull-down assay for the detection of activated RAS (GTP-Ras). As shown in Fig. 7A, GTP-Ras was increased threefold in AEC exposed to FGF-10 compared with control (P < 0.005). However, cyclic stretch did not increase Ras activation (Fig. 7A). Moreover, MAPK activation caused by FGF-10, but not cyclic stretch, was reduced in A549 cells expressing a Ras dominant/negative protein (N17 cells) compared with wild-type A549 cells (Fig. 7B). Together, these data strongly implicate that the protective effects of FGF-10 are mediated by MAPK activation via the Grb2-SOS/RAS/Raf-1 pathways.

**Cyclic Stretch Induces AEC Apoptosis**

To determine whether cyclic stretch induces AEC apoptosis, AEC were treated with FGF-10 (10 ng/ml) for 1 h and then exposed to cyclic stretch for 1 h. As shown in Fig. 3, cyclic stretch induced AEC apoptosis, as assessed by DNA fragmentation ELISA. Additionally, cyclic stretch-induced AEC apoptosis was assessed by TUNEL staining in AEC, as previously described (3). We noted that cyclic stretch increased AEC TUNEL staining by ~13% compared with static con-
control (control 5.4 ± 1.0% vs. stretch 18.0 ± 1.5%, P < 0.05, n = 3), whereas pretreatment of AEC with a Ras inhibitor, farnesyl transferase inhibitor (FTI 277, 10 μM), a Raf-1 inhibitor, forskolin (50 μM), or SH3 binding domain (SH3b-p; an agent that blocks Ras/Raf-1 signaling via Grb2-SOS for 15 min) blocked FGF-10 (200 ng/ml for 10 min)-induced AEC ERK1/2 phosphorylation (mean ± SE, n = 3, *P < 0.05, control vs. FGF-10; †P < 0.005 stretch vs. FGF + stretch; ‡P < 0.05, FGF-10 vs. FTI + FGF-10, forskolin + FGF-10, and SH3b-p + FGF-10).

Cyclic Stretch-Induced DNA Damage is in Part the Result of Oxidative Stress

To determine whether oxidants play a role in mediating cyclic stretch-induced DNA damage, we first exposed AEC to FGF-10 (10 ng/ml) for 1 h followed by cyclic stretch at 30% stretch, 30 cycles/min for 1 h, and then assessed DCF fluorescence. Compared with static controls, cyclic stretch increased AEC DCF fluorescence (25% increase vs. control; P < 0.05). Furthermore, FGF-10 completely blocked the increase in stretch-induced DCF fluorescence. Second, to determine whether iron-derived free radicals mediate cyclic stretch-induced DNA damage, we pretreated AEC with an iron chelator [phytic acid (500 mM) or deferroxime (1 mM)] or free radical scavenger, sodium benzoate (50 mM), for 15 min followed by cyclic stretch at 30% stretch, 30 cycles/min for 1 h. As shown in Fig. 8A, both iron chelators and a free radical scavenger attenuated cyclic stretch-induced DNA-SB formation. Furthermore, the combination of an iron chelator and a free radical scavenger with FGF-10 provided a modest increase in protection, but this did not reach statistical significance (Fig. 8A). Finally, to determine whether ROS derived from NAD(P)H oxidase contributes to cyclic stretch-induced AEC DNA damage, we exposed AEC to an NAD(P)H oxidase inhibitor (diphenyleneiodonium, 2 μM) for 30 min followed by cyclic stretch (30% strain at 30 cycles/min) for 1 h. As shown in Fig. 8B, diphenyleneiodonium partially blocked cyclic stretch-induced DNA damage, which suggests a role for NAD(P)H oxidase in generating ROS by cyclic stretch (50% reduction in DNA-SB formation, *P < 0.05 vs. cyclic stretch).

DISCUSSION

The major findings of this report are that mechanically stretched AEC undergo DNA damage, as assessed by both DNA-SB formation and a DNA fragmentation assay. Furthermore, we found that FGF-10 prevents cyclic stretch-induced DNA damage by mechanisms involving MAPK activation via the Grb2-SOS/RAS/Raf-1 pathway. Mechanical forces can have profound effects on AEC functions that alter lung development and induce pathological conditions (11, 24, 26, 34). In patients with respiratory failure, mechanical ventilation can reduce the work of breathing while allowing time for lung repair. However, it is also known that patients ventilated with high tidal volumes are at risk for ventilator-induced lung injury and a higher mortality rate (1, 23). Although it is unclear precisely how
mechanical forces cause lung injury, mechanisms that promote volutrauma and alveolar inflammation have been implicated (7, 11).

Our study supports the accumulating evidence showing that DNA damage is one mechanism by which mechanically stretched AEC are injured (12). DNA-SB formation is among the earliest abnormalities that occur in cells exposed to stress, and various stress signals can result in cell death if not repaired properly (27). The alkaline unwinding, ethidium bromide fluorescent technique for measuring DNA-SB formation is one of the most sensitive assays for detecting DNA damage, with a detection threshold of one break per chromosome (5). In our study, cyclic stretch (30 cycles/min with 30% strain amplitude) caused DNA-SB formation within 30 min. We also found that cyclic stretch caused DNA-SB, as assessed by the single-cell gel electrophoretic assay (Comet assay). Both assays use alkaline unwinding conditions that preferentially detect single-stranded DNA breakage and to a lesser extent double-stranded breaks. Notably, FGF-10 was protective against cyclic stretch-induced DNA-SB, as assessed by both assays. The DNA fragmentation ELISA preferentially detects ds-DNA breaks, as seen in apoptosis. In this study, we showed that cyclic stretch induced AEC DNA fragmentation and that FGF-10 was protective. Although we detected some apoptosis using the sensitive DNA nucleosomal fragmentation (Fig. 3) and TUNEL assays, the levels present under our experimental conditions likely represent a small proportion of the total number of cells (~13% over control conditions), since we were unable to detect significant levels of LDH release over 60 min (data not shown). The reasons why some groups (12, 13, 34), unlike others (37), detect significant levels of cyclic stretch-induced cell death are unclear. We suspect that this likely relates to differences in specific methods used in the various studies, including the cell type, strain protocol, time course, and measured end points of DNA damage and cell death. It is clear from the above studies and the present data that cyclic stretch induces DNA damage in a wide variety of cells, including AECs.

Using a cyclic stretch protocol similar to previous reports (10, 12, 37), we noted AEC DNA damage after 30–60 min but not after 15 min of cyclic stretch or under static conditions (Figs. 1–4). A well-recognized limitation of the Flexercell unit is that a nonuniform strain is applied to the cells, being highest in the periphery of the wells (20–30%). We estimate that the level of AEC strain under our experimental conditions corresponds to the levels that may be present during very high tidal volume mechanical ventilation that is associated with ventilator-induced lung injury (11, 32).

We found that FGF-10 decreased (cyclic stretch-induced) AEC DNA damage and DNA fragmentation (Figs. 2–4). The protective effects of FGF-10 against stretch-induced DNA damage observed in this study are comparable to the beneficial effects of KGF against oxidant-induced AEC DNA damage that we have described previously (43). Also, the beneficial effects of FGF-10 are unlikely because of the proliferative effects of FGF-10, since we used a short FGF-10 treatment period (1 h) that does not alter the total number of AEC (data not shown). The role of FGF-10 in preventing lung injury has not been investigated, but some evidence suggests that it could prove to be important. As mentioned earlier, FGF-10, similar to KGF, is a potent AT2 cell mitogen that is essential for lung development and unique among the FGF family members for their binding to FGFR2IIIb located exclusively on epithelial cells (20). Second, numerous in vitro and in vivo studies have established that KGF attenuates lung injury.
after exposure to a variety of noxious agents, including ventilator-induced lung injury in a rat (42). Data presented in this study demonstrating that FGF-10 reduces cyclic stretch-induced DNA damage suggest a role for FGF-10 in preventing ventilator-induced lung injury. Finally, growth factors may decrease oxidant-induced lung damage by inhibiting apoptosis, a central pathway regulating cellular homeostasis (22). Although there is no evidence that directly addresses this possibility with FGF-10 and KGF, there are some supportive data with other FGFs. FGF-2 induces translational regulation of Bcl-xl and Bcl-2, two antiapoptotic proteins that localize to the mitochondria, by an MAPK-dependent mechanism (22).

There is no information regarding the signaling mechanisms activated by FGF-10. However, MAPK, protein kinase C, and inositol 1,4,5-trisphosphate are important signaling pathways that mediate the protective effects of KGF and other FGF family members (Fig. 9; Refs. 22, 40, 43). The present study implicates a role for MAPK activation via the Grb2-SOS/Ras/Raf-1 pathway in mediating the protective effects of FGF-10. First, we found that MAPK inhibitors blocked the protective effects of FGF-10 against cyclic stretch-induced AEC DNA damage and DNA fragmentation (Figs. 3 and 4). Second, we provide evidence that FGF-10 caused ERK1/2 phosphorylation in the AEC and was inhibited by U-0126 (Fig. 5, A and B). Third, inhibitor experiments suggest a role for the Grb2-SOS, Ras, and Raf-1 in mediating FGF-10-induced, but not stretch-induced, ERK1/2 phosphorylation. Fourth, using a Ras activation assay, we found that FGF-10, but not cyclic stretch, activated Ras (Fig. 7A), and, using a clone of A549 cells expressing a Ras dominant/negative protein (N17 cells), we provide evidence that FGF-10 failed to cause ERK1/2 phosphorylation (Fig. 7B).

Although stretch and FGF-10 both caused MAPK phosphorylation (Fig. 7B), FGF-10-induced ERK1/2 phosphorylation was significantly higher (fivefold) than that of stretch (twofold). Furthermore, FGF-10 caused MAPK phosphorylation via the Grb2-SOS/Ras/Raf-1 pathway, whereas stretch did not. The above data suggest that involvement of the upstream Grb2-SOS/Ras/Raf-1 pathway has an important role in mediating the protective effect of FGF-10.

Together, MAPK activation is essential in mediating the protective effects of FGF-10 in preventing cyclic stretch-induced DNA damage. This was based on our finding that MAPK inhibitors (U-0126 and PD-98059) blocked the protective effect of FGF-10, as assessed by both the DNA-SB and DNA fragmentation assays (Figs. 3 and 4), and FGF-10-induced MAPK activation, as assessed by Western analysis (Fig. 5B). Because MAPK inhibitors did not affect cyclic stretch-induced DNA damage in the absence of FGF-10, we cannot implicate the MAPK pathway in preventing cyclic stretch-induced DNA damage.

The mechanisms of cyclic stretch-induced DNA damage are not known. There is some evidence that cyclic stretch may induce oxidative stress (2, 13). In this study, we provide evidence that cyclic stretch-induced DNA damage is mediated by oxidative stress. This conclusion is based on our observation that cyclic stretch increased DCF fluorescence and that iron chelators, a free radical scavenger, and an NAD(P)H oxidase inhibitor, each blocked cyclic stretch-induced DNA damage (Fig. 8, A and B). Collectively, these data suggest that ROS derived from NAD(P)H oxidase is one mechanism accounting for cyclic stretch-induced AEC DNA damage (2, 13).

There is some evidence that growth factors, such as KGF, may limit oxidant-induced DNA damage by in-

![Fig. 9. Schematic diagram of FGF signaling pathways. FGF bind to activated FGF receptors (FGFR), which in turn facilitates activation of Grb2-SOS, Ras, Raf-1, ERK1/2 pathways. MEK, mitogen/extracellular signal-regulated kinase.](http://ajplung.physiology.org/)
creasing DNA repair. Our group has previously demonstrated that KGF prevents oxidant-induced DNA damage from H$_2$O$_2$ or radiation by enhancing DNA repair (32, 43). In these studies, DNA polymerase inhibitors and ice-cold conditions blocked the protective effects of KGF (43). Given the above data and the shared receptor binding by KGF and FGF-10 (FGFR2iiib), one possible mechanism for the protective effects of FGF-10 may involve enhanced DNA repair.

In the present study, we focused on the signaling mechanisms accounting for the protective effects of FGF-10. Future studies are necessary to address the role of FGF-10 in AEC DNA repair.

In summary, our data suggest that cyclic stretch induces AEC DNA damage and apoptosis that is in part the result of oxidative stress. Furthermore, FGF-10 is protective against cyclic stretch-induced DNA damage by mechanisms involving ERK1/2 phosphorylation via the Grb2-SOS/Ras/Raf-1 pathway. These data provide insight into the mechanisms caus-


