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European Journal of Pharmacology 378 (1999) 109–114

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Impairment of epithelium-dependent relaxation in coaxial bioassay by reactive oxygen species

Ismailoglu U. Burcin [a], Inci Sahin-Erdemli [a,*], Sungur Arzu [b], Mustafa Ilhan [c]

[a] *Hacettepe University, Faculty of Pharmacy, Department of Pharmacology and Faculty of Medicine, 06100, Ankara, Turkey*
[b] *Hacettepe University, Faculty of Pharmacy, Department of Pathology, Ankara, Turkey*
[c] *Hacettepe University, Faculty of Pharmacy, Department of Pharmacology, Ankara, Turkey*

Received 21 January 1999; received in revised form 18 May 1999; accepted 21 May 1999

Abstract

The purpose of the present study was to investigate the effects of reactive oxygen species on the activity of epithelium-derived relaxant factor (EpDRF) released by guinea-pig tracheal epithelium. Reactive oxygen species were generated by the electrolysis of the physiological buffer in which the tissues were bathed. Epithelium-dependent relaxation induced by acetylcholine in precontracted rat anococcygeus muscle that was placed in epithelium-intact guinea-pig trachea (coaxial bioassay system) was significantly attenuated when the tissues were exposed to electrolysis. Impairment of the acetylcholine response was prevented by incubation with free radical scavengers prior to electrolysis. In isolated rings of guinea-pig trachea, the contractile responses elicited by acetylcholine, histamine and 5-hydroxytryptamine were not altered after electrolysis of the bathing solution. The results of the present study suggested that exposure to reactive oxygen species impaired EpDRF release from guinea-pig trachea epithelium but did not alter the contractility of tracheal smooth muscle.

Keywords: EpDRF (epithelium-derived relaxant factor); Airway epithelium; Trachea, guinea pig; Anococcygeus muscle, rat; Reactive oxygen species; Coaxial bioassay

1. Introduction

The airway epithelium participates in the regulation of bronchial reactivity and this has been attributed to several factors including its barrier and metabolic functions and the release of relaxing and constricting substances (Bertrand and Tschirhart, 1993; Sparrow et al., 1995; Folkerts and Nijkamp, 1998). The pharmacological relevance of relaxing substances released by the airway epithelium, such as nitric oxide and prostaglandin E_2 as well as epithelium-derived relaxing factor (EpDRF) detected by the coaxial bioassay system, has been studied extensively (Ilhan and Sahin, 1986; Butler et al., 1987; Nijkamp et al., 1993; Folkerts and Nijkamp, 1998).

Bronchial hyper-responsiveness, as shown in several experimental models of airway diseases and in humans with bronchial asthma, is commonly associated with epithelial dysfunction or damage (Hogg and Eggleston, 1984; Laitinen et al., 1985; Jeffery et al., 1989). Allergens and respiratory viruses as well as ozone inhalation cause inflammatory changes in the airways and epithelial damage in experimental animals (Buckner et al., 1985; Chung, 1986; Holroyde and Norris, 1988). The inflammation of the airways involves infiltration of neutrophils and other inflammatory cells, activation of alveolar macrophages and overproduction of the reactive oxygen species, superoxide anion, hydrogen peroxide and hydroxyl radical (Brigham, 1986; Djukanovic et al., 1990). These species can cause lipid peroxidation of the cellular membranes and thus alter both membrane structure and function, producing cellular injury (Kellogg and Fridovich, 1975; Halliwell and Gutteridge, 1984).

The present study was designed to investigate the effects of reactive oxygen species on guinea-pig tracheal EpDRF-mediated relaxation in a coaxial bioassay system and on the contractile responses elicited by various agonists in tracheal rings. The reactive oxygen species were generated by electrolysis of the physiological buffer in which the tissues were bathed (Jackson et al., 1986a,b; Gumusel et al., 1996).

* Corresponding author. Hacettepe University, Faculty of Pharmacy, Department of Pharmacology, 06100, Ankara, Turkey. Tel.: +90-312-305-2131; fax: +90-312-311-4777; E-mail: moe01-k@tr-net.net.tr

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00390-8

2. Materials and methods

In the present study, rats and guinea-pigs were cared and used in accordance with the guidelines of Hacettepe University Animal Care Unit.

2.1. Coaxial bioassay system

Male rats and guinea-pigs (200–300 g) were killed by a sharp blow to the head. Rat anococcygeus muscle was isolated and prepared as described by Gillespie (1972) and guinea-pig trachea was isolated and used in its original tubular shape. In this bioassay model, anococcygeus muscle was the assay tissue and the trachea was the donor organ for the assays (Guc et al., 1988). The anococcygeus muscle was passed through the trachea and mounted under a resting tension of 1 g in a 40-ml organ bath filled with Krebs–Henseleit solution at 37°C and gassed with 95% O_2–5% CO_2. The composition of the Krebs–Henseleit solution was (in mM): NaCl, 95; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25.0; glucose, 11.6. The Krebs–Henseleit solution also contained indomethacin (10 μM) in all experiments to prevent the possible involvement of cyclooxygenase products.

The tissues were allowed to equilibrate 45 min before each experimental procedure. Isometric changes in the tension were recorded with an isometric force transducer and ''MAY 95-transducer data acquisition system'' (Commat, Ankara, Turkey) on an IBM-compatible personal computer.

The rat anococcygeus muscle that was mounted either alone or passed through the trachea was precontracted by 1 μM phenylephrine (60–80% of the maximum contraction), and the relaxation response to increasing concentrations of acetylcholine (1 μM–1 mM) was obtained.

Some experiments were conducted after the removal of the tracheal epithelium by rubbing the mucosal surface with a probe coated with cotton wool. Successful removal of the epithelium was confirmed by histological examination.

The anococcygeus muscle mounted with or without trachea was exposed to reactive oxygen species generated by electrolysis of the bathing solution by means of two platinum electrodes (constant DC current of 20 mA for 5 min). The electrodes were 1 cm away from the tissue holder in the organ bath so that the tissues were not exposed to field stimulation. After electrolysis, the baths were washed out and the tissues were equilibrated for 5 min before concentration–response curves for acetylcholine were repeated.

In a group of experiments with the coaxial system, the tissues were incubated with the free radical scavenging agents dimethylsulfoxide (100 μM), superoxide dismutase (150 U/ml) and catalase (1000 U/ml), added to the bath 30 min prior to electrolysis. Then the experiments were repeated as described above. In the preliminary experiments, incubation of the tissues with these free radical scavengers and then the washout process in the absence of electrolysis did not alter the relaxation response elicited by acetylcholine.

In another group of experiments, the anococcygeus muscle that was mounted alone was contracted with phenylephrine (0.1–10 μM), and cumulative concentration–response curves for acetylcholine were obtained before and after electrolysis of the bathing solution as described above.

The direct effect of hydrogen peroxide on acetylcholine-induced relaxation in the coaxial bioassay system was also investigated. Rat anococcygeus muscle mounted with epithelium-intact trachea was incubated with hydrogen peroxide (10 μM) for 30 min and then the tissues were washed and equilibrated for 10 min. The acetylcholine-induced relaxation response was elicited as described above. The effect of hydrogen peroxide on the acetylcholine response was also studied in the presence of catalase (1000 U/ml).

2.2. Guinea-pig tracheal open ring preparation

The guinea-pig trachea was cut into rings each consisting of two to three rings of cartilage. The rings were then cut longitudinally through the cartilage opposite the smooth muscle and suspended in 40-ml organ baths filled with Krebs–Henseleit solution at 37°C and bubbled with 95% O_2 and 5% CO_2. A resting tension of 1 g was applied to the open rings and isometric tension was recorded as described above. The rings were equilibrated for 45 min before any experimental procedure.

The tracheal open rings were contracted by increasing concentrations of acetylcholine (0.1–100 μM), histamine (0.1–100 μM) and 5-hydroxytryptamine (0.1–100 μM), added to the organ bath in a cumulative manner. The concentration–response curves for these agonists were remeasured after electrolysis of the bathing solution as described above. The contractile responses to these agonists were also obtained after the removal of the trachea epithelium. Each tracheal ring preparation was used to evaluate the response to one agonist.

2.3. Histological examination

Specimens from the trachea subjected to either electrolysis or epithelium denudation were stained with haematoxylin and eosin for histological examination.

2.4. Statistical analysis

In the coaxial bioassay system, relaxant responses to acetylcholine are expressed as percentages of the phenylephrine (1 μM)-induced contraction. In rat anococcygeus muscle responses to phenylephrine and in guinea-pig tracheal rings responses to acetylcholine, histamine and 5-hy-

droxytryptamine are expressed as ''g'' contraction. The maximum response elicited by the agonist (E_{max}) and the concentration required to achieve half-maximum contraction (EC_{50}) were obtained from individual concentration–response curves. EC_{50} values are given as pD_2 values, which are defined as the negative logarithm of EC_{50} ($pD_2 = -\log EC_{50}$).

All data are expressed as means ± standard error of the mean (S.E.M.). Statistical analysis was performed by using analysis of variance (ANOVA) with repeated measurements and Bonferroni test; paired samples were compared by Student's *t*-test. A *P*-value of less than 0.05 was considered significant.

2.5. Drugs used

Phenylephrine, acetylcholine, histamine, 5-hydroxytryptamine, catalase, dimethylsulfoxide, superoxide dismutase and hydrogen peroxide were purchased from Sigma (USA).

3. Results

Rat anococcygeus muscle precontracted with phenylephrine (1 μM) was not relaxed by acetylcholine (1 μM–1 mM) when mounted alone in the organ bath. However, when the same preparation was placed in epithelium-intact guinea-pig trachea, acetylcholine induced concentration-dependent relaxation (Fig. 1). Removal of the epithelium from the tracheal tube abolished this relaxation of rat anococcygeus muscle (Fig. 1).

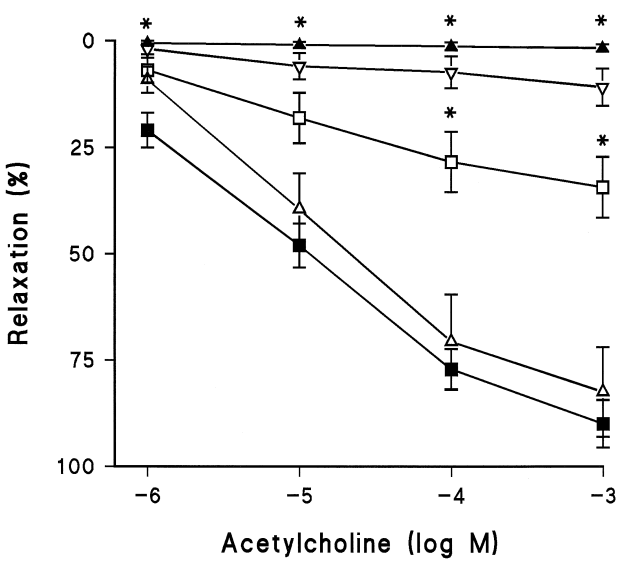

Fig. 1. Acetylcholine-induced relaxation response in rat anococcygeus muscle precontracted with phenylephrine (1 μM) when it was mounted alone (▲); when it was placed into guinea-pig trachea with intact epithelium before (control, ■) and after electrolysis (□); after removal of the epithelium (▽) and when it was exposed to electrolysis before being placed in epithelium intact trachea (△). The data are expressed as percentages of the phenylephrine-induced contraction and shown as mean ± S.E.M. (* significantly different from control, $P < 0.05$) ($n = 4$–17).

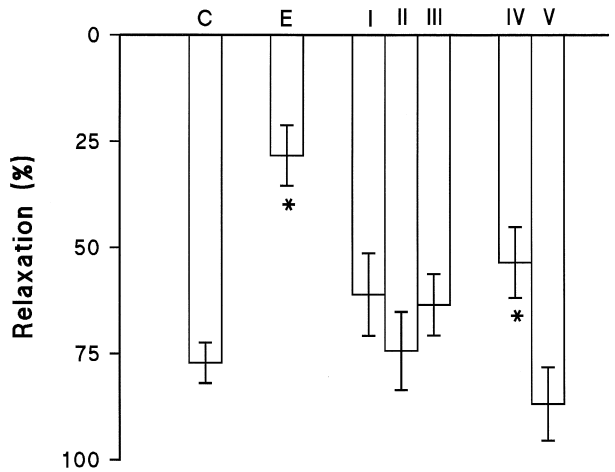

Fig. 2. Effect of electrolysis on the acetylcholine (100 μM)-induced relaxation response in precontracted rat anococcygeus muscle placed in guinea-pig trachea before (E) ($n = 6$) and after incubation with superoxide dismutase 150 U/ml (I) ($n = 5$); catalase 1000 U/ml (II) ($n = 5$); dimethylsulfoxide 100 μM (III) ($n = 6$); and the effect of 10 μM hydrogen peroxide before (IV) ($n = 6$) and after incubation with catalase 1000 U/ml (V) ($n = 4$). Control acetylcholine response (C) ($n = 17$). The data are expressed as percentages of the phenylephrine-induced contraction and shown as means ± S.E.M. (* significantly different from control, $P < 0.05$).

Electrolysis of the bathing solution resulted in inhibition of the relaxation response elicited by acetylcholine in the rat anococcygeus muscle mounted in epithelium-intact trachea (Fig. 1). However, the inhibition of the acetylcholine response was not observed when anococcygeus muscle was subjected to electrolysis alone, before it was put in epithelium-intact trachea (Fig. 1).

Incubation with the free radical scavengers, superoxide dismutase (150 U/ml), catalase (1000 U/ml), and dimethylsulfoxide (100 μM), prior to electrolysis prevented the impairment of the acetylcholine (100 μM)-induced relaxation response in the coaxial system (Fig. 2).

The effect of hydrogen peroxide (10 μM) on the acetylcholine-induced relaxation in the coaxial bioassay system was also studied. Incubation with hydrogen peroxide inhibited the relaxation response elicited by acetylcholine (100 μM), significantly. The inhibition of the acetylcholine response was prevented by incubation with catalase (1000 U/ml) (Fig. 2).

In a group of experiments the anococcygeus muscle was mounted alone and contractile responses to phenylephrine (0.1–10 μM) were determined before and after electrolysis of the bathing solution. Exposure to electrolysis did not significantly alter the phenylephrine concentration–response curve: the pD_2 and E_{max} values were 6.34 ± 0.06 and 6.73 ± 0.33 g before electrolysis, and 6.47 ± 0.05 and 7.18 ± 0.33 g after electrolysis, respectively ($n = 8$).

In tracheal open ring preparations, acetylcholine (0.1–100 μM), histamine (0.1–100 μM) and 5-hydroxytryptamine (0.1–100 μM) induced concentration-dependent contractile responses which were not altered by exposure

Table 1
Acetylcholine, histamine and 5-hydroxytryptamine-induced contractile responses in guinea-pig tracheal open rings before (control) and after electrolysis

	Before electrolysis		After electrolysis	
	pD_2	E_{max}	pD_2	E_{max}
Acetylcholine	5.55 ± 0.04	2.08 ± 0.50	5.42 ± 0.06	2.35 ± 0.56
Histamine	5.37 ± 0.10	1.44 ± 0.19	5.16 ± 0.08	1.81 ± 0.33
5-Hydroxytryptamine	5.85 ± 0.25	0.87 ± 0.10	5.66 ± 0.15	0.84 ± 0.12

E_{max}, maximum contraction is expressed as ''g'' of tension.
pD_2, negative logarithm of concentration required to achieve half-maximum contraction ($n = 5$–7).

to electrolysis (Table 1). The mechanical denudation of trachea epithelium also had no effect on the contractility of tracheal open rings: the pD_2 and E_{max} values for acetylcholine were 5.44 ± 0.06 and 2.03 ± 0.26 g, respectively ($n = 7$) and were not different from control values (Table 1).

The histological evaluation revealed that exposure to electrolysis did not cause any morphological change in the epithelial layer of the guinea-pig trachea. The epithelium was intact in all cross-sections of specimens from tracheas subjected to electrolysis ($n = 4$).

4. Discussion

In a coaxial bioassay system consisting of guinea-pig trachea as the donor organ and rat anococcygeus muscle as the assay tissue, it has been shown that tracheal epithelium releases EpDRF in response to acetylcholine (Guc et al., 1988). The same model was also used in the present study and our findings further supported the release of EpDRF from trachea epithelium.

Cyclooxygenase products (i.e., prostaglandin E_2) and nitric oxide are smooth muscle relaxing substances released by the airway epithelium (Butler et al., 1987; Fernandes and Goldie, 1990; Nijkamp et al., 1993; Folkerts and Nijkamp, 1998). However, the EpDRF detected by coaxial bioassay is probably neither a cyclooxygenase product nor nitric oxide. The present experiments were performed in the presence of indomethacin, thus, excluding the role of cyclooxygenase products in the EpDRF response. Previous studies using hyperosmotic stimuli or coaxial bioassay assemblies to demonstrate airway epithelium-dependent relaxation suggested that EpDRF was not a prostanoid substance. Furthermore, the lack of effect of the soluble guanylate cyclase inhibitor methylene blue and nitric oxide synthesis inhibitors in these models indicates that EpDRF is not identical to nitric oxide (Ilhan and Sahin, 1986; Fernandes and Goldie, 1990; Munakata et al., 1990; Bertrand and Tschirhart, 1993).

In the present experiments, electrolysis of the bathing solution resulted in inhibition of the acetylcholine-induced relaxation response in rat anococcygeus muscle mounted in epithelium-intact trachea. Electrolysis of a physiological buffer has been reported to produce a mixture of reactive oxygen species (Jackson et al., 1986a). These species are most probably responsible for the impairment of the acetylcholine response in the present study. Furthermore, direct exposure to hydrogen peroxide similarly impaired the acetylcholine response in the coaxial bioassay system.

The free radical scavengers, superoxide dismutase, catalase, and dimethylsulfoxide, offered varying degrees of protection when incubated with the tissues prior to electrolysis, suggesting the involvement of hydroxyl radical, hydrogen peroxide and superoxide anion, respectively, in this system. The generation of these species by electrolysis has been shown previously, using a luminol chemiluminescence assay (Jackson et al., 1986a).

Reactive oxygen species are known to modify membrane lipids and to cause lipid peroxidation and the oxidation of proteins, carbohydrates and DNA (Halliwell and Gutteridge, 1984; Carson et al., 1986; Halliwell, 1996). Furthermore, they may also cause cellular damage and death, or activate apoptosis (Halliwell and Gutteridge, 1984; Jacobson, 1996; Clutton, 1997). In the present study, histological examination did not give any morphological evidence of epithelial damage in the guinea-pig trachea subjected to electrolysis. Impairment of the acetylcholine-response could not be demonstrated when the anococcygeus muscle was exposed to electrolysis separately, before it was put in the epithelium-intact trachea. This finding suggests that the trachea, as donor organ of the coaxial bioassay system was selectively affected by the reactive oxygen species. Dysfunction of the epithelium and, thus, reduced production and/or release of EpDRF can be proposed as a potential mechanism for the reactive oxygen species-induced impairment of acetylcholine response in the present model.

This study demonstrated that reactive oxygen species impaired EpDRF release by trachea epithelium. The interaction of reactive oxygen species with endothelium-dependent relaxing factor (EDRF)/nitric oxide, leading to inhibition of nitric oxide-mediated functions, has been reported (Grylewski et al., 1986; Rubanyi and Vanhoutte, 1986a,b; Lamb et al., 1987; Lawson et al., 1990; Gumusel et al., 1996). Unlike nitric oxide, EpDRF has been suggested to be insensitive to reactive oxygen species (Fernandes and Goldie, 1990; Spina and Page, 1991). This suggestion was based on the findings that EpDRF-mediated responses in the coaxial bioassay system were not affected by free radical scavengers. However, in the present experimental model, EpDRF was found to be sensitive to reactive oxygen species generated by electrolysis.

The effects of reactive oxygen species on the responsiveness of tracheal smooth muscle to various agonists were also evaluated in the present study. The contractile responses elicited by acetylcholine, histamine or 5-hydroxytryptamine were not modified in the guinea-pig

tracheal rings exposed to electrolysis. Reactive oxygen species-induced alterations of receptor-mediated responses have been reported in various tissues including airway smooth muscle. Reduction of β-adrenoceptor responses of guinea-pig tracheal smooth muscle in vitro (Engels et al., 1985) and increased airway responsiveness of anaesthetised cats to inhaled acetylcholine in vivo (Katsumata et al., 1988) have been demonstrated after exposure to reactive oxygen species. However, confirming the present data, it has also been reported that the responses of guinea-pig tracheal smooth muscle to histamine and isoprenaline are unaffected by incubation with hydrogen peroxide (Rhoden and Barnes, 1989).

Enhanced responsiveness of airway smooth muscle after the removal of epithelium has been demonstrated in several studies (Folkerts and Nijkamp, 1998). However, epithelium denudation of guinea-pig tracheal rings did not alter the contractile response to acetylcholine, histamine and 5-hydroxytryptamine in the present study. Thus, the present data may suggest that EpDRF, which is detected by the coaxial bioassay system, does not play a role in modulating the reactivity of guinea-pig tracheal rings. However, the role of cyclooxygenase products can not be ruled out as the experiments were conducted in the presence of indomethacin. Moreover, the method used to prepare the isolated trachea may have influenced the evaluation of smooth muscle reactivity as well (Folkerts and Nijkamp, 1998). When tracheal strips or rings were used instead of tubular preparations, epithelium-derived relaxing substances including EpDRF could have easily diffused away, and therefore their role on smooth muscle responsiveness could not be detected.

In conclusion, the present study demonstrated that exposure to reactive oxygen species resulted in impairment of EpDRF release in the coaxial bioassay system but did not alter the reactivity of tracheal smooth muscle to various agonists.

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