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# Mechanisms of epidermal growth factor-induced contraction of guinea pig airways

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**Abstract**

We investigated the functional effects of epidermal growth factor (EGF) on guinea pig airways in vitro. EGF (3 ng/ml to 1 μg/ml) induced a concentration-dependent contraction in epithelium-denuded strips. The average maximal contraction was 0.64 ± 0.1 g (mean ± S.E., for $n = 27$), which was 72.0 ± 9.5% of the 100 mM KCl-induced contraction. The $EC_{50}$ was 12.3 ± 1.6 ng/ml. The presence of the epithelium significantly suppressed the EGF-induced contraction ($P < 0.01$). EGF-induced contraction was abolished by cyclooxygenase inhibitors (indomethacin and ibuprofen) and a 5-lipoxygenase inhibitor, 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone (AA-861). It was also inhibited by a leukotriene-receptor antagonist, 8-[*p*-(4-phenylbutyloxy)benzoyl]amino-2-(tetrazol-5-yl)-4-oxo-4*H*-1-benzopyran hemihydrate (ONO-1078) but not affected by a thromboxane $A_2$-synthetase inhibitor, (*E*)-3-[4-(1-imidazolylmethyl)phenyl]-2-propenoic acid (OKY-046) or a thromboxane $A_2$-receptor antagonist, 9,11-epithio-11,12-methano-thromboxane $A_2$ (ONO-3708). A phospholipase $A_2$ inhibitor (mepacrine) inhibited the EGF-induced contraction but a diacylglycerol-lipase inhibitor, 1,6-di-(*O*-(carbamoyl)cyclohexanone oxime)hexane (U-57908) and a phospholipase D inhibitor (wortmannin) did not affect it. A tyrosine kinase inhibitor (genistein) abolished it. Measurement of prostanoids showed that EGF (300 ng/ml) did not increase the prostaglandin $F_{2\alpha}$ level in either epithelium-intact or epithelium-denuded strips. In epithelium-intact strips, EGF significantly increased the prostaglandin $E_2$ concentration ($P < 0.01$). These results suggest that EGF causes contraction of guinea pig airway smooth muscle by activating tyrosine kinase followed by phospholipase $A_2$ activation, and that arachidonic acid metabolites, especially leukotrienes, may have important roles in this contraction.

*Keywords:* EGF (epidermal growth factor); Smooth muscle, airway; Leukotriene; Tyrosine kinase; Phospholipase $A_2$

## 1. Introduction

In the atherosclerotic regions of arteries, relations among smooth muscle hyperplasia and hypertrophy, abnormal vasoreactivity, and growth factors have been widely studied (Kalsner and Richards, 1984; Reidy, 1992). Now, epidermal growth factor (EGF) and platelet-derived growth factor are known to have direct effects on vascular smooth muscle tone in rat and dog arteries (Berk et al., 1985, 1986; Muramatsu et al., 1985, 1986). Both the growth and vasospastic activities of these growth factors are speculated to have a relation with the altered vascular responses at sites of atherosclerosis (Berk and Alexander, 1989).

Airway remodeling including smooth muscle hyperplasia and hypertrophy in the asthmatic airway has also been a subject of great interest (Ebina et al., 1993; Wang et al., 1993). Hypertrophy and hyperplasia of airway smooth muscle are considered to be possible causes of non-specific bronchial hyper-responsiveness, a hallmark of bronchial asthma. Since EGF is known to promote growth activity in smooth muscle of the lung, and EGF and contractile agonists of airway smooth muscle share membrane signaling mechanisms involv-

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ing phosphoinositide hydrolysis and $Ca^{2+}$ mobilization, we were interested in studying the effects of EGF on airway smooth muscle.

In a guinea pig tracheal preparation, Patel and his associates (Patel et al., 1988) reported the contractile activity of EGF, but the mechanisms of contraction have not been clarified fully. In vascular and gastric smooth muscles, the mechanisms of contraction elicited by EGF vary depending on the species and the type of muscle (Berk and Alexander, 1989; Hollenberg, 1993; Yang and Hollenberg, 1991; Yang et al., 1991). In this study, we examined the effect of EGF on guinea pig airway smooth muscle, investigated the precise mechanism of EGF-induced contraction, and measured arachidonic acid metabolites.

## 2. Materials and methods

### 2.1. Isolation of tissue for bioassay

Male Hartley guinea pigs weighing 350–550 g (Hokudo, Japan) were killed by cervical dislocation. The tracheas were removed, dissected free of extraneous tissue and cut into rings containing two cartilage rings. Tracheal strips were prepared by cutting at the site opposite the smooth muscle. In some cases, the epithelium was removed by gently rubbing the luminal surface of the strip with a cotton swab. The preservation of airway epithelium in epithelium-intact strips and elimination of airway epithelium in epithelium-denuded strips were confirmed by histological examination of these strips. Both sides of each preparation were tied with nylon threads. The upper thread was attached to an isometric transducer (Nihon Kohden, Japan), and the lower one was clamped at the bottom of the organ bath. Each preparation was placed in an organ bath containing 5 ml of Krebs-Henseleit solution of the following composition (mM): NaCl, 115; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25.0; $NaH_2PO_4$, 1.2; and glucose, 10.0; in distilled deionized water, equilibrated with 95% $O_2$ and 5% $CO_2$, and maintained at 37°C. A resting tension of 2 g was applied. After equilibration for 60 min, during which time the tissue was washed at 10-min intervals, drugs were added directly to the bath.

### 2.2. Experimental protocols

The magnitude of the response to 100 mM KCl was measured as a reliable prognostic indicator of a good response to EGF, and was taken as a standard response. After strips showed maximal responses, they were washed sufficiently. EGF (3 ng/ml to 1 $\mu$g/ml) was added cumulatively at 10-min intervals.

In epithelium-denuded strips, to investigate the mechanism of the effect of EGF, we compared the magnitude of the response to 30 ng/ml EGF before and after the 30-min incubation with several drugs as described below. Preliminary studies indicated that EGF did not cause tachyphylaxis in guinea pig trachea. After the first EGF-induced response, the tissue was washed at 10-min intervals for 60 min again, and before the second addition of EGF the tension was readjusted to 2 g. The magnitude of the response to 30 ng/ml EGF after incubation with a drug was expressed as a percentage of the initial response and was compared to the vehicle control.

### 2.3. Measurement of prostaglandin $F_{2\alpha}$ and prostaglandin $E_2$

Guinea pig tracheal strips were incubated in a tube filled with 2.5 ml of Krebs-Henseleit solution, equilibrated with 95% $O_2$ and 5% $CO_2$, and maintained at 37°C. Each tube contained 16 strips from two guinea pigs and the total weight of strips in each tube was adjusted to be the same. In some groups, the epithelia of all strips were removed. After a 90-min incubation, the whole medium was changed and strips were re-incubated for 30 min. After a 30-min incubation, 2.0 ml of medium was taken as the first sample. After replacement of the medium, 300 ng/ml of EGF or vehicle (20 $\mu$l of distilled water) was added and the strips were re-incubated for another 30 min. After incubation, 2.0 ml of medium was taken again as the second sample. 12 tubes were separated into four groups: epithelium-intact EGF ($n = 3$) and vehicle ($n = 3$) groups, and epithelium-denuded EGF ($n = 3$) and vehicle ($n = 3$) groups.

The samples were purified on Sep-pak C18 (Waters, Japan) and Bond Elut DEA columns (Varian, USA). Then samples were subjected to thin-layer chromatography for further purification. After the purification, the concentrations of prostaglandin $F_{2\alpha}$ and prostaglandin $E_2$ were measured by radioimmunoassay with specific antibodies (Ono Pharmaceutical Co., Japan). Results were expressed as nanograms per milliliter.

### 2.4. Reagents

The reagents used in this study and the sources were as follows: EGF culture grade (mouse submaxillary glands) (Becton Dickinson Lab, Ware, USA), genistein (ICN Biochemicals, USA), ibuprofen, calphostin C, wortmannin, mepacrine (Sigma Chemical Co., USA), OKY-046 ((*E*)-3-[4-(1-imidazolylmethyl)-phenyl]-2-propenoic acid), ONO-3708 (9,11-epithio-11,12-methano-thromboxane $A_2$), ONO-1078 (8-[*p*-(4-phenylbutyloxy)benzoyl]amino-2-(tetrazol-5-yl)-4-oxo-4*H*-1-benzopyran-hemihydrate), prostaglandin $F_{2\alpha}$, leukotriene $D_4$ (gifts from Ono Pharmaceutical Co.,

Japan), indomethacin (Wako Junyaku Co., Japan), AA-861 (2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone) (a gift from Takeda Chemical Industries, Japan), U-57908 (1,6-di(*O*-(carbamoyl)cyclohexanoneoxime)hexane) (a gift from Upjohn Co., USA) and H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride) (Seikagaku Co., Japan). U-57908, wortmannin, calphostin C and genistein were dissolved in dimethyl sulfoxide (DMSO). EGF, OKY-046, ONO-3708 and H-7 were dissolved in distilled water. The other drugs were dissolved in ethanol.

### 2.5. Statistics

The results are expressed as means ± S.E. Statistical analysis was performed by Student's *t*-test for unpaired observations. A paired *t*-test was applied for the comparison of prostanoid concentrations before and after incubation with EGF. Probability was established when the value was less than 0.05.

## 3. Results

### 3.1. Responses of tracheal strips to EGF

In epithelium-denuded strips, mouse EGF (3 ng/ml to 1 μg/ml) induced a concentration-dependent contraction (Fig. 1). The average maximal contraction was 0.64 ± 0.1 g (mean ± S.E., for $n = 27$), which was 72.0 ± 9.5% of the contraction induced with 100 mM KCl (Fig. 2). The concentration of EGF that induced a half-maximal contraction ($EC_{50}$) was 12.3 ± 1.6 ng/ml, and a maximal contraction was obtained at 100–300 ng/ml (Fig. 3). To eliminate the possibility that the contraction induced by mouse EGF is due to other biologically active peptides which might exist in EGF from mouse submaxillary glands, the contractile activity of human recombinant EGF (Boehringer Mannheim) was also examined. Human recombinant EGF (3–300 ng/ml) also contracted epithelium-denuded guinea pig airway strips ($n = 4$, data not shown). The effect of mouse EGF on epithelium-intact strips was examined using 19 tracheal strips. The results were variable. In

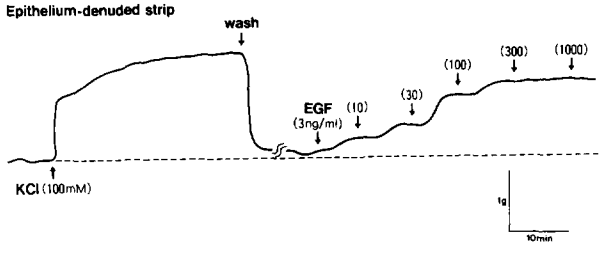


Fig. 1. A typical response to EGF (3 ng/ml to 1 μg/ml) in an epithelium-denuded strip.

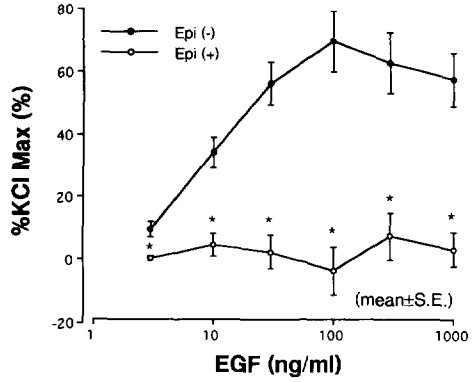


Fig. 2. Response to EGF (3 ng/ml to 1 μg/ml) in both epithelium-denuded (Epi(−)) (closed circles, $n = 27$) and -intact (Epi(+)) (open circles $n = 19$) strips. The contraction induced with 100 mM KCl was taken as 100% for each response. In epithelium-intact strips, as a mean, the response to each concentration was significantly attenuated. * $P < 0.01$, compared to the responses of epithelium-denuded strips for each concentration of EGF.

10 strips, concentration-dependent relaxation was observed. In three strips, concentration-dependent contractions were observed (the magnitude of the contraction was much smaller than that of epithelium-denuded strips). In six strips, no significant effect was observed with EGF (3 ng/ml to 1 μg/ml). However, as a mean, the presence of epithelium significantly suppressed the contractile activity of EGF ($P < 0.01$) (Fig. 2).

### 3.2. Actions of inhibitors of arachidonic acid metabolism (Table 1)

The effects of cyclooxygenase inhibitors, a 5-lipoxygenase inhibitor, a leukotriene-receptor antagonist, a thromboxane $A_2$-synthetase inhibitor and a thromboxane $A_2$-receptor antagonist are shown in Fig. 4 and Table 1. The EGF (30 ng/ml)-induced contraction was almost completely abolished by the cyclooxygenase inhibitors, indomethacin (1 and 10 μM) and ibuprofen

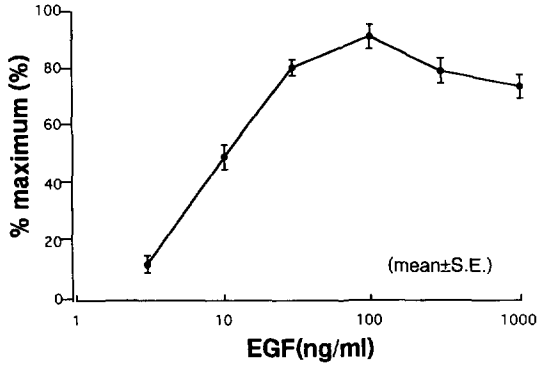


Fig. 3. Response to EGF (3 ng/ml to 1 μg/ml) in epithelium-denuded strips ($n = 27$). The maximal contraction of each preparation was taken as 100% for each response.

Table 1
Actions of inhibitors of arachidonic acid metabolism

| Drug | n | Mean ± S.E. (%) |
|---|---|---|
| Ethanol (5 μl) | 6 | 102.0 ± 23.8 |
| Indomethacin (1 μM) | 5 | 6.7 ± 6.7 [a] |
| Indomethacin (10 μM) | 5 | 0.0 ± 0.0 [a] |
| Ibuprofen (10 μM) | 5 | 0.0 ± 0.0 [a] |
| AA-861 (3 μM) | 5 | 13.8 ± 9.8 [a] |
| ONO-1078 (10 μM) | 5 | 0.0 ± 0.0 [a] |
| Distilled water (5 μl) | 4 | 110.5 ± 17.5 |
| OKY-046 (10 μM) | 5 | 103.4 ± 25.7 |
| ONO-3708 (100 nM) | 5 | 111.5 ± 7.3 |

Means ± S.E.: the magnitude of a response to 30 ng/ml EGF after incubation with a drug is expressed as a percentage of the initial response and compared to the time (vehicle) control. [a] $P < 0.01$, compared to the vehicle (ethanol, 5 μl) control.

(10 μM). A specific inhibitor of 5-lipoxygenase, AA-861 (3 μM) (Ashida et al., 1983; Yoshimoto et al., 1982), and a leukotriene $C_4$, $D_4$-receptor antagonist, ONO-1078 (10 μM) (Yamaguchi et al., 1992), almost completely inhibited the EGF-induced contraction. A thromboxane $A_2$-synthetase inhibitor, OKY-046 (10 μM) (Naito et al., 1983) and a thromboxane $A_2$-receptor antagonist, ONO-3708 (100 nM) (Kondo et al., 1989), did not affect the EGF-induced contraction at all. As indicated in Table 1, statistical analysis revealed that the inhibitory effects of indomethacin, ibuprofen, AA-861 and ONO-1078 were significant when compared to the vehicle control (ethanol, 5 μl), and that the effects of OKY-046 and ONO-3708 were not significant when compared to the vehicle control (distilled water, 5 μl).

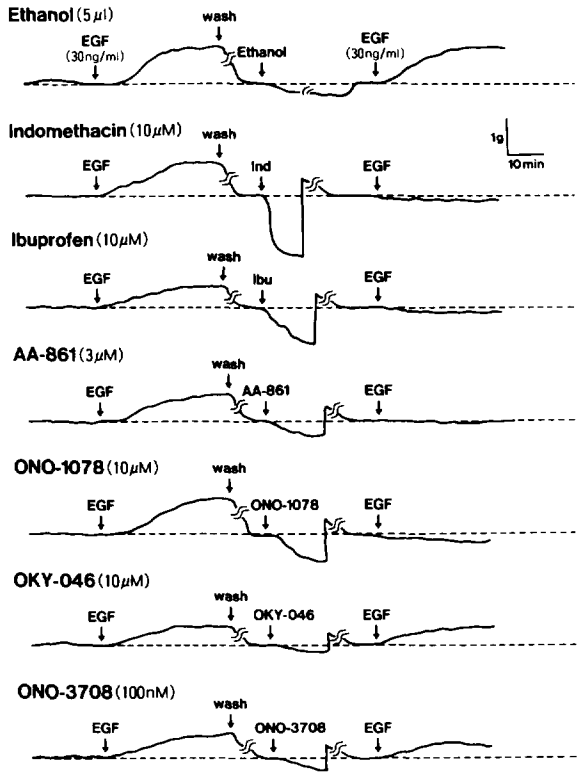


Fig. 4. Effects of drugs used to determine the arachidonic acid metabolites responsible for the EGF-induced contraction in epithelium-denuded strips. Strips were exposed to EGF (30 ng/ml) before and after a 30-min incubation with drug. Ethanol was used as the time and vehicle control for indomethacin, ibuprofen, AA-861, and ONO-1078.

Table 2
Effects of other drugs

| Drug | n | Mean ± S.E. (%) |
|---|---|---|
| Ethanol (5 μl) | 6 | 102.0 ± 23.8 |
| Mepacrine (10 μM) | 5 | 105.0 ± 26.2 |
| Mepacrine (100 μM) | 5 | 0.0 ± 0.0 [a] |
| DMSO (5 μl) | 4 | 104.9 ± 7.8 |
| U-57908 (20 μM) | 5 | 145.0 ± 25.2 |
| Wortmannin (100 nM) | 5 | 132.0 ± 17.1 |
| Genistein (3 μM) | 5 | 0.0 ± 0.0 [b] |
| Calphostin C (100 nM) | 5 | 89.5 ± 13.7 |
| Distilled water (5 μl) | 4 | 110.5 ± 17.5 |
| H-7 (10 μM) | 6 | 6.7 ± 6.7 [c] |

Means ± S.E.: the magnitude of a response to 30 ng/ml EGF after incubation with a drug is expressed as a percentage of the initial response and compared to the time (vehicle) control. [a] $P < 0.01$, compared to the vehicle (ethanol, 5 μl) control. [b] $P < 0.01$, compared to the vehicle (DMSO, 5 μl) control. [c] $P < 0.01$, compared to the vehicle (distilled water, 5 μl) control.

### 3.3. Actions of inhibitors of phospholipase $A_2$, phospholipase D and diacylglycerol lipase (Table 2)

From the above results, we considered endogenous arachidonic acid metabolites to be important in the mechanism of EGF-induced contraction. In response to EGF, arachidonic acid is produced by activation of phospholipase $A_2$ (Margolis et al., 1988), phospholipase D (Kaszkin et al., 1992), and diacylglycerol lipase (Majerus et al., 1986).

To investigate the role of phospholipase $A_2$, we examined the effect of mepacrine. Mepacrine at 100 μM completely abolished the EGF-induced contraction, but 10 μM mepacrine did not affect it. U-57908 (20 μM), which inhibits diacylglycerol lipase but does not affect phospholipase $A_2$, phospholipase C, or diacylglycerol kinase (Sutherland and Amin, 1982; Chuang and Severson, 1990), did not affect the EGF-induced contraction. The phospholipase D inhibitor, wortmannin (100 nM) (Reinhold et al., 1990), also showed no significant effect on the EGF-induced contraction.

### 3.4. Actions of a tyrosine kinase inhibitor and protein kinase C inhibitor (Table 2)

Since the first step in the action of EGF is the activation of receptor-intrinsic tyrosine kinase (Ullrich

and Schlessinger, 1990), we investigated the effect of tyrosine kinase by using genistein, a specific inhibitor of this enzyme (Akiyama et al., 1987). As shown in Table 2, genistein (3 $\mu$M) completely abolished the EGF-induced contraction.

Since protein kinase C is related to some kinds of cell-growth proliferation induced by EGF (Susa et al., 1992), and protein kinase C regulates phospholipase $A_2$ in glomerular mesangial cells when it is stimulated by hormones (Pfeilschifter, 1990), we investigated the role of protein kinase C in the EGF-induced contraction. A potent inhibitor of protein kinase C, H-7 (Hidaka et al., 1984) (10 $\mu$M), almost completely abolished the EGF-induced contraction, but a more specific protein kinase C inhibitor, calphostin C (Kobayashi et al., 1989) (100 nM to 3 $\mu$M) did not affect it.

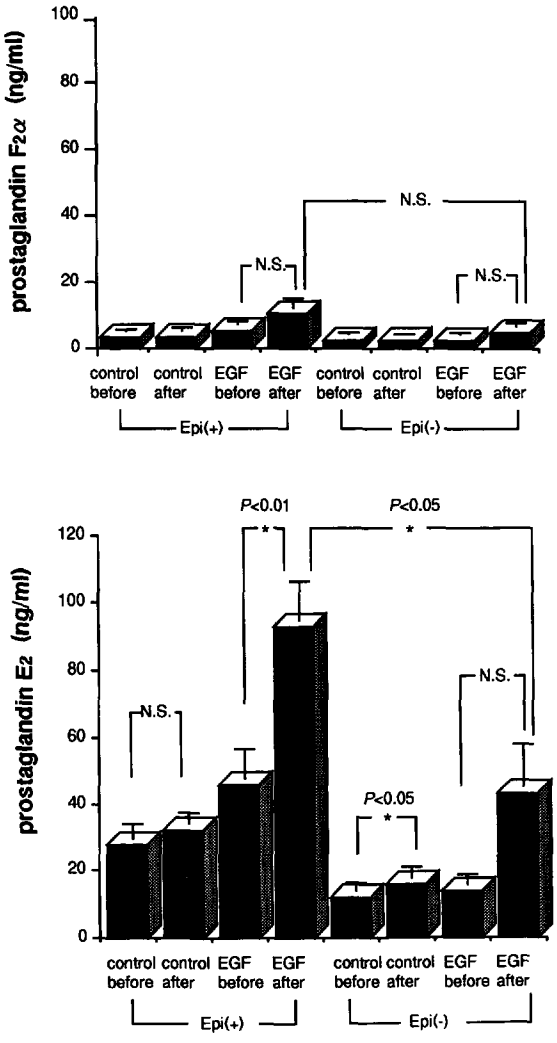


Fig. 5. Concentration of prostaglandin $F_{2\alpha}$ (upper) and prostaglandin $E_2$ (lower) in guinea pig tracheal strips incubated with EGF (300 ng/ml) or vehicle control. Epi(−) and Epi(+) show epithelium-denuded and -intact strips, respectively. Values are expressed as means ±S.E.M. for the concentration of prostaglandin $F_{2\alpha}$ or prostaglandin $E_2$ (ng/ml) in 3 tubes including 16 strips each.

### *3.5. Measurement of prostaglandin $F_{2\alpha}$ and prostaglandin $E_2$ (Fig. 5)*

In epithelium-intact strips, the concentrations of prostaglandin $F_{2\alpha}$ before and after incubation with EGF were $5.54 \pm 0.57$ and $10.75 \pm 2.68$ ng/ml (means ± S.E., for $n = 3$, $P = 0.14$), respectively. Those before and after vehicle were $3.51 \pm 0.20$ and $3.60 \pm 0.72$ ng/ml ($n = 3$, $P = 0.88$), respectively. In epithelium-denuded strips, concentrations before and after incubation with EGF were $2.41 \pm 0.29$ and $4.79 \pm 1.23$ ng/ml ($n = 3$, $P = 0.13$), respectively. Those before and after vehicle were $2.18 \pm 0.68$ and $2.22 \pm 0.31$ ng/ml ($n = 3$, $P = 0.92$), respectively. However, there was no significant difference in prostaglandin $F_{2\alpha}$ production before and after incubation with EGF.

In epithelium-intact strips, concentrations of prostaglandin $E_2$ before and after incubation with EGF were $45.93 \pm 8.58$ and $92.93 \pm 11.48$ ng/ml (means ± S.E., for $n = 3$), respectively. There was a significant increase in prostaglandin $E_2$ production ($P < 0.01$) after EGF treatment. No significant increase was observed in the vehicle group ($27.93 \pm 4.19$ and $32.35 \pm 3.44$ ng/ml ($n = 3$), respectively, $P = 0.57$). In epithelium-denuded strips, concentrations before and after incubation with EGF were $14.17 \pm 2.70$ and $43.16 \pm 13.00$ ng/ml ($n = 3$), respectively. However, this difference was not statistically significant ($P = 0.12$). Those before and after vehicle were $11.83 \pm 2.56$ and $16.04 \pm 3.00$ ng/ml ($n = 3$), respectively, and there was a significant difference ($P < 0.05$). Furthermore, after incubation with EGF, the prostaglandin $E_2$ concentration of the epithelium-intact group was significantly higher than that of the epithelium-denuded one ($P < 0.05$).

## 4. Discussion

In this study, the contractile effect of EGF on epithelium-denuded strips of guinea pig trachea was confirmed. The $EC_{50}$ ($12.3 \pm 1.6$ ng/ml) was almost the same as the value observed in previous studies for rat artery (Berk and Alexander, 1989) and guinea pig trachea (Patel et al., 1988). Since it might be possible that mouse EGF extracted from submaxillary glands contains other biologically active peptides, we also examined the effects of human recombinant EGF on epithelium-denuded strips. Human recombinant EGF (3 ng/ml to 300 ng/ml) also contracted guinea pig tracheal strips, suggesting that the contractile activity of mouse EGF observed in this study is specific to EGF. The presence of epithelium in the strips significantly suppressed the contraction induced by EGF. This suggests that the effect of EGF on airway smooth muscle is basically contractile, and stimulation of air-

way epithelium with EGF may modify the smooth muscle response in a way which suppresses the contraction. To examine this possibility, we measured prostaglandin $E_2$ in epithelium-intact and -denuded strips stimulated with EGF. EGF significantly increased prostaglandin $E_2$ production in epithelium-intact strips but not in epithelium-denuded ones. Further, after incubation with EGF, the concentration of prostaglandin $E_2$ was significantly higher in epithelium-intact strips than in epithelium-denuded ones. We may, therefore, reasonably conclude that, in epithelium-intact strips, prostaglandin $E_2$ from epithelium stimulated with EGF suppresses the EGF-induced contraction.

As reported previously (Patel et al., 1988), the contractile activity of EGF was completely inhibited by 1 and 10 $\mu$M indomethacin. To confirm the specificity of the effect of indomethacin, we examined the effect of another cyclooxygenase inhibitor, ibuprofen. Ibuprofen (10 $\mu$M) also abolished the EGF-induced contraction, suggesting that cyclooxygenase activation is essential for EGF-induced contractions. However, in this study, a 5-lipoxygenase inhibitor, AA-861, also significantly suppressed the EGF-induced contraction. We also observed that a leukotriene-receptor antagonist, ONO-1078, completely abolished the EGF-induced contraction. We examined the effects of AA-861 and ONO-1078 on prostaglandin $F_{2\alpha}$-induced contraction in guinea-pig trachea, but they did not affect it (data not shown). A thromboxane $A_2$-synthetase inhibitor and a thromboxane $A_2$-receptor antagonist did not affect the EGF-induced contraction. The concentrations of prostaglandin $F_{2\alpha}$ before and after incubation with EGF were $2.41 \pm 0.29$ and $4.79 \pm 1.23$ ng/ml ($n = 3$), respectively, in epithelium-denuded strips. The $P$ value for the difference was 0.12. However, in our bio-assay system, the minimum concentration of prostaglandin $F_{2\alpha}$ required to induce contraction of guinea pig tracheal strips was 30–40 ng/ml, which is well above the concentrations of prostaglandin $F_{2\alpha}$ after EGF stimulation. Furthermore, exogenous arachidonic acid is reported to contract guinea pig trachea in epithelium-denuded strips and this contraction is abolished by a 5-lipoxygenase inhibitor but not by indomethacin. Therefore leukotrienes are considered responsible for the contraction induced by exogenous arachidonic acid (Farmer et al., 1987; Tschirhart et al., 1987). Guinea pig trachea is more sensitive to leukotrienes than human or rat trachea (Bakhle and Ferreira, 1985). For these reasons, leukotrienes seem to be important as end products responsible for EGF-induced contraction.

Abolishment of the EGF-induced contraction by cyclooxygenase inhibitors raises the following three possibilities. First, some of the effects of leukotrienes are mediated through prostaglandins. Second, both prostaglandins and leukotrienes work synergistically in airway smooth muscles during EGF-induced contraction. Third, a cyclooxygenase inhibitor-sensitive enzyme might be involved in the pathway of EGF-induced release of arachidonic acid. The effects of leukotrienes on guinea pig trachea have been reported to be decreased markedly in the presence of a cyclooxygenase inhibitor (Bakhle and Ferreira, 1985). However, we observed enhancement of leukotriene $D_4$-induced contraction by indomethacin (data not shown). This is in opposition to the first possibility. From our results, it is difficult to reach a clear conclusion and further investigation will be needed to clarify the effect of cyclooxygenase inhibitors on EGF-induced contractions.

To clarify the source of arachidonic acid in the EGF-induced contraction, we examined the effects of mapacrine (a phospholipase $A_2$ inhibitor), U-57908 (a diacylglycerol lipase inhibitor) and wortmannin (a phospholipase D inhibitor). In guinea pig gastric smooth muscle, EGF induces a contraction through the metabolism of diacylglycerol by diacylglycerol lipase in the longitudinal muscle, but not in the circular muscle (Yang et al., 1992). Even in the same organ of the same species, the effect of EGF is mediated through different pathways. The report of Patel et al. (1988) examined only the effect of 10 $\mu$M mepacrine, which caused partial (36%) inhibition in guinea pig trachea. In our study, 100 $\mu$M mepacrine completely abolished the EGF-induced contraction. However, neither the diacylglycerol-lipase inhibitor nor the phospholipase D inhibitor affected it. These results support the hypothesis that phospholipase $A_2$ activation is essential for arachidonic acid generation in guinea pig trachea stimulated by EGF. Further, since wortmannin has recently been reported to inhibit phosphatidylinositol 3-kinase at the concentration of 50 nM (Kanai et al., 1993), our results also rule out the contribution of phosphatidylinositol 3-kinase activity in the action of EGF in guinea pig airway.

That genistein completely inhibited the effect of EGF is in agreement with a study on guinea pig gastric smooth muscle (Yang et al., 1992), and suggested the contribution of EGF receptor kinase activation in EGF-induced contractions. Akiyama et al. (1987) reported that genistein has a half-maximal effect at 2.6 $\mu$M against EGF receptor kinase. However, they reported that genistein also inhibit other tyrosine kinases with the same potency. Because of this, it seems difficult to eliminate the possible contribution of a tyrosine kinase apart from the EGF receptor kinase. The pathway by which tyrosine kinase activates phospholipase $A_2$ has not been fully elucidated. Although there is a report that tyrosine kinase activates phospholipase $A_2$ through phospholipase C-independent stimulation and not through the activation of protein kinase C (Gold-

berg et al., 1990), protein kinase C is considered to be an important enzyme in this pathway in general (Pfeilschifter, 1990). In this study, a potent protein kinase C inhibitor, H-7, completely inhibited the EGF-induced contraction, however, a more specific protein kinase C inhibitor, calphostin C, did not affect it. These results suggest that protein kinase C activation may not be essential for the EGF-induced activation of phospholipase $A_2$.

In conclusion, the results of our study suggest that EGF causes contraction of guinea pig airway smooth muscle by activating tyrosine kinase followed by phospholipase $A_2$ activation, and that arachidonic acid metabolites, especially leukotrienes, may have important roles in this contraction.

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## References

Akiyama, T., J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh and M. Shibuya, 1987, Genistein, a specific inhibitor of tyrosine-specific protein kinases, J. Biol. Chem. 262, 5592.

Ashida, Y., T. Saijo, H. Kuriki, H. Makino, S. Terao and Y. Maki, 1983, Pharmacological profile of AA-861, a 5-lipoxygenase inhibitor, Prostaglandins 26, 955.

Bakhle, Y.S. and S.H. Ferreira, 1985, Lung metabolism of eicosanoids: prostaglandins, prostacyclin, thromboxane, and leukotrienes, in: Handbook of Physiology. The Respiratory System. Circulatory and Nonrespiratory Functions, ed. A.P. Fishman and A.B. Fisher (Am. Physiol. Soc., Bethesda) p. 365.

Berk, B.C. and R.W. Alexander, 1989, Vasoactive effects of growth factors, Biochem. Pharmacol. 38, 219.

Berk, B.C., T. Brock, R. Webb, M. Taubman, W. Atkinson, M.J. Gimbrone and R. Alexander, 1985, Epidermal growth factor, a vascular smooth muscle mitogen, induces rat aortic contraction, J. Clin. Invest. 75, 1083.

Berk, B.C., R. Alexander, T. Brock, M.J. Gimbrone and R. Webb, 1986, Vasoconstriction: A new activity for platelet-derived growth factor, Science 232, 87.

Chuang, M. and D.L. Severson, 1990, Inhibition of diacylglycerol metabolism in isolated cardiac myocytes by U-57908 (RHC 80267), a diacylglycerol lipase inhibitor, J. Mol. Cell. Cardiol. 22, 1009.

Ebina, M., T. Takahashi, T. Chiba and M. Motomiya, 1993, Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma, Am. Rev. Respir. Dis. 148, 720.

Farmer, S., D. Hay, D. Raeburn and J. Fedan, 1987, Relaxation of guinea-pig tracheal smooth muscle to arachidonate is converted to contraction following epithelium removal, Br. J. Pharmacol. 92, 231.

Goldberg, H., M. Viegas, B. Margolis, J. Schlessinger and K. Skorecki, 1990, The tyrosine kinase activity of the epidermal-growth-factor receptor is necessary for phospholipase $A_2$ activation, Biochem. J. 267, 461.

Hidaka, H., M. Inagaki, S. Kawamoto and Y. Sasaki, 1984, Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C, Biochemistry 23, 5036.

Hollenberg, M.D., 1993, Growth factors and signal transduction in smooth muscle systems, in: Lung Biology in Health and Disease, Vol. 65, Signal Transduction in Lung Cells, ed. J.S. Brody, D.M. Center, V.A. Tkachuk (Marcel Dekker, New York) p. 369.

Kalsner, S. and R. Richards, 1984, Coronary arteries of cardiac patients are hyperreactive and contain stores of amines: a mechanism for coronary spasm, Science 223, 1435.

Kanai, F., K. Ito, M. Todaka, H. Hayashi, S. Kamohara, K. Ishii, T. Okada, O. Hazeki, M. Ui and Y. Ebina, 1993, Insulin-stimulated GLUT4 translocation is relevant to the phosphorylation of IRS-1 and the activity of PI3-kinase, Biochem. Biophys. Res. Commun. 195, 762.

Kaszkin, M., L. Seidler, R. Kast and V. Kinzel, 1992, Epidermal-growth-factor-induced production of phosphatidylalcohols by HeLa cells and A431 cells through activation of phospholipase D, Biochem. J. 287, 51.

Kobayashi, E., H. Nakano, M. Morimoto, T. Tamaoki, 1989, Calphostin C (UCN-1028C), A novel microbial compound, is a highly potent and specific inhibitor of protein kinase C, Biochem. Biophys. Res. Commun. 159, 548.

Kondo, K., M. Naka, T. Kitagawa, K. Wakitani, M. Sakata, H. Kira, T. Okegawa and A. Kawasaki, 1989, Effects of ONO-3708, an antagonist of the thromboxane $A_2$/prostaglandin endoperoxide receptor, on platelet aggregation and thrombosis, Eur. J. Pharmacol. 163, 253.

Majerus, P., T. Connolly, H. Deckmyn, T. Ross, T. Bross, H. Ishii, V. Bansal and D. Wilson, 1986, The metabolism of phosphoinositide-derived messenger molecules, Science 234, 1519.

Margolis, B., B. Holub, D. Troyer and K. Skorecki, 1988, Epidermal growth factor stimulates phospholipase $A_2$ in vasopressin-treated rat glomerular mesangial cells, Biochem. J. 256, 469.

Muramatsu, I., M.D. Hollenberg and K. Lederis, 1985, Vascular actions of epidermal growth factor-urogastrone: possible relationship to prostaglandin production, Can. J. Physiol. Pharmacol. 63, 994.

Muramatsu, I., M.D. Hollenberg and K. Lederis, 1986, Modulation by epidermal growth factor-urogastrone of contraction in isolated canine helical mesenteric arterial strips, Can. J. Physiol. Pharmacol. 64, 1561.

Naito, J., H. Komatsu, A. Ujiie, S. Hamano, T. Kubota and M. Tsuboshima, 1983, Effects of thromboxane synthetase inhibitors on aggregation of rabbit platelets, Eur. J. Pharmacol. 91, 41.

Patel, P., H. Itoh, K. Lederis and M.D. Hollenberg, 1988, Contraction of guinea pig trachea by epidermal growth factor – urogastrone, Can. J. Physiol. Pharmacol. 66, 1308.

Pfeilschifter, J., 1990, Regulatory functions of protein kinase C in glomerular mesangial cells, Klin. Wochenschr. 68, 1134.

Reidy, M.A., 1992, Factors controlling smooth-muscle cell proliferation, Arch. Pathol. Lab. Med. 116, 1276.

Reinhold, S.L., S.M. Prescott, G.Y. Zimmerman and T.M. McIntyre, 1990, Activation of human neutrophil phospholipase D by three separable mechanisms, FASEB J. 4, 208.

Susa, M., D. Vulevic, H.A. Lane and G. Thomas, 1992, Inhibition or down-regulation of protein kinase C attenuates late phase $p70^{s6k}$ activation induced by epidermal growth factor but not by platelet-derived growth factor or insulin, J. Biol. Chem. 267, 6905.

Sutherland, C.A. and D. Amin, 1982, Relative activities of rat and dog platelet phospholipase $A_2$ and diglyceride lipase, J. Biol. Chem. 257, 14006.

Tschirhart, E., N. Frossard, C. Bertrand and Y. Landry, 1987, Arachidonic acid metabolites and airway epithelium-dependent relaxant factor, J. Pharmacol. Exp. Ther. 243, 310.

Ullrich, A. and J. Schlessinger, 1990, Signal transduction by receptors with tyrosine kinase activity, Cell 61, 203.

Wang, C.G., T. Du, L.J. Xu and J.G. Martin, 1993, Role of leukotriene D4 in allergen-induced increases in airway smooth muscle in the rat, Am. Rev. Respir. Dis. 148, 413.

Yamaguchi, T., H. Kohorogi, I. Honda, O. Kawano, M. Sugimoto, S. Araki and M. Ando, 1992, A novel leukotriene antagonist, ONO-1078, inhibits and reverses human bronchial contraction induced by leukotrienes C4 and D4 and antigen in vitro, Am. Rev. Respir. Dis. 146, 923.

Yang, S.G. and M.D. Hollenberg, 1991, Distinct receptors for epidermal growth factor-urogastrone in cultured gastric smooth muscle cells, Am. J. Physiol. 260, G827.

Yang, S.G., M. Saifeddine, M. Chuang, D. Severson and M.D. Hollenberg, 1991, Diacylglycerol lipase and the contractile action of epidermal growth factor-urogastrone: evidence for distinct signal pathways in a single strip of gastric smooth muscle, Eur. J. Pharmacol. 207, 225.

Yang, S.G., M. Saifeddine and M.D. Hollenberg, 1992, Tyrosine kinase inhibitors and the contractile action of epidermal growth factor-urogastrone and other agonists in gastric smooth muscle, Can. J. Physiol. Pharmacol. 70, 85.

Yoshimoto, T., C. Yokoyama, K. Ochi, S. Yamamoto, Y. Maki, Y. Ashida, S. Terao and M. Shiraishi, 1982, AA861, a selective inhibitor of the 5-lipoxygenase reaction and the biosynthesis of slow-reacting substance of anaphylaxis, Biochim. Biophys. Acta 713, 470.