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Effects of Various Reactive Oxygen Species on the Guinea Pig Trachea and Its Epithelium

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ABSTRACT—Reactive oxygen species (ROS) are key factors playing important roles in tissue damage of airways under different pathological conditions. Effects of ROS (superoxide anion, H_2O_2 and hydroxyl radical) were recorded on isometric tension of intact and epithelium denuded, not precontracted guinea pig trachea. Superoxide anion was produced by xanthine/xanthine oxidase and hydroxyl radical either by $FeSO_4/H_2O_2$ or $FeSO_4$/ascorbic acid. In intact preparations, the muscle tension was unaffected by superoxide anion, while H_2O_2 and hydroxyl radical produced a biphasic response, contraction followed by relaxation. Both the amplitude and duration of contractions evoked by H_2O_2 were larger than those caused by hydroxyl radical producing systems. On denuded tracheal strips, superoxide anion elicited also a biphasic response, and the H_2O_2 and hydroxyl radical produced contractions were of higher amplitude and of longer duration than in intact tissues. Indomethacin pretreatment enhanced or slightly reduced the amplitude of contractions evoked by both H_2O_2 and hydroxyl radical on the intact and denuded preparations, respectively. Moreover, the duration of contractions of the trachea induced by oxidative systems was prolonged. Indomethacin did not affect the action of superoxide anion on the intact tissues and reduced the amplitude of the biphasic response on denuded ones. Nordihydroguaiaretic acid pretreatment did not alter the responses elicited by ROS in intact preparations and reduced their action on the denuded ones. Our results suggest that a) various ROS contract tracheal smooth muscle with simultaneous release of epithelium derived relaxing factors, b) epithelium possesses superoxide anion scavenging capacity which is high enough to protect smooth muscle from its actions, and c) cyclooxygenase products participate in relaxation and lipoxygenase products in contraction caused by ROS in the guinea pig trachea.

Keywords: Superoxide anion, H_2O_2, Hydroxyl radical, Guinea pig trachea, Arachidonic acid metabolite

Many pathological processes in the human organism are associated with increased production of reactive oxygen species (ROS), such as superoxide anion, H_2O_2, hydroxyl radical and singlet oxygen. In recent years, numerous studies reported a close relationship between the increased ROS production and the incidence or development of pathological processes in the airways, such as bronchial hyperreactivity, asthma, adult and newborn respiratory distress syndromes, etc. (1 – 6).

The impairment and destruction of the epithelium, along with the increased airway tone and sensitivity to some contractile mediators, such as histamine or acetylcholine (7), is an important factor of bronchial hyperreactivity and asthma. It was shown that hypochlorous acid, ozone, H_2O_2 and a hyperoxic environment can impair the function of the airway epithelium and its morphology in a dose- and time-dependent manner (8 – 13). Apart from the contractile factors, relaxant factors are also present and released upon various stimuli from the epithelium (9, 14, 15).

Rhoden and Barnes (16) described a biphasic response of isolated guinea pig trachea to H_2O_2. Later existence of species differences was demonstrated. H_2O_2 was shown to relax the rabbit (17) and dog (18) trachea, contract the cat trachea (19) and cause biphasic effect on the guinea pig trachea (20). The effects of H_2O_2 are generally considered to be modulated by cyclooxygenase inhibitors or epithelium removal (16, 21 – 25). Several studies investigating the role of prostaglandins in the mediation of the effects of H_2O_2 came to contradictory conclusions (16, 20) and pointed to marked interspecies differences (16, 18, 20, 26, 27).

Although there are many studies focusing on the effect of H_2O_2 on isolated trachea or bronchi of various animal species: rat (22), cow (21), ferret (28), rabbit (17), guinea

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pig (16, 17, 20, 29), human (25), less were done on the effects of ozone (guinea pig trachea (30), feline bronchi (31)), hypochlorous acid (guinea pig trachea (32, 33)), hydroxyl radical (rabbit trachea (26)) and xanthine/xanthine oxidase (guinea pig airways in vivo (34) and cat bronchi in vitro (35)). The lack of comprehensive studies that would compare the effects of long-lasting contact of systems generating hydroxyl radical and superoxide anion on the tone of the guinea pig trachea in the presence or absence of epithelium with that of H_2O_2 on the same tissue of a single animal species prompted us to compare them in guinea pig trachea. We analyzed also the possible involvement of arachidonic acid metabolites in the mediation of the long-lasting effects of ROS.

MATERIALS AND METHODS

Experimental animals and organ preparation

The experiments were done on the isolated tracheas of male Trik guinea pigs (300 – 400 g). After killing the animals by cervical dislocation and exsanguination, the tracheae were excised and cleaned thoroughly of surrounding connective tissue. They were then cut transversely into two equal parts and opened longitudinally on the ventral side. Then the trachea was cut alternately from one and the other side to obtain a preparation where the axes of individual strips of the tracheas overlapped with the axis of the whole preparation (36). Such preparations were mounted into a 20-ml organ bath filled with Krebs-Henseleit solution at 37°C and gassed with 5% CO_2 and 95% O_2. The Krebs-Henseleit solution contained: 118 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.64 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$ and 5.5 mmol/l glucose (pH = 7.4). The isometric tension was recorded using a strain gauge transducer (TDA 100; Mikrotechna, Prague, Czech Republic). One end of the tissues was tied to the bottom of a 20-ml tissue bath and the other to a transducer. Before administration of substances, the preparations were equilibrated for 15 min at 20-mN load and for another 30 min at 10-mN load. The experiments were carried out on intact and epithelium-denuded, not precontracted preparations at the initial tension of 10 mN. The epithelium was removed with moist cotton wool by gentle rubbing. Tissue sections stained with hematoxyline and eosin were examined histologically to control the effectiveness of epithelium removal. Only few broken epithelium cells were noticed in the denuded preparations in contrast to the controls. Control and experimental responses of the trachea to ROS, indomethacin and nordihydroguaiaretic acid (NDGA) were obtained in the upper and lower part of the trachea alternatively to minimize possible differences in the response of these two parts.

The effects of ROS on muscle tone

The contraction and relaxation amplitudes, the area under the curve of the contractile part of the 30-min lasting responses (AUC), and the time when muscle tone returns back to the initial tension (t_o) were evaluated.

Various concentrations of H_2O_2 (1 μmol/l – 10 mmol/l applied in single doses and cumulatively) and systems generating superoxide anion radical (1 μmol/l – 1 mmol/l xanthine with 0.1 – 5 mU/ml xanthine oxidase) or hydroxyl radical (1 μmol/l – 1 mmol/l $FeSO_4$ with 0.1 and 1 mmol /l H_2O_2, or 0.1 mmol/l $FeSO_4$ with 1 μmol/l – 1 mmol/l ascorbic acid) were tested in preliminary experiments. The chosen concentrations of H_2O_2 (1 mmol/l) and of $FeSO_4$ (0.1 mmol/l) with H_2O_2 (1 mmol/l) caused roughly equieffective contractions. $FeSO_4$ (0.1 mmol/l), ascorbic acid (0.1 mmol/l), xanthine (10 μmol/l) and xanthine oxidase (2 mU/ml) were applied in concentrations that did not affect the tracheal tone when administered alone.

To study the involvement of the arachidonic acid metabolites in the action of ROS, the tissues were pretreated for 60 min either by the cyclooxygenase inhibitor indomethacin (1 μmol/l) or NDGA (10 μmol/l).

Assay of superoxide anion

Generation of superoxide anion by xanthine/xanthine oxidase system was monitored by a method based on cytochrome c reduction (37). The reaction mixture consisted of xanthine (10 μmol/l), cytochrome c (60 μmol/l) and phosphate-buffered saline of the following composition: 136 mmol/l NaCl, 2.6 mmol/l KCl, 0.6 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$, 8 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4 and 5.5 mmol/l glucose (pH = 7.4). Addition of xanthine oxidase (2 mU/ml) to this solution started the reaction. In some experiments, superoxide dismutase (200 U/ml) was used to remove superoxide anion. The change of the absorbance at 550 nm against the control (the mixture without xanthine oxidase) was recorded continually on an HP 8953 1A spectrophotometer over the period of 15 min and analyzed by Hewlett Packard software. To calculate the produced superoxide anion, a molar extinction coefficient of 21000 $mol^{-1} \cdot l \cdot cm^{-1}$ was used.

Chemicals and solutions

Chemicals used were: H_2O_2 and $FeSO_4$ (Lachema, Brno, Czech Republic); cytochrome c from bovine heart, indomethacin, nordihydroguaiaretic acid, catalase from bovine liver (Fluka, Buchs, Switzerland); superoxide dismutase from human erythrocytes, xanthine sodium salt and xanthine oxidase from buttermilk, chromatographically purified (Sigma, Deisenhofen, Germany); isoprenaline hydrochloride (Léčiva, Praha, Czech Republic); ascorbic acid (Slovakofarma, Hlohovec, Slovak Republic); stobadine (IEPh SAS, Bratislava, Slovak Republic).

Indomethacin was dissolved in 0.1 mol/l Na_2CO_3 and NDGA in 0.1 mol/l NaOH to create a 1 mmol/l stock solution. Under this condition, the pH value of the physiological salt solution was not altered at their final concentration of 1 μmol/l. All the other substances were dissolved in distilled water and administered in a volume not exceeding 1% of organ bath volume.

Statistical analyses

The data in the text and figures are expressed as the mean ± S.E.M. of at least seven trials. If a higher number of trials was performed, *n* is given in the text or legend to figures. The values were compared with the unpaired two-tailed Student's *t*-test and probability values $P<0.05$ were considered significant.

RESULTS

The effect of ROS on muscle tone

H_2O_2 (1 mmol/l) contracted the intact trachea with a maximum of 2.39 ± 0.36 mN in the 2nd min. The muscle tone then slowly declined (t_o 21 ± 3.2 min) and within 30 min, it reached the value of –0.56 ± 0.18 mN; i.e., a tone below its initial one (Fig. 1A). The contraction AUC was 18.7 ± 3.4. In tissues with removed epithelium, the H_2O_2 induced maximum of tracheal contraction was twice that of the intact tissue (4.23 ± 0.57 mN) and AUC was 85.4 ± 18.7. The muscle tone then slowly decreased but even after 30 min, lasting contact with the tissues was still at the level of 2.26 ± 0.29 mN (Fig. 1B). The effect of H_2O_2 was inhibited by catalase (250, 500 and 1000 U/ml, n = 6) in a concentration-dependent manner and was not affected by the hydroxyl radical scavenger stobadine (1 and 10 μmol/l, n = 8).

Two systems producing hydroxyl radical were used. The first hydroxyl radical generating system (0.1 mmol/l of $FeSO_4$ with 1 mmol/l of H_2O_2) elicited after a short-lived (5 – 7 min) increase (with maximum of 2.58 ± 0.30 mN within 2 – 3 min and AUC = 9.89 ± 1.63) a reduction of the muscle tone that reached the level of –3 to –4 mN within 10 – 30 min (t_o 6.5 ± 1.2 min) (Fig. 2A). On the epithelium denuded tracheas, this system elicited an at least three times bigger contraction (reaching 8.46 ± 1.08 mN in the 4th min, AUC = 93.2 ± 19.5) than in the tissues with intact epithelium. The decline of this contraction resulted between the 15th and 20th min in a muscle tone that did not differ significantly from the initial one (0.61 ± 0.66 mN) (Fig. 2B). The qualitative alterations of the muscle tone produced by the second hydroxyl radical generating system (0.1 mmol/l of $FeSO_4$ with 0.1 mmol/l ascorbic acid) were similar to those produced by $FeSO_4/H_2O_2$. The amplitude of the initial contraction reached a lower maximum (0.54 ± 0.17 mN in the 2nd min) and AUC (2.09 ± 0.36) than that produced by $FeSO_4/H_2O_2$. The muscle tone then decreased below the initial level to –1.43 ± 0.32 mN in the 30th min, and t_o was 6.5 ± 1.2 min (Fig. 3A). Epithelium-denuded preparations were responding again with roughly three times bigger contractions (amplitude = 1.69 ± 0.34 mN, AUC = 32.2 ± 6.2) compared to those with intact epithelium. Moreover, the contractions of denuded preparations elicited by $FeSO_4$/ascorbic acid (Fig. 3B) persisted longer (t_o 28.3 ± 2.4) than those elicited by $FeSO_4/H_2O_2$ (Fig. 2B). The effect of hydroxyl radical generating systems was significantly (n = 8, $P<0.01$) decreased by stobadine (10 μmol/l, data not shown).

In contrast to previous ROS generating systems, xanthine (10 μmol/l) with xanthine oxidase (2 mU/ml) generating superoxide anion did not change significantly the tone

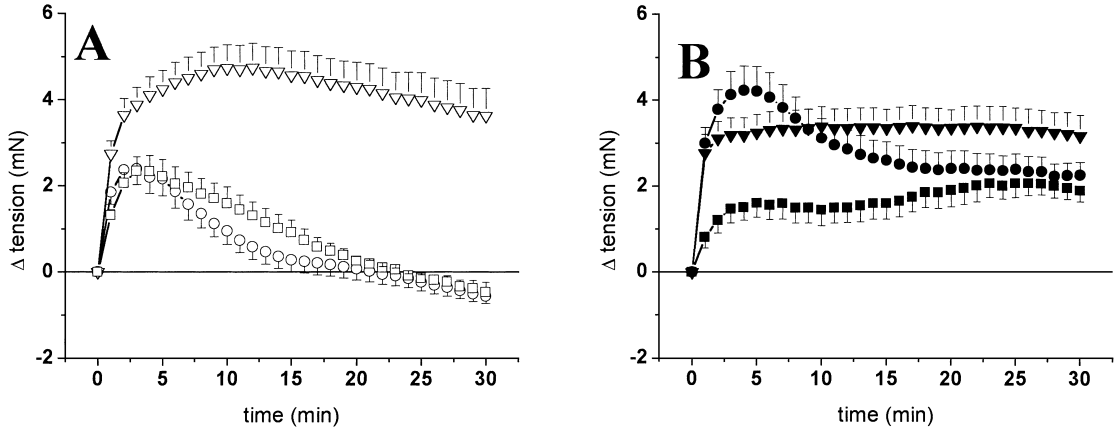

Fig. 1. Time course (30 min) of the effects of H_2O_2 (1 mmol/l) in the absence (circles) and presence of indomethacin (triangles: 1 μmol/l) or NDGA (squares: 10 μmol/l) on the intact (A) and epithelium-denuded guinea pig trachea (B). Indomethacin and NDGA were added into the bathing fluid 60 min before the application of H_2O_2. Results are expressed as the mean ± S.E.M. of 7 – 14 trials.

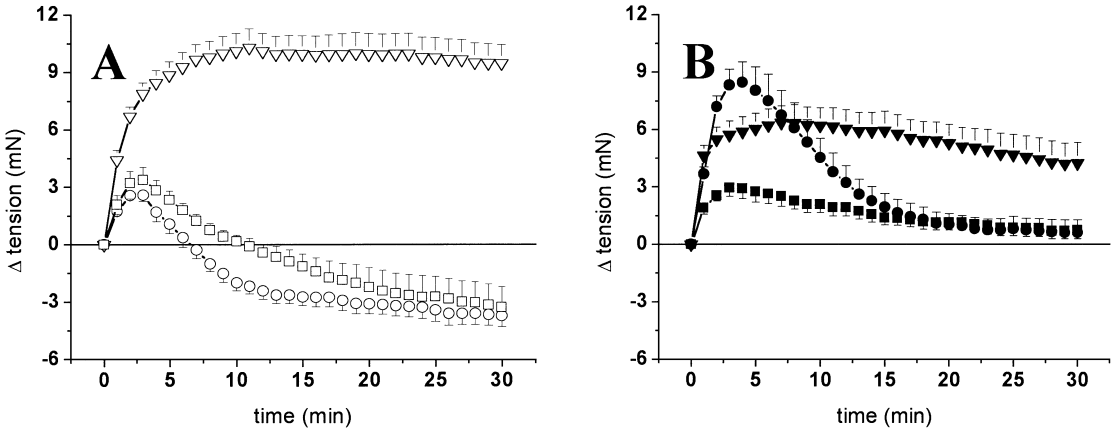

Fig. 2. Time course (30 min) of the effects of the system of H_2O_2 (1 mmol/l) with $FeSO_4$ (0.1 mmol/l) in the absence (circles) and presence of indomethacin (triangles: 1 μmol/l) and NDGA (squares: 10 μmol/l) for 60 min on the intact (A) and epithelium-denuded guinea pig trachea (B). Indomethacin and NDGA were added into the bathing fluid 60 min before the application of H_2O_2 with $FeSO_4$. Results are expressed as the mean ± S.E.M. of 8 trials.

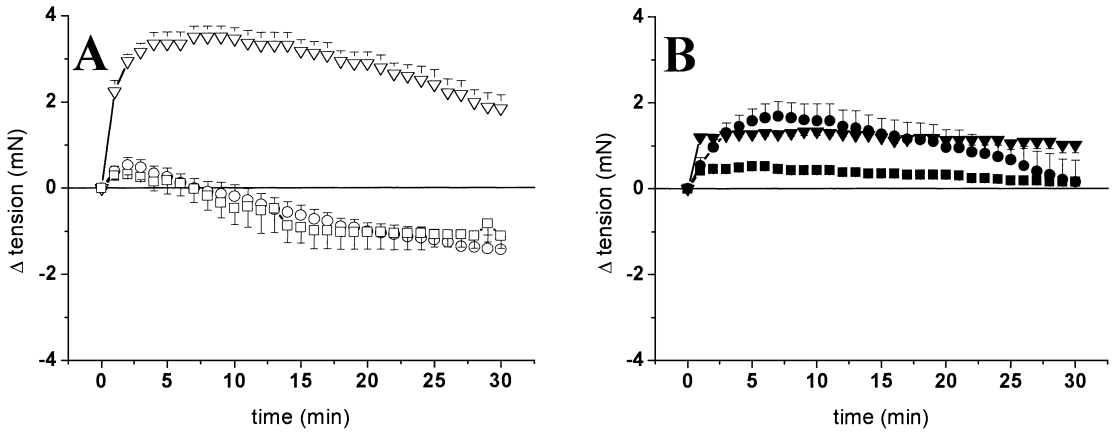

Fig. 3. Time course (30 min) of the effects of the system of ascorbic acid (0.1 mmol/l) with $FeSO_4$ (0.1 mmol/l) in the absence (circles) and presence of indomethacin (triangles: 1 μmol/l) and NDGA (squares: 10 μmol/l) for 60 min on the intact (A) and epithelium-denuded guinea pig trachea (B). Indomethacin and NDGA were added into the bathing fluid 60 min before the application of ascorbic acid with $FeSO_4$. Results are expressed as the mean ± S.E.M. of 8 trials.

of the trachea with intact epithelium (amplitude = 0.09 ± 0.04 mN, AUC = 0.58 ± 0.09, Fig. 4A). To verify whether the effect of this system was not due to an inadequate generation of superoxide anion, its production was also measured. Xanthine (10^{-5} mol/l) with xanthine oxidase (2 mU/ml) elicited a superoxide dismutase (200 U/ml)-sensitive cytochrome c reduction for roughly 10 min; thereafter, the oxidation of cytochrome c overcame its reduction, leading to a reversal in the change of absorbance and apparent discontinuation of cytochrome c reduction. An example is given in Fig. 5.

Removal of epithelium unmasked the biphasic effect of the superoxide anion produced by xanthine/xanthine oxidase. The initial contraction with maximum amplitude of 1.24 ± 0.23 mN within 10 min (AUC = 7.46 ± 1.52) was followed by a decrease of the tone (t_o 8.6 ± 1.9) to a level of −1.12 ± 0.20 mN in the 30th min (Fig. 4B). The effect of xanthine/xanthine oxidase was almost completely inhibited (n = 5, data not shown) by simultaneous administration of superoxide dismutase (100 U/ml) and catalase (1000 U/ml).

Effects of indomethacin and NDGA on the action of ROS

The cyclooxygenase inhibitor, indomethacin (1 μmol/l) and the lipoxygenase inhibitor NDGA (10 μmol/l), administered 60 min prior the respective ROS application, were present in the bathing fluid during their action. Indomethacin decreased the initial tone of the intact trachea by 5.85 ± 0.88 (n = 40) and that of the epithelium-denuded trachea by 2.03 ± 0.08 mN (n = 40). In contrast, NDGA

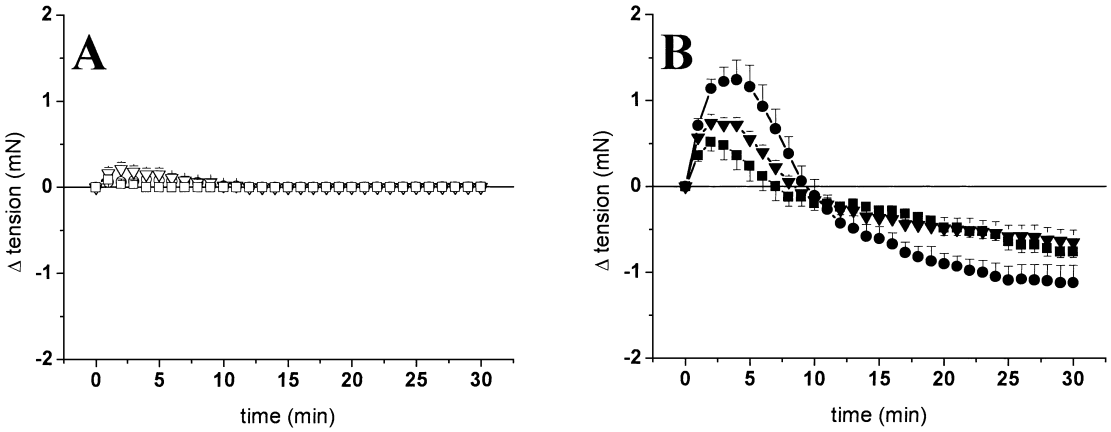

Fig. 4. Time course (30 min) of the effects of the system xanthine (10 μmol/l) with xanthine oxidase (2 mU/ml) in the absence (circles) and presence of indomethacin (triangles: 1 μmol/l) and NDGA (squares: 10 μmol/l) for 60 min on the intact (A) and epithelium-denuded guinea pig trachea (B). Indomethacin and NDGA were added into the bathing fluid 60 min before the application of xanthine with xanthine oxidase. Results are expressed as the mean ± S.E.M. of 7 – 14 trials.

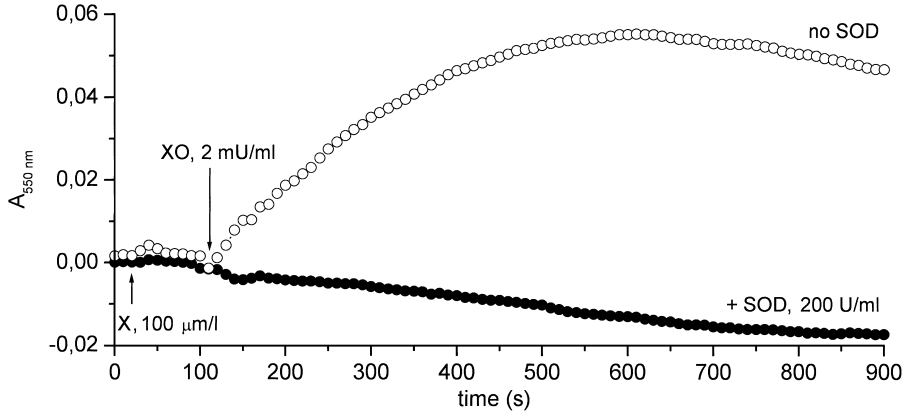

Fig. 5. Reduction of cytochrome c by superoxide anion. Superoxide anion is produced by xanthine (X: 10 μmol/l) present in the bathing fluid and xanthine oxidase (XO: 2 mU/ml) added as indicated by the arrow. In experiments where superoxide anion dismutase (SOD: 200 U/ml) was included in the bathing fluid, there was no reduction of cytochrome c. Open circles: control response, closed circles: response in the presence of SOD.

almost equally decreased the initial tone of both the intact (by 5.85 ± 0.88 mN, n = 29) and epithelium-denuded trachea (by 5.42 ± 0.08 mN, n = 29).

The characteristics of changes in responses of the intact trachea to H_2O_2 (Fig. 1A), $FeSO_4/H_2O_2$ (Fig. 2A) and $FeSO_4$/ascorbic acid (Fig. 3A) after pretreatment of the tissues with indomethacin (1 μmol/l) were similar. The maximum contraction amplitude reached was augmented significantly (4.73 ± 0.54 mN in the 10th min by H_2O_2, 10.3 ± 0.98 mN in the 11th min by $FeSO_4/H_2O_2$ and 3.51 ± 0.25 mN in the 7th min by $FeSO_4$/ascorbic acid). In contrast to the untreated tissue, the tone was still at the level of 50 – 90% of the maximum contraction amplitude in the 30th min (Figs. 1A, 2A and 3A). To exclude the role of the reduced resting tone in the indomethacin induced increase of the contractile effect of H_2O_2, its action was also studied in isoprenaline (1 μmol/l) relaxed tissues. Under these conditions, the contraction amplitude and the time course of the H_2O_2 induced response did not differ significantly from the control (n = 8). NDGA (10 μmol/l) pretreatment did not affect the time course and amplitude of responses to H_2O_2, $FeSO_4/H_2O_2$ and $FeSO_4$/ascorbic acid on the intact trachea (Figs. 1A, 2A and 3A).

On denuded preparations, indomethacin pretreatment slightly but not significantly reduced the H_2O_2 (Fig. 1B) and $FeSO_4$/ascorbic acid (Fig. 3B) induced initial contraction amplitude and significantly (P<0.005) reduced the $FeSO_4/H_2O_2$ (Fig. 2B) induced ones. In contrast to the untreated tissues, the contractions in all three systems were long lasting and did not differ significantly in the 30th min from the maxima reached. NDGA pretreatment of denuded preparations significantly attenuated the initial contractile

phase of the H_2O_2 (Fig. 1B), $FeSO_4/H_2O_2$ (Fig. 2B) and the $FeSO_4$/ascorbic acid (Fig. 3B) effects. In the case of H_2O_2, the tone remained elevated even in the 30th min (Fig. 1B).

In contrast to the effects of the above-mentioned ROS generating systems, the initial tone of the intact trachea was not significantly altered by xanthine (10 μmol/l) with xanthine oxidase (2 mU/ml) even when the tissue was pretreated by indomethacin or NDGA (Fig. 4A). The amplitude of the biphasic effect of xanthine/xanthine oxidase system on denuded preparations was, however, reduced by indomethacin or NDGA pretreatment by about 50% (Fig. 4B).

DISCUSSION

The effect of long lasting (30 min) contact of ROS with the guinea pig trachea in the presence and absence of epithelium was analyzed under in vitro conditions. The ROS were administered either into the bath solution directly (H_2O_2) or as ROS-generating systems: $FeSO_4/H_2O_2$ or $FeSO_4$/ascorbic acid for hydroxyl radical or xanthine /xanthine oxidase for superoxide anion radical (38 – 42).

As described earlier, there are species variations among the responses of airways to H_2O_2: contraction in bovine (21), cat (19) and horse (27) trachea, relaxation in rabbit (17) (also by hydroxyl radical (26)) and dog trachea (18) and biphasic response in guinea pig trachea (16, 20). On long lasting contact of tissues with H_2O_2, $FeSO_4/H_2O_2$ and $FeSO_4$/ascorbic acid, the contraction not only faded progressively with time but also a significant relaxation appeared, suggesting a biphasic character of the response in the present study. In untreated preparations the contraction dominated in the action of H_2O_2 and the relaxation in the action of $FeSO_4$/ascorbic acid generating hydroxyl radical in the intact trachea. The response to the system $FeSO_4/H_2O_2$, which was intermediate between the two mentioned above, was probably due to the simultaneous presence of both hydroxyl radical and H_2O_2.

In contrast, the superoxide anion generating system xanthine/xanthine oxidase did not affect the intact trachea, although the presence of superoxide anion was confirmed spectrophotometrically by superoxide dismutase sensitive reduction of cytochrome c. It is known that the airway epithelium contains non-enzymatic low-molecular weight antioxidants (43, 44), high-molecular weight antioxidant enzymes (15, 45, 46) and a putative unidentified antioxidant system (47). Since removal of the epithelium unmasked the biphasic response of trachea to superoxide anion, it seems likely that the antioxidant mechanisms of the epithelium are sufficient to eliminate superoxide anion before it reaches smooth muscle cells and elicits changes of the tracheal tone.

The contractile phase preceding the relaxatory one, on the long lasting contact of ROS with the guinea pig trachea, suggests that the mechanisms associated with the sequence of processes leading to contraction have faster kinetics or a lower threshold than those associated with relaxation. It might be also due to a difference in activation pathways; e.g., contraction being caused by direct action and relaxation by indirect (epithelium-, nerve-, etc. mediated) effect. Fast inactivation of the contractile components is unlikely because in indomethacin pretreated tissues, ROS induced more or less sustained contractions.

The difference between the intensity of contraction and relaxation caused by H_2O_2 and hydroxyl radical generating systems in intact tissues is probably due to release of a smaller amount of the relaxing factors by H_2O_2 than by hydroxyl radical. This hypothesis is supported by a more pronounced increase of hydroxyl radical- than H_2O_2-induced contraction by indomethacin.

Gao and Vanhoutte (20) and Rhoden and Barnes (16) described an increase in contractile response to H_2O_2 of the epithelium denuded guinea pig trachea. Our findings show augmentation not only of H_2O_2- but also of hydroxyl radical-induced contractions on epithelium removal. Under such a condition, the increased tone was persistent and the relaxant component of the response was absent. Removal of the epithelium was found to cause partial loss of relaxant factors following H_2O_2 administration also in other animal species (17, 18). Deepithelisation of the trachea is presumably also removing superoxide anion-eliminating mechanisms of the epithelium, thus unmasking direct actions of superoxide anion on the smooth muscle. This is in agreement with the suggestion that the epithelium is the source of a factor(s) (48) responsible for reduction of the contractile effect of ROS and protection of smooth muscle from their action. Thus an impaired epithelium plays an important role in development of persisting high tone induced by ROS under pathological conditions of the airways.

Findings of Charette et al. (49) demonstrated that in isolated guinea pig trachea, prostanoid tone is dependent on cyclooxygenase (COX)-2 activity. Moreover, it was found that of the two isoforms, the constitutive COX-1 and the inducible COX-2, only the latter is activated by H_2O_2 (50, 51) and superoxide (50). The COX inhibitor indomethacin relaxed both the intact and epithelium-denuded trachea in the present as well as in the earlier experiments (20). The indomethacin induced relaxation of the intact trachea was significantly larger than that of the epithelium-denuded trachea. This difference suggests that both epithelial and smooth muscle cells continuously produce contractile COX metabolites, which prevail over the relaxant ones (52). In contrast to COX, the contractile lipoxygenase products almost exclusively originated from smooth muscle, because inhibition of lipoxygenase by NDGA reduced the

tone of both the intact and epithelium-denuded trachea to a similar degree. Rhoden and Barnes (16) on the epithelium denuded and Gao and Vanhoutte (20) on the intact and denuded guinea pig trachea observed attenuation of H_2O_2-induced contraction in the presence of indomethacin. In contrast, Gupta and Prasad (17) found significant attenuation of H_2O_2-induced relaxation, and Prasad and Gupta (26) described a reversal of the relaxant effect of hydroxyl radical to contraction on the rabbit trachea after indomethacin pretreatment. Our results are supporting those of Prasad and Gupta (26), because in intact tissue, indomethacin pretreatment changed the biphasic character of tracheal responses to H_2O_2 and hydroxyl radical into a monophasic prolonged and increased contraction. Although isoprenaline, NDGA and indomethacin relaxed the preparations roughly to the same extent, the difference among their actions against ROS on intact tissues (enhancement of contraction by indomethacin and no effect of NDGA and isoprenaline) suggests that enhancement of contractions is not due to the tone decreased by indomethacin. This suggested a more pronounced ROS-induced release or synthesis of relaxant than contracting prostanoids in intact tissue.

Indomethacin reduced the peak contraction but prolonged the contractions elicited by H_2O_2 and hydroxyl radical on epithelium denuded preparations, suggesting that smooth muscle itself produces both relaxant and contractile prostanoids upon stimulation by these ROS. Furthermore, the ability of NDGA to suppress the initial contraction of denuded preparations suggests that lipoxygenase products are also involved in this initial part of the smooth muscle response. In light of the difference between the characteristics of the indomethacin effect on contraction to H_2O_2 and hydroxyl radical in the presence and absence of epithelium, it can be assumed that epithelial cells mainly produce relaxant prostaglandins, while smooth muscle cells produce both relaxant and contractile prostaglandins.

Superoxide anion in the epithelium denuded trachea, however, activates the production of both contracting and relaxing prostaglandins since indomethacin pretreatment attenuates both phases of the superoxide anion action. The fact that indomethacin did not unmask the response to xanthine/xanthine oxidase, suggests that it does not interact with the superoxide anion eliminating antioxidants produced by the epithelium.

Sporn and Peters-Golden (53) observed a slight activation of alveolar macrophages lipoxygenase by H_2O_2 in low concentrations and its inhibition by higher concentrations. On the other hand, Burghuber et al. (54, 55) and Gurtner et al. (56) observed an increased leukotriene production following oxidative stress of the lung (57). Our results suggest that lipoxygenase products of smooth muscle cells could participate at least partially in the mediation of the effects of ROS on airways.

In conclusion our results showed that hydrogen peroxide and hydroxyl radical induced changes in smooth muscle tone of the same tissue and of the same animal species were probably due to the closely related processes involving the arachidonic acid cascade. The time course and the action of ROS scavangers exclude the simple intracellular conversion of H_2O_2 to hydroxyl radical. The systems generating hydroxyl radical seem to be able to release more epithelium derived relaxatory prostaglandins than H_2O_2. Thus the present study, along with the relevant data from the literature, suggests participation of metabolites of the arachidonic acid cascade in the mediation of the effects of ROS (Fig. 6). The lack of the action of superoxide in the presence of epithelium suggests that epithelium possesses high superoxide scavenging capacity.

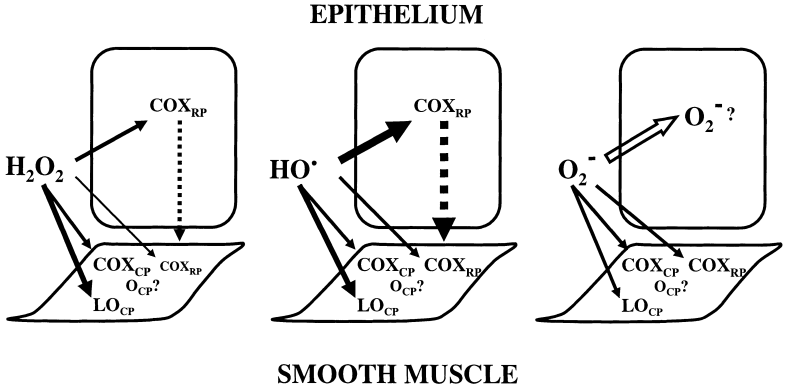

Fig. 6. Summarization of our suggestion on the possible effects of reactive oxygen species on arachidonic acid metabolites in the epithelium and smooth muscle of the guinea pig trachea. COX_{CP}: cyclooxygenase contractile products, COX_{RP}: cyclooxygenase relaxatory products, LO_{CP}: lipoxygenase contractile products, O_{CP}: other contractile products, O_2^-: superoxide anion, H_2O_2: hydrogen peroxide, $^{\cdot}OH$: hydroxyl radical.

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REFERENCES

1 Lykens MG, Davis WB and Pacht ER: Antioxidant activity of bronchoalveolar lavage fluid in the adult respiratory distress syndrome. Am J Physiol **262**, L169 – L175 (1992)
2 Dohlman AW, Black HR and Royall AA: Expired breath hydrogen peroxide is a marker of acute airway inflammation in pediatric patients with asthma. Am Rev Respir Dis **148**, 955 – 960 (1993)
3 Metnitz PG, Bartens C, Fischer M, Fridrich P, Stelzer H and Drunl W: Antioxidant status in patients with acute respiratory distress syndrome. Int Care Med **25**, 180 – 185 (1999)
4 Quinlan TGJ, Lamb NJ, Tilley R, Evans TW and Gutteridge JM: Plasma hypoxanthine levels in ARDS: implications for oxidative stress, morbidity, and mortality. Am J Respir Crit Care Med **155**, 479 – 484 (1997)
5 Vachier I, Chanez P, Le Doucen C, Damon M, Descomps B and Godard P: Enhancement of reactive oxygen species formation in stable and unstable asthmatic patients. Eur Respir J **7**, 1585 – 1592 (1994)
6 Plaza V, Prat J, Roselló J, Ballester E, Ramis I, Mullol J, Gelpi E, Vives-Corrons JLL and Picado C: In vitro release of arachidonic acid metabolites, glutathione peroxidase, and oxygen-free radicals from platelets of asthmatic patients with and without aspirin intolerance. Thorax **50**, 490 – 496 (1995)
7 Fish JE, Shaver JR and Peters SP: Airway hyperresponsivenesses in asthma. Is it unique? Chest **107**, Suppl 154S – 157S (1995)
8 Alpert SE, Kramer CM, Hayes MM and Dennery PA: Morphologic injury and lipid peroxidation in monolayercultures of rabbit tracheal epithelium exposed in vitro to ozone. J Toxicol Environ Health **30**, 287 – 304 (1990)
9 Kim KJ and Suh DJ: Assymetric effects of hydrogen peroxide on alveolar epithelial barrier properties. Am J Physiol **264**, L308 – L315 (1993)
10 Yoo JH, Erzurum SC, Hay JG, Lemarchand P and Crystal RG: Vulnerability of the human airway epithelium to hyperoxia. J Clin Invest **93**, 297 – 302 (1994)
11 Vesely KR, Schelegle ES, Stovall MY, Harkema JR, Green JF and Hyde DM: Breathin pattern response and epithelial labelling in ozone induced airway injury in neutrophil-depleted rats. Am J Resp Cell Mol Biol **20**, 699 – 709 (1999)
12 Guo Y, Schneider LA and Wangensteen OD: HOCl effects on tracheal epithelium: conductance and permeability measurements. J Appl Physiol **78**, 1330 – 1338 (1995)
13 Baeza-Squiban A, Boisvieux-Ulrich E, Delcher L and Marano F: Defense and repair mechanisms in the airway epithelium exposed to oxidative stress: effects of analogues of retionic acid. Toxicol Lett **96 – 97**, 245 – 251 (1998)
14 Cantin AM, Fells GA, Hubbard RC and Crystal RG: Antioxidant macromolecules in the epithelial lining fluid of the normal human lower respiratory tract. J Clin Invest **86**, 962 – 971 (1990)
15 Kozar RA, Weibel CJ, Cipolla J, Klein AJ, Haber MM, Abedin MZ and Trooskin SZ: Antioxidant enzymes are induced during recovery from acute lung injury. Crit Care Med **28**, 2486 – 2491 (2000)
16 Rhoden KJ and Barnes PJ: Effect of hydrogen peroxide on guinea-pig tracheal smooth muscle in vitro: role of cyclo-oxygenase and airway epithelium. Br J Pharmacol **98**, 325 – 330 (1989)
17 Gupta JB and Prasad K: Mechanism of hydrogen peroxide-induced modulation of airway smooth muscle. Am J Physiol **263**, L714 – L722 (1992)
18 Gao Y and Vanhoutte PM: Effects of hydrogen peroxide on the responsiveness of isolated canine bronchi: role of prostaglandin E2 and I2. Am J Physiol **263**, L402 – L408 (1992)
19 Bauer V, Oike M, Tanaka H, Inoue R and Ito Y: Hydrogen peroxide induced responses of cat tracheal smooth muscle cells. Br J Pharmacol **121**, 867 – 874 (1997)
20 Gao Y and Vanhoutte PM: Products of cyclooxygenase mediate the responses of the guinea pig trachea to hydrogen peroxide. J Appl Physiol **74**, 2105 – 2111 (1993)
21 Stewart RM, Weir EK, Montgomery MR and Niewoehner PE: Hydrogen peroxide contracts airway smooth muscle: a possible endogenous mechanism. Respir Physiol **45**, 333 – 342 (1981)
22 Szarek JL and Schmidt NL: Hydrogen peroxide-induced potentiation of contractile responses in isolated rat airways. Am J Physiol **258**, L232 – L237 (1990)
23 Gao Y and Vanhoutte PM: Hydrogen peroxide induces epithelium-dependent contraction of canine bronchi. Am Rev Respir Dis **143**, Suppl A341 (1991)
24 Hulsmann AR, Raatgeep HR, den Hollander JC, Stijnen T, Saxena PR, Kerrebijn KF and de Jongste JC: Oxidative epithelial damage produces hyperresponsiveness of human peripheral airways. Am J Respir Crit Care Med **149**, 519 – 525 (1994)
25 Rabe KF, Dent G and Magnussen H: Hydrogen peroxide contracts human airways in vitro: role of epithelium. Am J Physiol **269**, L332 – L338 (1995)
26 Prasad K and Gupta JB: Influence of hydroxyl radical on rabbit airway smooth muscle chronically exposed to hydrogen peroxide in vivo. Am J Physiol **264**, L566 – L574 (1993)
27 Olszewski MA, Robinson NE, Yu MF and Derksen FJ: Effects of hydrogen peroxide on isolated trachealis muscle of horses. Am J Veter Res **56**, 1479 – 1485 (1995)
28 Morikawa T, Webber SE and Widdicombe JG: The effect of hydrogen peroxide on smooth muscle tone, mucus secretion and epithelial albumin transport of the ferret trachea in vitro. Pulm Pharmacol **2**, 106 – 113 (1991)
29 Misawa M and Arai H: Airway inflammation induced by xanthine/xanthine oxidase in guinea pigs. Agents Actions **38**, 19 – 26 (1993)
30 Fedan JS, Millecchia LL, Johnston RA, Rengasamy A, Hubbs A, Dey RD, Yuan LX, Watson D, Goldsmith WT, Reynolds JS, Orsini L, Dortch-Carnes J, Cutler D and Frazer DG: Effect of ozone treatment on airway reactivity and epithelium-derived relaxing factor in guinea pigs. J Pharmacol Exp Ther **293**, 724 – 734 (2000)
31 Takata S, Aizawa H, Inoue H, Koto H and Hara N: Ozone exposure suppresses epithelium-dependent relaxation in feline airway. Lung **173**, 47 – 56 (1995)
32 Murlas CG, Murphy TP and Lang Z: HOCl causes airway substance P hyperresponsiveness and neutral endopeptidase hypoactivity. Am J Physiol **258**, L361 – L368 (1990)
33 Doelman CJA: Airway hyperreactivity and reduction of β-adrenoceptor responses induced by hypochlorite. *In* Reactive

Oxygen Species and Airway Hyperreactivity, pp 109 – 122, Febodruk, Enschede (1991)
34 Misawa M and Nakano E: Airway constriction by xanthine/xanthine oxidase in guinea pigs in vivo. J Toxicol Envir Health **39**, 193 – 205 (1993)
35 Bauer V, Nakajima T, Pucovsky V, Onoue H and Ito Y: Effects of superoxide generating systems on muscle tone, cholinergic and NANC responses in cat airways. J Autonom Nerv Syst **79**, 34 – 44 (2000)
36 Todorov S: New method for in vitro studies of the histaminergic receptors in tracheal smooth muscle of male and female guinea pigs. Acta Physiol Pharmacol Bulg **3**, 77 – 84 (1977)
37 Babior BM, Kipnes RS and Curnutte JS: Biological defense mechanisms: The production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest **52**, 741 – 744 (1973)
38 Wong SF, Halliwell B, Richmond R and Skowroneck WR: The role of superoxide anion and hydroxyl radicals in the degradation of hyaluronic acid induced by metal ions and by ascorbic acid. J Inorg Chem **14**, 127 – 134 (1981)
39 Kumagai Y, Ikeda Y and Toki S: Hydroxyl radical-mediated conversion of morphine to morphinone. Xenobiotica **22**, 507 – 513 (1992)
40 Bhatnagar A: Biochemical mechanism of irreversible cell injury caused by free radical-initiated reactions. Mol Cell Biochem **137**, 9 – 16 (1994)
41 Ohyashiki T, Kumada Y, Hatanaka N and Matsui K: Oxygen radical-induced inhibition of alkaline phosphatase activity in reconstituted membranes. Arch Biochem Biophys **313**, 310 – 317 (1994)
42 Takemura G, Onodera T and Ashraf M: Characterization of exogenous hydroxyl radical effects on myocardial function, metabolism and ultrastructure. J Mol Cell Cardiol **26**, 441 – 454 (1994)
43 Crystal RG: Oxidants and respiratory tract epithelial injury: pathogenesis and strategies for therapeutic intervention. Am J Med **91**, 39S – 44S (1991)
44 Kelly FJ, Mudway I, Blomberg A, Frew A and Sandstrom T: Altered lung antioxidant status in patients with mild asthma. Lancet **354**, 482 – 483 (1999)
45 Simon R, DeHart P and Nadeau D: Resistance of rat pulmonary alveolar epithelial cells to neutrophil- and oxidant-induced injury. Am J Respir Cell Mol Biol **1**, 221 – 229 (1989)
46 Kinnula V, Chang L, Everitt JI and Crapo JD: Oxidants and antioxidants in alveolar epithelial type II cells: in situ, freshly isolated, and cultured cells. Am J Physiol **262**, L69 – L77 (1992)
47 Cohn LA, Kinnula VL and Adler KB: Antioxidant properties of guinea pig tracheal epithelial cells in vitro. Am J Physiol **266**, L397 – L404 (1994)
48 Mulier B, Rahman I, Watchorn T and Donaldson K: Hydrogen peroxide-induced epithelial injury: the protective role of intracellular nonprotein thiols (NPSH). Eur Respir J **11**, 384 – 391 (1998)
49 Charette L, Misquitta C, Guay J, Reindeau D and Jones TR: Involvement of cyclooxygenase 2 (COX-2) in intrinsic tone of isolated guinea pig trachea. Can J Physiol Pharmacol **73**, 1561 – 1567 (1995)
50 Feng L, Xia Y, Garcia GE, Hwang D and Wilson CB: Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-alpha, and lipopolysacharide. J Clin Invest **95**, 1669 – 1675 (1995)
51 Adderley SR and Fitzgerald DJ: Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. J Biol Chem **19**, 5038 – 5046 (1999)
52 Joad JP, McDonald RJ, Giri SN and Bric JM: Ozone effects on mechanics and arachidonic acid metabolite concentrations in isolated rat lung. Environ Res **66**, 186 – 197 (1994)
53 Sporn PHS and Peters-Golden M: Hydrogen peroxide inhibits alveolar macrophage 5-lipoxygenase metabolism in association with depletion of ATP. J Biol Chem **263**, 14776 – 14783 (1988)
54 Burghuber OC, Strife RJ, Zirrolli J, Henson PM, Henson JE, Mathias MM, Reeves JT, Murphy RC and Voelkel NF: Leukotriene inhibitors attenuate rat lung injury induced by hydrogen peroxide. Am Rev Resp Dis **131**, 778 – 785 (1985)
55 Burghuber OC, Silberbauer K, Haber P, Sinzinger H, Elliott M and Leithner C: Pulmonary and antiaggregatory effects of prostacyclin after inhalation and intravenous infusion. Respiration **45**, 450 – 454 (1984)
56 Gurtner GH, Farrukh IS, Adkinson NF Jr, Sciuto AM, Jacobson JM and Michael JR: The role of arachidonate mediators in peroxide-induced lung injury. Am Rev Respir Dis **136**, 480 – 483 (1987)
57 Stevens WH, Lange CG, Woolwy MJ, Ellis R, Tagari P, Black C, Ford-Hutchinson A and O'Byrne PM: Effect of FLAP antagonist MK-0591 on leukotriene production and ozone-induced airway response in dogs. J Appl Physiol **76**, 1583 – 1588 (1994)