Nitrogen dioxide (NO₂) is a free radical formed by combustion processes in air. An increased incidence of airway inflammation and reactivity are associated with exposure to elevated indoor levels of this reactive gas (1–3). The toxicity of NO₂ has been attributed to the oxidation of cellular constituents and generation of reactive compounds that initiate cellular injury (4). Free radical oxidation of cellular constituents such as polysaturated fatty acids (PUFA) can lead to the generation of aldehydeic compounds. Oxidation of sugars may also be an important source of aldehydeic compounds (5). These aldehydes may bind with essential cellular proteins to impair or inhibit activity, ultimately leading to adverse effects on cell function and viability. In the present study, we have examined the effects of NO₂, a potent oxidant gas, on generation of aldehydeic compounds from airway epithelial monolayers and the actions of a predominant aldehydeic product on Na⁺,K⁺-ATPase (sodium pump), an enzyme that is vital to cellular function and viability.

The predominant reaction of NO₂ may be to react with PUFA by abstraction of an allylic hydrogen (6,7). The epithelium lining the lung airways, which possesses a significant content of PUFA (8), represents one of the first major targets of NO₂ and receives a large burden of the exposure (9–11). The mucin layer overlying airway epithelial cells also possesses a significant content of PUFA, as well as other compounds such as sugars, which may potentially interact with this gas (12). Lipid radicals, generated by hydroxide abstraction, in the presence of molecular oxygen initiate free radical chain autoxidation of membrane PUFA (5–7). The first product of autoxidation is a lipid hydroperoxide. These hydroperoxides may decompose to form a number of products, including reactive aldehydes (13). Aldehydes have longer half lives and possess significant cytotoxicity (13).

Cytotoxic effects attributed to aldehydes include depletion of intracellular glutathione; decreased protein thiol; onset of lipid peroxidation; disturbance of calcium homeostasis; inhibition of DNA, RNA, and protein synthesis; and inhibition of cellular respiration and glycolysis (13–15). Oxidation products derived from either the mucin layer or the apical airway epithelial cell plasma membrane may bind with cellular proteins and subsequently impair or inhibit cellular function.

In the present study, we have examined the generation of aldehydeic compounds from guinea pig tracheobronchial epithelial (GPTE) monolayers exposed to NO₂ levels associated with indoor exposure. GPTE cells were cultured in an air interface to form tight monolayers that actively transport ions and solutes vectorially (16–19). These cell monolayers were acutely exposed for 1 hr to NO₂ (1 or 5 ppm) and the release of aldehydes into the basolateral fluid was examined. We have identified glycolaldehyde, a two carbon α-hydroxyaldehyde, released into the extracellular medium from GPTE monolayers exposed to NO₂. We then evaluated the effects of exogenous glycolaldehyde on epithelial Na⁺,K⁺-ATPase activity through measurements of ouabain-sensitive ⁸⁶Rb⁺ uptake and specific [H]-ouabain binding and compared its actions with those observed for NO₂ exposure.

Materials and Methods

Materials

³H-ouabain (specific activity 24 Ci/mmol) and ⁸⁶RbCl (1.54 mCi/mg) were obtained from New England Nuclear (Boston, MA). Glycolaldehyde dimer and ouabain were obtained from Sigma Chemical Co. (St. Louis, MO). PC-1 (serum-free defined culture medium) was obtained from Hycor Biomedical (Irvine, CA). 2,4-dinitrophenylhydrazine (DNPH), acetaldehyde, and trans-2-nonenal were obtained from Aldrich Chemical Co. (Milwaukee, WI). NO₂ in air at levels of 1 or 5 ppm and compressed air (NO₂ ≤0.1 ppm) were obtained from Airgas (Los Angeles, CA). NO₂ levels in air were certified to be of ±5% analytical accuracy for a period of six months from the date of production. For some experiments, NO₂ levels in air were verified with an NO₂ analyzer, Model 1150SP (Interscan Corp. Chatsworth, CA). Other reagents were obtained from standard commercial sources.

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Preparation of Tracheobronchial Epithelial Cell Monolayers Cultured in an Air Interface
Male Hartley guinea pigs [specific pathogen-free; Crl:(HA)BR], weighing 250–300 g, were obtained from Charles River (Wilmington, MA). Animals were euthanized with sodium pentobarbital (300 mg/kg) given intraperitoneally. Tracheobronchial epithelial cells were isolated following established procedures (16–20). Isolated airway epithelial cells were resuspended in PC-1 medium (16–20), which was supplemented with 2 mM L-glutamine, 100 U penicillin G/ml, 100 μg streptomycin/ml, 0.25 μg amphotericin B/ml, and 50 μg gentamicin/ml. GPTE cells were plated at a density of 1.0 x 10⁶/cm² in 6.5- or 12-mm collagen-treated Transwells (Costar, Cambridge, MA) (16–19). Cells were allowed to adhere to the collagen-treated membrane substratum for 24 hr (16–19). On day 1 (24 hr), basolateral and apical media were removed. To grow cells in an air interface, 0.4 or 0.9 ml PC-1 medium was subsequently added only to the basolateral side of the cell monolayer for 6.5- or 12-mm Transwells, respectively (16–19). Everyday thereafter, fluid on the basolateral surface was replenished with PC-1 culture medium while the apical surface was essentially left nominally fluid free (16–19).

Exposure to Nitrogen Dioxide
Monolayers with similar spontaneous potential difference (SPD) and transepithelial resistance (RTE) values, as measured with the use of a Millicell ERS (Millipore, Bedford, MA), were paired into air or NO₂ exposure groups prior to actual exposure (18). Confluent GPTE monolayers in 12-mm Transwells were rinsed twice with 2 ml (0.5 ml added to the apical side and 1.5 ml to the basolateral side) of Krebs Ringers phosphate-buffered HEPES solution (KRPH) composed of 125 mM NaCl, 5 mM KCl, 1.0 mM MgSO₄, 8.5 mM Na₂HPO₄, 1.4 mM NaH₂PO₄, 5 mM glucose, 1.3 mM CaCl₂, and 10 mM HEPES, pH 7.4, at 37°C (18,19). Following the two rinses, KRPH (0.9 ml) was added to the basolateral side of the monolayer, while the apical side was left fluid free to facilitate contact with air or NO₂. Monolayers in 6.5-mm Transwells were rinsed twice with 1.25 ml (0.25 ml added to the apical side and 1.0 ml to the basolateral side) of KRPH and then 0.4 ml was added to the basolateral side alone. Monolayers were allowed to equilibrate in KRPH for 60 min at 37°C (18,19). GPTE monolayers were then exposed to NO₂ (1 or 5 ppm) in air or air alone using previously described procedures (18,19). Nominal NO₂ levels rather than actual levels have been provided; exposure time was 1 hr.

Identification and Measurement of DNP-carbonyl Compounds
Basolateral fluid or apical rinse fluid containing products liberated from exposed GPTE monolayers were derivatized with DNP using procedures previously described by Eserbauer et al. (21) and Poli et al. (22), as modified by Robison et al. (23). DNP-carbonyl compounds were separated into classes by thin layer chromatography, and individual aldehydes were quantitated through HPLC separation in conjunction with a Perkin Elmer LC 235 UV diode array detector (Perkin Elmer, Norwalk, CT). The HPLC eluate was monitored at 235 and 365 nm. Known amounts of 2-decadional dinitrophenylhydrazone were added as an internal standard for quantitation of DNP-derivatized carbonyl compounds. The concentrations of DNP derivatives were estimated by UV analysis at 365 nm using ε = 2.8 x 10⁴ M⁻¹cm⁻¹ at 365 nm (21). The concentration of the Di-DNP (Di-dinitrophenylhydrazone) derivative of glycolaldehyde was determined using ε = 4.14 x 10⁴ M⁻¹cm⁻¹ at 365 nm (personal communication, M. Thomas).

Effect of Glycolaldehyde on Airway Epithelial Bioelectric Properties
Airway epithelial viability in response to glycolaldehyde treatment was evaluated through measurements of bioelectric properties (short circuit current and RTE). Monolayers were mounted in Ussing chambers (Precision Instrument Design, Tahoe City, CA) and allowed to equilibrate for 15 min in 10 ml of KRPH on both sides (18). Glycolaldehyde was added to either the apical or basolateral bathing fluid at concentrations ranging from 0.1 to 20 mM. Short circuit current (SCC) was measured with an automatic voltage/current clamp (DVC-1000, World Precision Instruments, Sarasota, FL) (18). The monolayer was continuously short circuited except for a brief period every 30 sec when a constant voltage pulse (DV = 2 mV) was imposed for 3 sec to yield a current response (dI). Rₑ was estimated from the relation of DI/dt and SPD estimated from RTE x SCC (18). Bioelectric properties were monitored for 30 min following addition of glycolaldehyde.

Measurement of Airway Epithelial Ouabain-sensitive ⁸⁶Rb Uptake
To estimate the Na⁺,K⁺-ATPase activities of glycolaldehyde-treated airway epithelial monolayers, we measured ⁸⁶Rb (K⁺ surro-
with the Crunch Interactive Statistical Package (Crunch, San Francisco, CA). A comparison of factor means determined to be statistically different was compared with the post hoc Tukey A test. A p value ≤ 0.05 was considered significant.

Results

Generation of Carboxyl Compounds in Response to NO₂ Exposure

HPLC separation of zone 2 carbonyl compounds indicated that a major aldehydic compound released into the basolateral fluid from NO₂-exposed GPTE monolayers was the Di-DNP derivative of glycolaldehyde (Fig. 1). Derivatization with DNP, TLC separation, and HPLC retention time of this compound generated from GPTE monolayers are consistent with previous identification of glycolaldehyde in this laboratory using identical techniques and confirmation of structure with the use of gas chromatography/mass spectrometry (23,28). The Di-DNP derivative of glycolaldehyde was present as two peaks, the first with a retention time of 31.8 min, which was the oxidized form that has lost two hydrogens, and the second at 33.3 min, which was the reduced form (23). A DNP derivative of authentic glycolaldehyde was found to co-elute with these two peaks obtained from an extract of the basolateral fluid treated with DNP. The oxidized form of glycolaldehyde was observed in air controls; however, the reduced form was not present. The DNP derivative of decadienal, with a retention time at 47.2 min, was added as an internal standard for quantitation. Glycolaldehyde levels were significantly increased in response to a 1-hr exposure to 1 or 5 ppm NO₂, as compared with air controls (Fig. 2). Release of glycolaldehyde toward the apical side was below the detection limit.

Effect of Glycolaldehyde on Bioelectric Properties

The effects of exogenous glycolaldehyde on GPTE bioelectric properties (e.g., SCC and R_TE) were evaluated (Table 1). GPTE monolayers mounted in Ussing chambers were bathed with KRPH on both sides, and glycolaldehyde (2 to 20 mM) was added to the basolateral fluid. Bolus addition of glycolaldehyde at concentrations <1 mM had minimal effects on the SCC or R_TE.

Effect of Glycolaldehyde on Ouabain-sensitive ⁸⁶Rb Uptake

Treatment of the basolateral aspect of GPTE monolayers with 20 mM glycolaldehyde transiently increased ouabain-sensitive ⁸⁶Rb uptake at 5 min; however, at 15 and 30 min, uptake was significantly decreased below control levels (Fig. 3). Concentrations of 5 or 10 mM did not alter ⁸⁶Rb uptake at 5 min in comparison with control; however, the uptake at 30 min was significantly decreased.

Effect of Glycolaldehyde on Na⁺,K⁺-ATPase Density and Affinity

The specific ³H-ouabain binding capacity (B_max) and binding constant (K_D) for basolateral Na⁺,K⁺-ATPase were measured in response to basolateral glycolaldehyde treatment. A representative Scatchard plot for a 15-min treatment of GPTE monolayers with 0 or 20 mM glycolaldehyde is shown in Figure 4. The B_max value for monolayers treated with 20 mM glycolaldehyde at 15.4 ± 1.6 pmol/mg protein was unchanged from the control at 16.4 ± 0.5. The K_D value of 0.78 ± 0.15 μM for GPTE monolayers treated with 20 mM glycolaldehyde was unchanged from the control value of 0.85 ± 0.07 μM. Four binding analyses
were performed for the control and four for 20 mM glycolaldehyde.

Discussion

In the present study, a significant generation of aldehydes from airway epithelial monolayers exposed to NO₂ was detected. Glycolaldehyde, an α-hydroxyaldehyde, was a major aldehydeic product generated from exposure of GPTE monolayers to NO₂. Glycolaldehyde was also identified as a major aldehydeic product released from alveolar macrophages exposed to NO₂ (23,28). Preliminary studies using ³H-arachidonic acid-labeled monolayers suggest that glycolaldehyde may be derived from cellular lipids. A recent report has identified a number of α-hydroxyaldehydes derived from peroxidation of arachidonic acid (29). Glycolaldehyde may be formed from PUFA through a similar oxidative mechanism. Another possible route for the formation of glycolaldehyde may be through the decomposition of sugars (5,30,31); however, the reactivity of NO₂ with sugars is known to be much slower than that found with PUFA. Glycolaldehyde is reactive with proteins, rapidly forming Schiff base adducts with amino groups (14,15). These Schiff base adducts can undergo Amadori rearrangement to yield a new aldehyde moiety that can form a Schiff base adduct with another amino group (14). This process can modify the physical properties of proteins as well as generate intra- and intermolecular crosslinking in or between proteins, respectively (14).

Basolateral glycolaldehyde concentrations estimated in the present studies may be lower than cellular concentrations due to diffusion of this compound into the larger volume of the extracellular medium, as well as losses due to binding with cellular components, metabolism, and volatility (23). In our preliminary studies, treatment of GPTE monolayers with exogenous hexanal yielded a recovery of only 17%. Based upon a measure of approximately 5 × 10⁵ cells per confluent monolayer, the total cellular volume is estimated to be about 0.5 μl. Thus, the cellular glycolaldehyde concentration for a 1-hr exposure to 5 ppm NO₂ was calculated to reach a maximum of approximately 3 mM. Assuming continuous generation of glycolaldehyde during the 60-min exposure, the production rate could have been as much as 0.05 mM/min.

The effect of exogenous glycolaldehyde on GPTE ouabain-sensitive basolateral Na⁺,K⁺-ATPase was examined using approximated cellular concentrations of this compound. Treatment with 20 mM glycolaldehyde, transiently stimulated ouabain-sensitive ⁸⁶Rb uptake at 5 min; however, uptake was inhibited at 30 min with concentrations ≥5 mM. Changes of airway epithelial bioelectric properties (RTE and SCC) in response to glycolaldehyde treatment were small and do not suggest a major loss of cell viability. The inhibition of ouabain-sensitive ⁸⁶Rb uptake in response to glycolaldehyde treatment was not associated with any alterations of ouabain binding characteristics, suggesting that aldehyde binding directly to Na⁺,K⁺-ATPase was either not involved in the observed changes of activity or occurs at sites distant from ouabain binding. These data indicate that the effects of exogenous glycolaldehyde differ from that observed with a 1-hr exposure to 1 or 5 ppm NO₂ where a twofold increase of ouabain-sensitive ⁸⁶Rb uptake was observed for 2 hr postexposure (19) and glycolaldehyde was presumably generated at a continuous low level. The dynamics associated with bolus addition of glycolaldehyde appear to be quite different from those of cellular glycolaldehyde generation in response to NO₂ exposure. These differences may include alterations in access to essential cellular targets, binding to nonessential targets such as the collagen matrix, and metabolism by various cellular enzymes such as aldehyde dehydrogenase (32,33). These results with exogenous glycolaldehyde may suggest that other lipid peroxidation products are probably mediating the actions of this reactive gas at levels associated with elevated indoor exposure.
Glycolaldehyde may have significant long-term pathological consequences to cell function and viability with regard to the formation of glycation products (34). Through a process known as glycation, protein crosslinking occurs nonenzymatically and involves reaction of a compound such as glycolaldehyde with the amino group of a protein (34). These reactive Amadori products may then bind with amino groups on other proteins to form advanced glycation end products (AGEs) through intermolecular crosslinks (34). This process may interfere with cell-to-cell attachment as well as cell adhesion to the basement membrane, potentially resulting in significant disruption of the epithelial barrier and development of increased airway reactivity.

In summary, we have found that glycolaldehyde, an aldehydic compound, is generated in elevated levels from airway epithelial monolayers acutely exposed to NO₂⁻, in the range associated with elevated indoor levels. Exogenous glycolaldehyde does not appear to play a significant role in the previously observed NO₂⁻-induced enhancement of airway epithelial Na⁺,K⁺-ATPase activity, suggesting that the alteration of pump activity is most likely mediated by other lipid peroxidation products yet to be identified. Glycolaldehyde may have significant long-term pathological consequences with regard to disruption of the epithelial barrier through its known reactivity with cellular proteins.

REFERENCES

1. Frampton MW, Morrow PE, Cox G, Gibb FR, Speers DM, Utell MJ. Effects of nitrogen dioxide exposure on pulmonary function and airway reactivity in normal humans. Am Rev Respir Dis 143:522–527 (1991).
2. Mohsenin V. Airway responses to nitrogen dioxide in asthmatic subjects. J Toxicol Environ Health 22:371–380 (1987).
3. Mohsenin V. Airway responses to 2.0 ppm nitrogen dioxide in normal subjects. Arch Environ Health 43:242–246 (1988).
4. Thomas HV, Mueller PK, Lyman RL. Liperoxidation of lung lipids in rats exposed to nitrogen dioxide. Science 195:532–534 (1968).
5. Pearson AM, Chen C, Gray JJ, Aust SD. Mechanism(s) involved in meat mutagen formation and inhibition. Free Radical Biol Med 13:161–167 (1992).
6. Pryor WA, Lightsey JW. Mechanisms of nitrogen dioxide reactions: initiation of lipid peroxidation and the production of nitrous acid. Science 214:435–437 (1981).
7. Pryor WA, Lightsey JW, Church DF. Reaction of nitrogen dioxide with alkanes and polyunsaturated fatty acids: addition and hydrogen abstraction mechanisms. J Am Chem Soc 104:6685–6692 (1982).
8. Holtzman MJ, Grunberger D, Hunter JA. Phospholipid fatty acid composition of pulmonary airway epithelial cells: potential substrates for oxygenation. Biochim Biophys Acta 877:459–464 (1986).
9. Adler KB, Fischer BM, Wright DT, Cohn LA, Becker S. Interactions between respiratory epithelial cells and cytokines: relationship to lung inflammation. Ann N Y Acad Sci 725:128–145 (1994).
10. Postlethwait EM, Bidani A. Reactive uptake governs the pulmonary air space removal of inhaled nitrogen dioxide. J Appl Physiol 68:594–603 (1990).
11. Postlethwait EM, Langford SD, Bidani A. Reactive absorption of nitrogen dioxide by pulmonary epithelial lining fluid. J Appl Physiol 69:523–531 (1990).
12. Cavanagh DG, Morris JB. Mucus protection and airway peroxidation following nitrogen dioxide exposure in the rat. J Toxicol Environ Health 22:313–328 (1987).
13. Witz G. Biological interactions of α,β-unsaturated aldehydes. Free Rad Biol Med 7:333–349 (1989).
14. Acharya AS, Manning JM. Reaction of glycolaldehyde with proteins: latent crosslinking potential of α-hydroxycarboxaldehydes. Proc Natl Acad Sci USA 80:3590–3594 (1983).
15. Manning LR, Manning JM. Influence of ligation state and concentration of hemoglobin A on its cross-linking by glycolaldehyde: functional properties of cross-linked, carbosymethylated hemoglobin. Biochemistry 27:6640–6644 (1988).
16. Robinson TW, Dorio RD, Kim KJ. Formation of tight monolayers of guinea pig tracheobronchial epithelial cells cultured in an air-interface: bioelectric properties. Biotechniques 15:468–473 (1993).
17. Robinson TW, Kim KJ. Air-interface cultures of guinea pig airway epithelial cells: effects of active sodium and chloride transport inhibitors on bioelectric properties. Exp Lung Res 20:101–118 (1994).
18. Robinson TW, Kim KJ. Dual effect of nitrogen dioxide on barrier properties of guinea pig tracheobronchial epithelial monolayers cultured in an air interface. J Toxicol Environ Health 44:57–71 (1996).
19. Robinson TW, Kim KJ. Enhancement of airway epithelial Na⁺K⁺-ATPase activity by NO₂⁻ and the protective role of nordihydroguaiaretic acid. Am J Physiol: Lung Cell Mol Physiol 270:L266–L272 (1996).
20. Adler KB, Schwartz JE, Whitcutt MJ, Wu R. A new chamber system for maintaining differentiated guinea pig respiratory epithelial cells between air and liquid phases. Biotechniques 5:462–465 (1987).
21. Esterbauer H, Cheeseman KH, Dianzani MU, Poli G, Slater TF. Separation and characterization of the aldehydic products of lipid peroxidation stimulated by ADP·Fe²⁺ in rat liver microsomes. Biochem J 208:129–140 (1982).
22. Poli G, Dianzani MU, Cheeseman KH, Slater TF, Lang J, Esterbauer H. Separation and characterization of the aldehydic products of lipid peroxidation stimulated by carbon tetrachloride or ADP·iron in isolated rat hepatocytes and rat liver microsomal suspension. Biochem J 227:629–638 (1995).
23. Robison TW, Forman HJ, Thomas MJ. Generation of aldehydes from rat alveolar macrophages exposed to low concentrations of nitrogen dioxide. Biochim Biophys Acta 1256:334–340 (1995).
24. Kim KJ, Roh DJ. Asymmetric effects of H₂O₂ on alveolar epithelial barrier properties. Am J Physiol 264:L308–L315 (1993).
25. Hansen O. Facilitation of ouabain binding to (Na⁺+K⁺)-ATPase by vasodilate at in vivo concentrations. Biochim Biophys Acta 69:265–269 (1979).
26. Norgiaard A, Kjeldsen K, Hansen O, Clausen TA. Simple and rapid method for the determination of the number of H₂O₂-ouabain binding sites in biopsies of skeletal muscle. Biochem Biophys Res Commun 111:319–325 (1983).
27. Kumar S, Berg JA, Katz AI. Na,K pump abundance and function in MDCK cells: effect of low ambient potassium. Renal Physiol Biochem 14:19–27 (1991).
28. Thomas MJ, Robison TW, Samuel M, Forman HJ. Detecting and identifying volatile aldehydes as dinitrophenylhydrazones using gas chromatography mass spectrometry. Free Rad Biol Med 18:553–557 (1995).
29. Loidl-Stahlhufen A, Spiteller G. α-Hydroxyaldehydes, products of lipid peroxidation. Biochim Biophys Acta 1211:156–160 (1994).
30. Thorsen P, Tjem A. The production of free radicals during the autoxidation of monosaccharides by buffer ions. Carbohydr Res 134:191–204 (1984).
31. Thorsen P, Wolff S, Crabbé J, Stern A. The autoxidation of glyceraldehyde and other simple monosaccharides under physiological conditions catalyzed by buffer ions. Biochim Biophys Acta 797:276–287 (1984).
32. Henhech GTM, Tipton KF. Steady-state kinetic analysis of aldehyde dehydrogenase from human erythrocytes. Biochem J 287:145–150 (1992).
33. Ting HH, Crabbé MJ. Bovine lens aldehyde dehydrogenase. Kinetics and mechanism. Biochem J 215:361–368 (1983).
34. Fong Y, Edelstein D, Wang EA, Brownlee M. Inhibition of matrix-induced bone differentiation by advanced glycation end products in rats. Diabetologia 36:802–807 (1993).