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# Effect of hydrogen peroxide on guinea-pig tracheal smooth muscle *in vitro*: role of cyclo-oxygenase and airway epithelium

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**1** Hydrogen peroxide ($H_2O_2$) (0.1 $\mu$M–3 mM) induced variable contractions of guinea-pig isolated trachea which were attenuated by catalase (100 u ml$^{-1}$) and mannitol (15 mM) suggesting that contractions were induced by $H_2O_2$ and/or the hydroxyl anion.

**2** Epithelial removal potentiated contractile responses of tracheal preparations to $H_2O_2$ with a leftward shift of the concentration-response curve and an increase in the maximal response.

**3** Indomethacin (3 $\mu$M) inhibited contractions to $H_2O_2$ of intact preparations and preparations without epithelium suggesting that contractions may be mediated by cyclo-oxygenase products. Intact preparations (but not preparations without epithelium) contracted in response to high concentrations (>0.1 mM) of $H_2O_2$ in the presence of indomethacin suggesting that other excitatory factor(s) released by the epithelium may induce contraction.

**4** Preincubation of intact tracheal preparations with $H_2O_2$ (1 mM) for 1 h had no effect on responses to histamine or isoprenaline.

**5** These results suggest that hydrogen peroxide generated during the inflammatory process may play a role in bronchoconstriction.

## Introduction

Oxygen-derived free radicals are important mediators of cell and tissue injury during inflammatory processes. In the lung, activation of alveolar macrophages (Drath & Karnovsky, 1975), neutrophils (Babior *et al.*, 1973) and eosinophils (De Chatelet *et al.*, 1977) leads to the release of highly reactive oxygen metabolites including the superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical ($OH^\cdot$). These have been implicated in the pathogenesis of experimental lung injury in animals (Brigham, 1986) and *in vitro* are cytotoxic to a variety of isolated cells (Sacks *et al.*, 1978; Burman & Martin, 1986).

Effects of oxygen-derived free radicals on airway smooth muscle have been reported, including direct effects on muscle tone and alterations of receptor-mediated responses. $H_2O_2$ contracts canine parenchyma and bovine tracheal smooth muscle (Stewart *et al.*, 1981) and $OH^\cdot$ contracts guinea-pig tracheal smooth muscle (Nishida *et al.*, 1985). Macrophages induce a deterioration of $\beta$-adrenoceptor function in guinea-pig tracheal smooth muscle which can be blocked by free radical scavengers (Engels *et al.*, 1985). *In vivo*, inhalation of xanthine/xanthine oxidase (a free radical generating system) leads to an increase in airway responsiveness of anaesthetized cats to inhaled acetylcholine (Katsumata *et al.*, 1988).

In this study we have assessed the direct effect of $H_2O_2$ on guinea-pig tracheal smooth muscle tone and its pharmacological modulation. We have also examined the effect of $H_2O_2$ on relaxation induced by isoprenaline in order to determine whether this free radical species can induce a deterioration of $\beta$-adrenoceptor function *in vitro*.

## Methods

### *Tissue preparation*

Male Dunkin-Hartley guinea pigs (300–600 g) were killed by cervical dislocation and the tracheae were removed. Tracheae were dissected free of connective tissue and were slit longitudinally through the cartilage opposite the smooth muscle layer. Eight transverse segments, each comprising 3–4 opened

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cartilaginous rings, were cut and alternate segments were gently rubbed with a moistened cotton wool probe to remove the epithelium. Tracheal segments were suspended in 10 ml organ baths containing modified Krebs-Henseleit solution at 37°C and bubbled continuously with a 95% $O_2$ and 5% $CO_2$ mixture. The composition of the Krebs-Henseleit solution was as follows (mM): NaCl 118, KCl 5.9, $MgSO_4 \cdot 7H_2O$ 1.2, $CaCl_2 \cdot 6H_2O$ 2.5, $NaH_2PO_4 \cdot H_2O$ 1.2, $NaHCO_3$ 25.5 and glucose 5.6. The pH of the equilibrated solution was 7.4. Tension was measured isometrically by Grass FT.03 transducers (Grass Instrument Co., Quincy, Mass., U.S.A.) and responses were recorded on a Grass 7D Polygraph. An initial tension of 2 g, which was found to be optimal for measurement of changes in tension, was applied to the tissues. During an equilibration period of 1 h, tissues were washed 3–4 times with Krebs-Henseleit solution and tension readjusted to 2 g.

*Effect of hydrogen peroxide on resting tone*

Cumulative concentrations of $H_2O_2$ (0.1 $\mu$M–3 mM) were added to baths containing preparations with and without epithelium. Responses were allowed to plateau between successive additions of $H_2O_2$ or if no response was seen, 3 min contact time was allowed. To study the effect of free radical scavengers on responses to $H_2O_2$, intact preparations were incubated with either catalase 100 u $ml^{-1}$, which degrades $H_2O_2$, or 15 mM mannitol, an $OH^{\cdot}$ scavenger, for 10 min before the addition of $H_2O_2$. To study the effect of cyclo-oxygenase inhibition on responses to $H_2O_2$, preparations were incubated with 3 $\mu$M indomethacin for 30 min before the addition of $H_2O_2$.

*Effect of hydrogen peroxide on responses to isoprenaline*

In order to study relaxation of guinea-pig tracheal smooth muscle to isoprenaline, tissues were precontracted with histamine. Preliminary experiments were therefore carried out to determine the effect of $H_2O_2$ on contractile responses to histamine. Intact preparations were incubated with and without 1 mM $H_2O_2$ for 1 h and cumulative-concentration response curves were constructed for histamine. Increasing concentrations of histamine were added to the baths, responses being allowed to reach a plateau between successive additions of histamine. Concentrations of histamine eliciting 30% of maximal contraction ($EC_{30}$) were determined from these curves ($EC_{30}$ = 1.8 $\mu$M untreated preparations, 1.0 $\mu$M pretreated preparations). In subsequent experiments intact preparations, incubated with and without 1 mM $H_2O_2$ for 1 h, were precontracted with the appropriate $EC_{30}$ for histamine and cumulative concentration-response curves constructed to isoprenaline.

*Drugs and solutions*

The following compounds were used: catalase, histamine dihydrochloride, hydrogen peroxide, indomethacin, (−)-isoprenaline bitartrate (Sigma). Indomethacin was dissolved in phosphate buffer pH 7.8 (0.02 M $KH_2PO_4$ and 0.12 M $Na_2HPO_4$) by heating to 40°C for 30 min. Isoprenaline was dissolved in distilled water containing 200 $\mu g/ml^{-1}$ ascorbic acid. Stock solutions of all other agents were dissolved in distilled water and diluted to appropriate concentrations in Krebs-Henseleit solution.

*Statistical analysis*

Contractile responses to $H_2O_2$ and histamine were expressed in mg of tension. Relaxant responses to isoprenaline were expressed as % inhibition of tone induced by the $EC_{30}$ of histamine used to precontract tissues. Contractile and relaxant responses under different conditions were compared by Student's $t$ test for paired and unpaired data where appropriate. Probability levels of $<0.05$ were considered to be significant. $pD_2$ values were defined as $-\log EC_{50}$ values. In all experiments $n$ represents the number of animals from which tissues were obtained.

## Results

*Effect of hydrogen peroxide on resting tone*

In 10 out of 17 intact tracheal preparations (Figure 1a) 0.1 $\mu$M–1 mM $H_2O_2$ produced a concentration-dependent contraction ($pD_2 = 4.09 \pm 0.19$, maximal contraction $1167 \pm 359$ mg). Higher concentrations caused a variable relaxation although tension remained above the baseline level. In 5 out of 17 intact preparations (Figure 1b) $H_2O_2$ at concentrations greater than 10 $\mu$M only induced relaxation with a maximal magnitude of $-371 \pm 81$ mg at 3 mM $H_2O_2$. In 2 out of 17 preparations $H_2O_2$ had no effect on resting tone at any concentration. An overall contractile response was apparent when changes in tension for all 17 preparations were averaged (Figure 1c), with a $pD_2$ of 3.90 and maximal contraction of $577 \pm 266$ mg. In all preparations there was no difference in the initial tone of the tissue and all preparations responded to a maximal concentration of histamine applied after washing out $H_2O_2$.

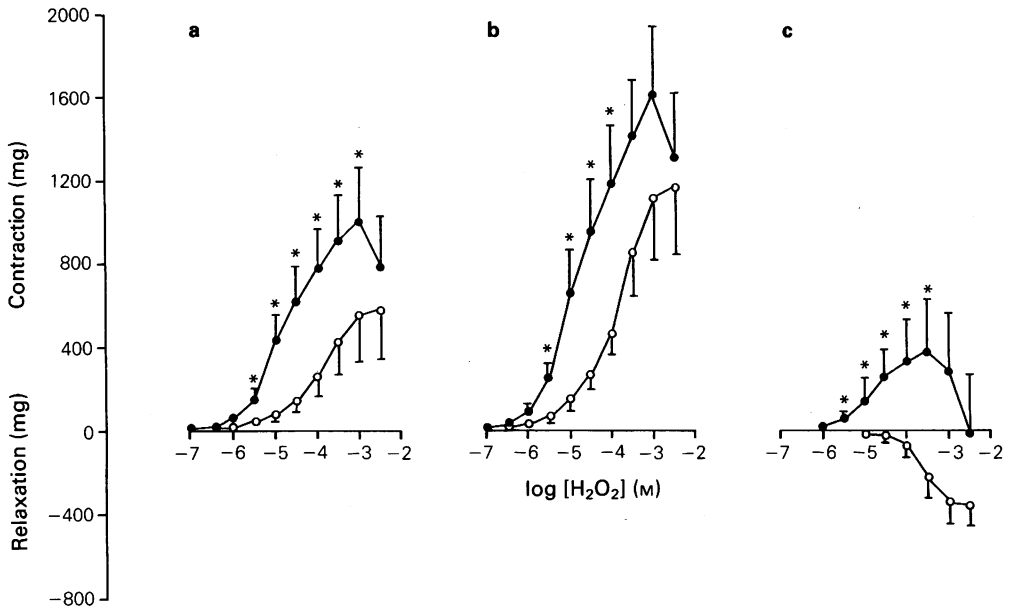


**Figure 1.** Changes in tension induced by $H_2O_2$ in intact tracheal preparations (○) and in preparations without epithelium (●). Figures show mean responses obtained from (a) preparations from all 17 tracheae studied, (b) preparations from 10 out of 17 tracheae where a contractile response was observed in intact preparations, and (c) preparations from 5 out of 17 tracheae where a relaxant response was observed in intact preparations. Points represent means with s.e. indicated by vertical bars. * $P < 0.05$ intact preparations versus those without epithelium.

*Effect of epithelium* Epithelial removal caused a potentiation of contractile responses to $H_2O_2$ manifested as a leftward shift of the concentration-response curve to $H_2O_2$. This was apparent in preparations from the 10 out of 17 tracheae which contracted to $H_2O_2$ ($pD_2$ 4.67 ± 0.14, $P < 0.05$ intact versus preparations without epithelium) or when considering averaged responses of preparations from all 17 tracheae ($pD_2$ 4.8), (Figures 1a and 1c). An increase in the maximal contraction induced by $H_2O_2$ was apparent only when averaged responses for all 17 tracheae were considered (993 ± 277 mg, $P < 0.05$ intact versus preparations without epithelium) but not when considering the 10 out of 17 tracheae which contracted to $H_2O_2$ (1604 ± 376 mg). Preparations with epithelium removed from the 5 out of 17 tracheae shown in Figure 1b also tended to contract in response to $H_2O_2$, with a maximum of 304 ± 275 mg at 0.3 mM $H_2O_2$. At higher concentrations tension tended to return to or relax below baseline.

*Effect of free radical scavengers* Catalase (100 u $ml^{-1}$) inhibited contractile responses of intact preparations to concentrations of up to 1 mM $H_2O_2$ ($n = 6$) (Figure 2). Mannitol (15 mM) reduced con-

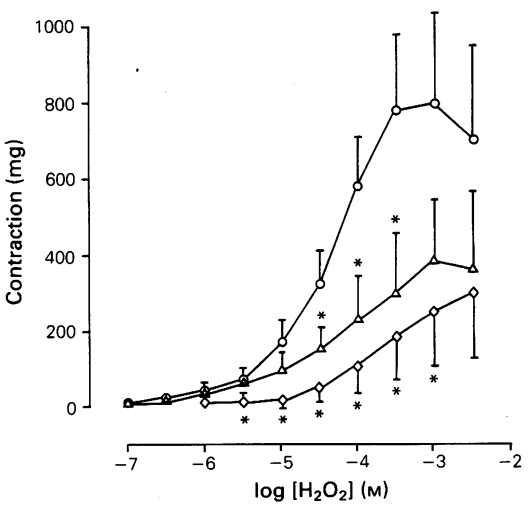


**Figure 2** Contractile responses of intact tracheal preparations to $H_2O_2$ in the presence of catalase 100 u $ml^{-1}$ (◇), mannitol 15 mM (△) or in the absence of free radical scavengers (○). Points represent mean with s.e. shown by vertical bars, $n = 6$. * $P < 0.05$ presence versus absence of free radical scavengers.

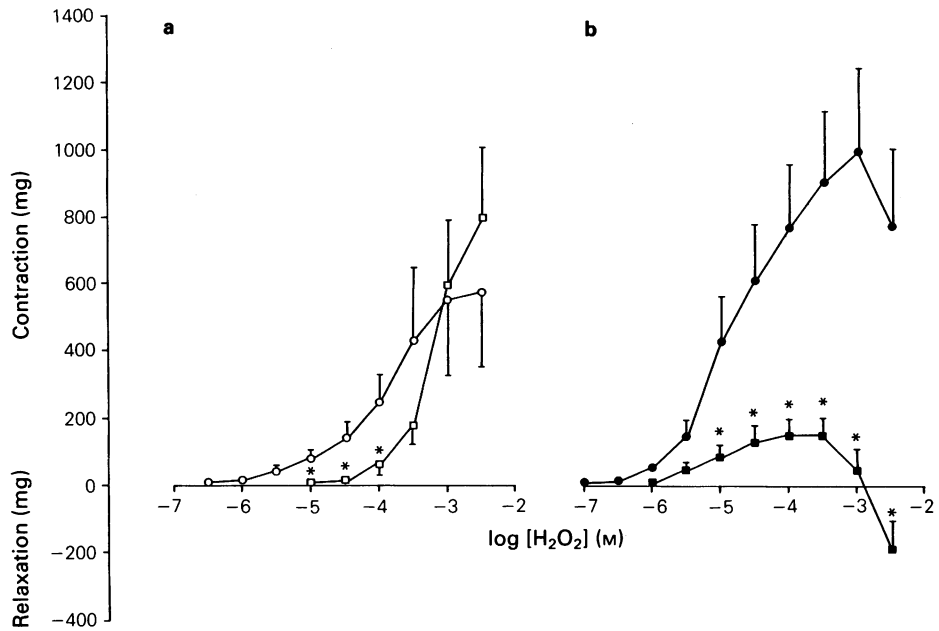


**Figure 3** Contractile responses of (a) intact preparations and (b) preparations without epithelium to $H_2O_2$ in the presence (squares) and absence (circles) of 3 $\mu$M indomethacin. Points represent mean with s.e.mean shown by vertical bars, $n = 13$. * $P < 0.05$ presence versus absence of indomethacin.

tractions induced by 30–300 $\mu$M $H_2O_2$ ($n = 6$) (Figure 2). Catalse and mannitol had no effect on resting tone.

*Effect of cyclo-oxygenase inhibition* The effect of indomethacin on responses of tracheal preparations to $H_2O_2$ are shown in Figure 3. Indomethacin (3 $\mu$M) inhibited contractile responses of intact preparations to low concentrations (up to 0.1 mM) of $H_2O_2$ ($n = 13$). Contractile responses of preparations without epithelium were inhibited at all concentrations of $H_2O_2$ ($n = 13$). In the presence of indomethacin a small relaxation was seen in preparations without epithelium in response to 3 mM $H_2O_2$.

### *Effect of hydrogen peroxide on responses to histamine and isoprenaline*

Contractile responses to histamine were unaffected by pretreatment with 1 mM $H_2O_2$ ($pA_2 = 5.36 \pm 0.17$ untreated preparations, $pA_2 = 5.54 \pm 0.13$ pretreated preparations; $n = 6$). Responses to isoprenaline were unaffected by pretreatment with 1 mM $H_2O_2$ ($pA_2 = 8.50 \pm 0.13$ untreated preparations, $pA_2 = 8.50 \pm 0.10$; $n = 6$). Contractions induced by $H_2O_2$ during the incubation period were transient and resting tension was at the baseline level prior to subsequent experimental procedures.

## Discussion

This study demonstrates that $H_2O_2$ can induce concentration-dependent contraction and/or relaxation of guinea-pig tracheal smooth muscle. Similar concentrations of $H_2O_2$ contract canine parenchyma and bovine tracheal smooth muscle (Stewart *et al.*, 1981). However, in our study responses of intact tracheal preparations to $H_2O_2$ were variable with only 10/17 preparations contracting to $H_2O_2$. Of the remaining preparations, 2 did not respond at all to $H_2O_2$ and 5 relaxed but only at high concentrations of $H_2O_2$. The nature of this variability is unclear since all preparations were equilibrated to a constant tension and all tissues responded to a maximal concentration of histamine at the end of the experiment. Contractile responses to $H_2O_2$ were inhibited by catalase, which degrades $H_2O_2$, and to a lesser degree by mannitol, an $OH^{\cdot}$ scavenger, suggesting that both active oxygen metabolites may contribute to contractile responses.

Mechanical removal of the epithelium potentiates contractile responses to a variety of agonists (Flavahan *et al.*, 1985; Barnes *et al.*, 1985; Fedan *et al.*, 1988) and indeed contractile responses to $H_2O_2$ were also potentiated by epithelial removal. It is unlikely that the epithelium should represent a diffusional barrier to $H_2O_2$ reducing its access to the

underlying smooth muscle since molecules larger than $H_2O_2$, such as mannitol, are able to diffuse freely across the epithelium (Boucher & Gatzy, 1978). $H_2O_2$ is a highly reactive molecule and it is possible that it may be inactivated by the epithelium reducing its effective concentration at the smooth muscle layer. Alternatively, $H_2O_2$ may induce the release of a relaxant factor from the epithelium. By use of a modified 'sandwich' preparation, release of a relaxant factor from guinea pig tracheal epithelium has been demonstrated in response to ovalbumin (Hay *et al.*, 1987).

Contractile responses of intact preparations and preparations without epithelium to low concentrations of $H_2O_2$ were attenuated by indomethacin suggesting that they may be mediated at least in part by cyclo-oxygenase products. Contractions of canine parenchyma and bovine trachea induced by $H_2O_2$ were also inhibited by indomethacin (Stewart *et al.*, 1981). Vasoconstriction in isolated saline-perfused rabbit lungs induced by oxygen-derived free radicals is associated with thromboxane production (Tate *et al.*, 1984). In the presence of indomethacin high concentrations of $H_2O_2$ induced contractions of intact preparation but not preparations without epithelium suggesting that other excitatory factor(s) released by the epithelium may also mediate the response to $H_2O_2$. In the presence of indomethacin, high concentrations of $H_2O_2$ induced small relaxations of preparations without epithelium which may reflect release of a relaxant substance from smooth muscle cells or a direct effect on smooth muscle cells. The response of guinea-pig tracheal smooth muscle may therefore represent a composite of excitatory and inhibitory responses induced by cyclo-oxygenase products and other factors released by the epithelium and possibly smooth muscle cells.

Responses of tracheal smooth muscle to isoprenaline were unaffected by pretreatment with $H_2O_2$. Incubations with 1 mM cumene hydroperoxide for 1 h has been shown to cause a reduction in the number of $\beta$-adrenoceptors in lung membrane preparations (Kramer *et al.*, 1986). Pulmonary macrophages induce a reduction in $\beta$-adrenoceptor responses of guinea-pig tracheal smooth muscle which can be blocked by catalase and thiourea (an $OH^{\cdot}$ scavenger) suggesting that $H_2O_2$ and/or $OH^{\cdot}$ may mediate this effect (Engels *et al.*, 1985). In our study tissues were incubated over a similar period of time (1 h) with $H_2O_2$, however it is possible that the local concentration of oxygen-derived free radicals generated by macrophages may be greater than was possible in our study. Alternatively, other inflammatory mediators released by macrophages in addition to free radicals may be required to induce deterioration of $\beta$-adrenoceptor function.

In conclusion, $H_2O_2$ released by inflammatory cells may induce contraction and/or relaxation of airway smooth muscle which may be mediated partly by cyclo-oxygenase products and partly by factor(s) released by the epithelium and possibly smooth muscle cells.

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## References


BABIOR, B.M., KIPNES, R.S. & CURNUTTE, J.T. (1973). The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.*, **52**, 741–744.

BARNES, P.J., CUSS, F.M. & PALMER, J.B. (1985). The effect of epithelium removal on smooth muscle contractility in bovine trachea. *Br. J. Pharmacol.*, **86**, 685–691.

BOUCHER, R.C. & GATZY, J.T. (1978). Tracheal epithelial permeability to nonelectrolytes. *Physiologist*, **21**, 11A.

BRIGHAM, K.L. (1986). Role of free radicals in lung injury. *Chest*, **89**, 859–863.

BURMAN, W.J. & MARTIN, W.J. (1986). Oxidant-mediated ciliary dysfunction: possible role in airway disease. *Chest*, **89**, 410–413.

DE CHATELET, L.R., SHIRLEY, P.S., MC PHAIL, L.C., HUNTLEY, C.C., MUSS, H.B. & BASS, D.A. (1977). Oxidative metabolism of the human eosinophil. *Blood*, **50**, 526–535.

DRATH, D.B. & KARNOVSKY, M.L. (1975). Superoxide production by phagocytic leukocytes. *J. Exp. Med.*, **141**, 257–262.

ENGELS, F., OOSTING, R.S. & NIJKAMP, F.P. (1985). Pulmonary macrophages induce deterioration of guinea-pig tracheal $\beta$-adrenergic function through release of oxygen radicals. *Eur. J. Pharmacol.*, **111**, 143–144.

FEDAN, J.S., HAY, D.W.P., FARMER, S.G. & RAEBURN, D. (1988). Epithelial cells: modulation of, airway smooth muscle reactivity. In *Asthma: Basic Mechanisms and Clinical Management*. ed. Barnes, P.J., Rodgers, I.W. & Thomson, N.C. pp. 143–159. London: Academic Press.

FLAVAHAN, N.A., AARHUS, L.L., RIMELE, T.J. & VANHOUTTE, P.M. (1985). Respiratory epithelium inhibits bronchial smooth muscle tone. *J. Appl. Physiol.*, **58**, 834–838.

HAY, D.W.P., MUCCITELLI, R.M., HORSTEMEYER, D.L., WILSON, K.A. & RAEBURN, D. (1987). Demonstration of the release of an epithelium-derived inhibitory factor from a novel preparation of guinea-pig trachea. *Eur. J. Pharmacol.*, **136**, 247–250.

KATSUMATA, U., ICHINOSE, M., MIURA, M., KIMURA, K., INOUE, H. & TAKISHIMA, T. (1988). Reactive oxygen exposure produces airway hyperresponsiveness. *Am. Rev. Resp. Dis.*, **137**, 285A.

KRAMER, K., RADEMAKER, B., ROZENDAL, W.H.M., TIMMERMAN, H. & BAST, A. (1986). Influence of lipid peroxidation on $\beta$-adrenoceptors. *FEBS Lett.*, **198**, 80–84.

NISHIDA, Y., SUZUKI, S. & MIYAMOTO, T. (1985). Biphasic contraction of isolated guinea-pig tracheal chains by superoxide radical. *Inflammation*, **9**, 333–337.

SACKS, T., MOLDOW, C.F., CRADDOCK, P.R., BOWERS, T.K. & JACOB, H.S. (1978). Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes. *J. Clin. Invest.*, **61**, 1161–1167.

STEWART, R.M., WEIR, E.K., MONTGOMERY, M.R. & NIEWOHMER, D.E. (1981). Hydrogen peroxide contracts airway smooth muscle: a possible endogenous mechanism. *Respir. Physiol.*, **45**, 333–342.

TATE, R.M., MORRIS, H.G., SCHROEDER, W.R. & REPINE, J.E. (1984). Oxygen metabolites stimulate thromboxane production and vasoconstriction in isolated saline-perfused rabbit lungs. *J. Clin. Invest.*, **74**, 608–613.

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