Amiloride-induced contraction of isolated guinea pig, mouse, and human fetal airways

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Christ, Michael J., Lynn M. Iwamoto, Asoka de Silva, Sarah L. Lavallee, and Kenneth T. Nakamura. Amiloride-induced contraction of isolated guinea pig, mouse, and human fetal airways. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R209–R213, 1998.—Nebulized amiloride has been proposed as therapy in cystic fibrosis to block Na+/H+ exchange and prevent dehydration of secretions. Patients with cystic fibrosis often have reactive airways. Bovine and canine trachea relax to amiloride in vitro, suggesting another benefit as a bronchodilator, whereas guinea pig trachea, a useful model of human airways, does not. We hypothesized that human airways would respond like guinea pig airways. Airway ring segments from guinea pigs, mice, and human fetuses were constricted with the concentration of acetycholine producing 50–75% maximum contraction. Subsequent changes in isometric tension to cumulative additions of amiloride (10–6–10–4 M) were measured. Guinea pig airways contracted 29 ± 5%, mouse airways contracted 23 ± 6%, and human fetal airways contracted 30 ± 8%. Contraction to amiloride was mimicked by dimethylamiloride, a more selective inhibitor of the Na+/H+ antiporter, and was attenuated by protein kinase C (PKC) inhibition with GF109203X and staurosporine. The present study indicates that amiloride-induced airway contraction in guinea pigs and mice closely parallels the response in isolated human airways and that the mechanism may involve the Na+/H+ antiporter and PKC.

Airway smooth muscle; cystic fibrosis; diuretics; Na+/H+ exchange; protein kinase C

Amiloride, a K+-sparking diuretic, inhibits Na+/H+ exchange throughout the body, including airway epithelium. It has been proposed as aerosolized therapy in patients with cystic fibrosis to block Na+/H+ hyperabsorption in airway epithelium and prevent dehydration of secretions (13), thus preventing dehydration of mucus and improving ciliary clearance (32). Patients with cystic fibrosis often have reactive airways (27), and amiloride produces relaxation of adult bovine (15) and canine (17) airways in vitro, suggesting another potential benefit as a bronchodilator. However, amiloride does not relax guinea pig trachea (6). Because of occasional species-specific differences, it is important to study human airway directly, when possible, before applying information learned from animal models to human conditions (2).

Guinea pig trachea is a good model for human central airways (4, 23), and it has been useful in studying the role of other ion transporters in regulating airway smooth muscle reactivity (11, 18, 31). Therefore, we hypothesized that the effect of amiloride on human airway tone would parallel that in guinea pigs. Because there is now a transgenic mouse model for cystic fibrosis that may be useful in future experiments (28), we also collected baseline data on mice.

Thus the present study was designed to meet several objectives: 1) to further examine the effect of amiloride on guinea pig and mouse airway tone in vitro, 2) to compare that to the effect on human airways, and 3) to further define amiloride’s cellular mechanism of action. We found that guinea pig, mouse, and human fetal airways all contracted to amiloride. Contraction to amiloride was mimicked by dimethylamiloride, a more selective inhibitor of the Na+/H+ antiporter, and was attenuated by inhibition of protein kinase C (PKC) with GF109203X and staurosporine.

METHODS

This study was approved by the Animal Care and Use Committee, Tripler Army Medical Center, and the Human Use Committees at Tripler Army Medical Center, Kapiolani Medical Center for Women and Children, and the University of Hawaii. Procedures involving guinea pigs and mice were performed in accordance with the National Institutes of Health policies, Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205], and the Animal Welfare Act and Amendments. Tripler Army Medical Center is accredited by the American Association for Accreditation of Laboratory Animal Care.

Tissue preparation. Dunkin-Hartley albino guinea pigs were obtained from the Charles River Breeding Laboratory (Wilmington, MA) and studied at 4–9 days (newborns) or 6 wk (young adults) of age. C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and studied at 4–9 days (newborns) or 6–10 mo (adults) of age. Human fetal tissue between 11 and 16 wk gestation was obtained from the Laboratory for Human Embryology (University of Washington, Seattle) and from Kapiolani Medical Center for Women and Children (University of Hawaii, Honolulu). Fetuses with known congenital or chromosomal abnormalities were excluded. Samples were shipped on ice in sterile Hanks’ balanced salt solution, arriving in our laboratory within 36 h postmortem.

Guinea pigs were sedated with 25 mg/kg ketamine hydrochloride (Vetalar; Parke-Davis, Morris Plains, N J.), and animals were euthanized with either 1 g/kg (newborns) or 500 mg/kg (adults) pentobarbital sodium (Wyeth, Philadelphia, PA). Airway tissues (extrathoracic trachea from guinea pigs and mice, intrathoracic trachea and mainstem bronchi from human fetuses) were removed, dissected free of connective tissue, cut into 3- to 4-mm ring segments, and placed into a physiological buffer solution [pH 7.4, containing (in mM) 5 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 140
NaCl, 4.5 KCl, 10 d-glucose, 1.5 CaCl$_2\cdot$2H$_2$O, and 1 MgCl$_2\cdot$6H$_2$O.

In some tissues, epithelium was denuded by gentle rubbing with a wooden probe (30). Ring segments were mounted lengthwise on two fine stainless steel wires passed through the lumen and then connected to a Grass FT.03 force transducer (Grass, Quincy, MA) coupled to a Gould 2600S pen recorder (Gould, Cleveland, OH) for continuous measurement of isometric tension. Ring segments were suspended in 25 ml organ baths (37°C), bubbled continuously with 100% O$_2$, and equilibrated for 1–2 h, with bath changes every 15 min. Segments were primed with a concentration producing 50–75% maximum contraction (EC$_{50-75}$) of acetylcholine (ACh) during the last two bath changes (20).

Experimental protocol. Initially, a concentration-response curve to 10$^{-6}$-10$^{-4}$ M ACh was constructed for each tissue for determination of the EC$_{50-75}$. The EC$_{50-75}$ for each species was relatively constant and reproducible within that species (guinea pigs and mice: 3 x 10$^{-6}$ M; human fetuses: 10$^{-5}$ M). For guinea pigs, this value is consistent with reports published previously (11, 18, 30, 31). When EC$_{50-75}$ values were determined to be reproducible and consistent with previous values, these concentrations were then used in subsequent experiments without repeating the concentration-response curves for each tissue.

Airway ring segments were stretched to their optimum resting tension (ORT) as determined by maximal force developed to an EC$_{50-75}$ of ACh. Preparations were washed and ORT was reestablished before all procedures. Ring segments were constricted with an EC$_{50-75}$ of ACh, and, after stable tension was obtained, changes in isometric tension to cumulative additions of amiloride (10$^{-8}$-10$^{-4}$ M) were measured. In some tissues, changes in isometric tension to amiloride were measured after selective inhibition of PKC with either 10 µM GF109203X (33) or 0.1 µM staurosporine (35). In others, changes in isometric tension to cumulative additions of dimethylamiloride (10$^{-8}$-10$^{-4}$ M) were measured. Tissues were then blotted dry, and weights were determined after drying in a 59°C oven for at least 5 days.

Drugs. The following drugs were used: ACh chloride, amiloride, staurosporine (Sigma Chemical, St. Louis, MO); GF109203X (Research Biochemicals International, Natick, MA); and dimethylamiloride (Merck Research Laboratories, Rahway, NJ). All aliquots of amiloride and dimethylamiloride were dissolved in dimethyl sulfoxide and frozen for storage. Solutions were prepared in distilled water on the day of each experiment. Solutions of ACh were kept on ice. Doses were administered in 100 µl aliquots, and drug concentrations are expressed as the final molarity in the 25-ml bath.

Statistical analysis. Data analysis was performed using a commercial software package (SigmaStat, Jandel, San Rafael, CA). Normality of data distribution was determined by the Kolmogorov-Smirnov test, and concentration-response data were compared using two-way analysis of variance (ANOVA) for repeated measures with Newman-Keuls multiple-comparison procedure. If parametric parameters were not met, data were ranked and then analyzed. Several airway segments (trachea and mainstem bronchi) from each fetus were studied. Responses for each fetus were averaged, and only the averaged value for each fetus was included in the data analysis. Responses to single-dose amiloride were compared using one-way ANOVA. P values < 0.05 were considered significant, and values are expressed as means ± SE.

RESULTS

In newborn guinea pig airways stretched to ORT without preconstriction, amiloride produced contraction at concentrations of 10 µM or greater, increasing tension from baseline to a maximum of 0.4 ± 0.1 g/mg dry wt (data not shown). After inducing airway tension with an EC$_{50-75}$ of ACh (Fig. 1A), amiloride produced contraction in all three species. Newborn guinea pig airways contracted 29 ± 5% at maximum (n = 16), and newborn mouse airways contracted 23 ± 6% (n = 7). Human fetal airways also contracted, despite a baseline loss of ACh-induced tension. When tension at 10 µM is normalized to baseline in human fetal airways, segments contracted 30 ± 8% to higher concentrations of amiloride (n = 9 airway segments from 4 fetuses). Control tissues to which amiloride was not added continued to lose tension over time (Fig. 1B).

Among guinea pigs and mice, airway segments from newborns and adults contracted similarly. Newborn and young adult guinea pig airways contracted 41 ± 10 (n = 9) and 46 ± 5% [n = 6, not significant (NS)], respectively, and newborn and adult mouse airways contracted 16 ± 9 (n = 7) and 23 ± 7% (n = 5, NS), respectively. There was no difference in contraction after removal of airway epithelium. Newborn guinea pig airways with intact epithelium contracted 29 ± 5% (n = 16), and those without epithelium contracted 40 ± 9% (n = 9, NS). Similarly, newborn guinea pig airways with intact epithelium contracted 59 ± 10% (n = 6), and those without epithelium contracted 46 ± 5% (n = 6, NS).

Airway contraction to cumulative additions of amiloride was significantly attenuated by selective inhibition of PKC (Fig. 2) with GF109203X (10 µM) and staurospo-
rine (0.1 µM). After incubation with GF109203X and staurosporine (n = 6; B) in newborn guinea pigs. Changes in airway tone are expressed as a percent of the contraction produced by an EC_{50–75} of ACh, and values are expressed as means ± SE. *P < 0.05 compared with controls, using 2-way ANOVA for repeated measures with Newman-Keuls multiple-comparison procedure.

In addition, inhibition of PKC with GF109203X attenuated contraction to amiloride in a dose-dependent manner (Fig. 3). Newborn guinea pig airways contracted 57 ± 5% (n = 5) to a single dose of 100 µM amiloride, but contraction was inhibited by incubation with GF109203X. This inhibition achieved statistical significance at 10 µM concentrations, which contracted only 33 ± 7% (n = 6, P < 0.04).

The contractile effect of amiloride on newborn guinea pig airways was mimicked by dimethylamiloride, a more selective inhibitor of the Na^+/H^+ antiporter (Fig. 4). After constriction with an EC_{50–75} of ACh, newborn guinea pig airways contracted 41 ± 5% to amiloride (n = 10) and 46 ± 4% to dimethylamiloride (n = 10).

**DISCUSSION**

Knox and co-workers (15) found that amiloride causes concentration-dependent relaxation of adult bovine tracheal strips constricted with carbachol, and Krampetz and Bose (17) demonstrated relaxation of adult canine tracheal smooth muscle strips contracted isometrically with carbachol. These findings have suggested that amiloride may have an additional benefit as a bronchodilator in patients with reactive airways. In contrast, Cortijo and coworkers (6) show that guinea pig tracheal strips stretched to ORT contract to amiloride. In the present study, we found that amiloride produced contraction of guinea pig airways stretched to ORT, as well as contraction of guinea pig and mouse airways contracted isometrically with ACh. Thus we felt it was important to study human airway directly.

Airway responsiveness in neonates remains poorly investigated, in part because newborn human airway cannot be obtained as readily as specimens from adults (9). Although animal airway tissues are usually studied immediately after removal, human airway tissue is often obtained after some delay, and the issue of anoxic damage may be problematic (10). We attempted to attenuate these changes by incubating the airway segments for up to 2 h in an oxygenated buffer solution before each experiment. There was no difference in contraction between human fetal tissue obtained from the Laboratory for Human Embryology (Seattle) and studied within 36 h postmortem and that obtained from Kapiolani Medical Center for Women and Children (Honolulu) and studied within 1–2 h postmortem (data not shown). Similar to De Jongste and co-workers (7), we found that anoxic changes were largely reversible using this method, without losing tissue viability.

However, a baseline inability of human fetal airways to sustain ACh-induced contraction was noted and may be related to the extreme prematurity of the human fetuses. Compared with human fetal controls to which
amiloride was not added (Fig. 1B), both groups lost tone similarly up to 10^{-5} M amiloride. At higher concentrations, airway tone increased in the experimental group, but the control group continued to lose tension. Taken together, these results indicate that the loss of baseline tone in the human fetuses was not an amiloride effect, and that amiloride produced reproducible airway contraction at concentrations of 10 µM or greater in all three species.

Significant differences in airway responsiveness to several agonists have been noted during ontogeny (3, 8, 30, 31). Fetal, newborn, and adult responses may not always be comparable. Although this was not a true ontogenetic study, there was no difference in amiloride-induced airway contraction between newborns and adults for guinea pigs and mice.

Much has been learned about the role of ion channels in regulating airway smooth muscle tone, but the mechanisms by which amiloride exerts its effect remain unclear despite speculation that specific ion channels are involved (14–16). Amiloride is known to inhibit several cellular processes, including the amiloride-sensitive Na+ channel, several protein kinases, the Na+/H+ antiporter, and Na+/Ca2+ exchange (12). The role of each of these in regulating airway tone remains uncertain.

There is convincing evidence that the amiloride-sensitive Na+ channel does not regulate airway tone. A half-maximal inhibition (IC_{50}) of only 1 µM amiloride is needed to inhibit this channel (12), but at least 10 µM was needed to affect airway tone. The channel is also located in airway epithelium, but contraction to amiloride was unaffected by removal of epithelium. In addition, the gene for this channel was recently cloned, and although no specific transcripts were identified in first- or second-trimester human fetuses (19), we observed airway contraction to amiloride as early as 11 wk gestation.

Studies also suggest that amiloride inhibits several protein kinases, including PKC (14, 29), which are involved in phosphorylation of smooth muscle contractile proteins to maintain contraction. Thus inhibition of PKC is expected to produce smooth muscle relaxation. This mechanism has been suggested to explain the relaxation to amiloride that has been described in bovine and canine airways. However, we found that amiloride produced contraction in guinea pig, mouse, and human fetal airways, and that inhibition of PKC with GF109203X and staurosporine attenuated this contraction. Taken together, these findings suggest that amiloride-mediated airway contraction may be, at least in part, mediated by PKC activation in these species.

Other researchers suggest that neither Na+/H+ nor Na+/Ca2+ exchange is directly involved in regulating airway tone. The IC_{50} for these channels is ~10 µM, consistent with observations that at least 10 µM amiloride is needed to affect airway tone, but amiloride analogs that are more specific for each of these channels do not affect airway tone in bovine airways in vitro (14). In addition, amiloride is only a weak inhibitor of Na+/Ca2+ exchange, and this mechanism is unlikely to be important in regulating airway tone (12). However, airway contraction to amiloride was mimicked by dimethylamiloride, a more specific analog for the Na+/H+ antiporter, suggesting that the Na+/H+ antiporter may be, at least in part, involved. Evidence has been presented elsewhere showing that Na+/H+ exchange may be linked to PKC (5) activation, which may be a potential mechanism of amiloride-mediated airway contraction. High concentrations of amiloride were studied to examine the full concentration-response pattern that might be achieved clinically (34). It is important to consider that airway contraction to amiloride was only noted at concentrations of 10 µM or greater, where the specificity of amiloride’s mechanism of action is less clear. Thus, although the Na+/H+ antiporter and PKC may partially account for the airway contractile effects, other mechanisms may also be involved.

It is also important to note that human in vivo studies have not demonstrated airway constriction to amiloride in normal or asthmatic subjects, or in patients with cystic fibrosis (1, 21, 22, 24–26, 32). However, these studies were not designed to specifically examine changes in airway resistance. Knox and co-workers (16) performed a double-blind, placebo-controlled study in normal and asthmatic patients, showing no change in forced expiratory volume in one second to either oral or inhaled amiloride. However, the authors note that they may not have obtained sufficient drug levels in bronchial smooth muscle cells to produce an effect on airway tone (at least 10 µM). They used an oral dose of 20 mg amiloride, which should produce plasma concentrations of only 1 µM. They also used a nebulized dose of 10 mM amiloride, which should produce a concentration of 100 µM in airway surface liquid (34), but the amount of drug that is ultimately absorbed across the bronchial mucosa is unknown. Therefore, additional studies may be indicated to determine the level of amiloride actually achieved in bronchial smooth muscle cells after nebulization to more fully evaluate the potential effect of amiloride on airway tone.

In summary, we found that 1) unlike bovine and canine airways, amiloride produced contraction in guinea pig, mouse, and human fetal airways; 2) removal of epithelium did not alter the contraction; 3) contraction was attenuated by inhibition of PKC with GF109203X and staurosporine; and 4) contraction was mimicked by dimethylamiloride, a more selective amiloride analog for the Na+/H+ antiporter. Taken together, these results suggest that the mechanism of amiloride-mediated airway contraction in these species may involve, at least in part, both the Na+/H+ antiporter and PKC activation.

Perspectives

Although the use of animal models is important in providing knowledge about mechanisms of airway smooth muscle physiology and pharmacology, extrapolation of animal findings to human conditions may be limited by species-specific differences in airway smooth muscle reactivity, unless a direct comparison to humans is made.
REFERENCES


