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European Journal of Pharmacology 513 (2005) 219–224
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Hydrogen peroxide and endothelium-dependent hyperpolarization in the guinea-pig carotid artery

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Received 25 January 2005; received in revised form 14 February 2005; accepted 24 February 2005
Available online 15 April 2005

Abstract

This study was designed to determine whether or not endothelium-dependent hyperpolarizations evoked by acetylcholine in the isolated guinea-pig carotid artery involve hydrogen peroxide. Membrane potential was recorded in the vascular smooth muscle cells of that artery. Under control conditions, acetylcholine induced endothelium-dependent hyperpolarization of the vascular smooth muscle cells which was not affected by the presence of catalase, superoxide dismutase or their combination. Neither the superoxide dismutase mimetic, tiron nor the thiol-reducing agent *N*-acetyl-L-cysteine modified the hyperpolarization evoked by 0.1 μM acetylcholine but each produced a partial and significant inhibition of the hyperpolarization induced by 1 μM acetylcholine. Neither 10 nor 100 μM hydrogen peroxide influenced the resting membrane potential of the smooth muscle cells and the higher concentration did not significantly influence the hyperpolarization elicited by acetylcholine. These data indicate that, in the guinea-pig isolated carotid artery, hydrogen peroxide is unlikely to contribute to the endothelium-dependent hyperpolarization evoked by acetylcholine.

Keywords: Endothelium; Smooth muscle; Endothelium-derived hyperpolarizing factor; Hydrogen peroxide; Membrane potential

1. Introduction

The endothelium controls blood vessel tone by releasing nitric oxide (Furchgott and Zawadzki, 1980) and prostacyclin (Moncada and Vane, 1979) as well as by a third pathway that involves the hyperpolarization of the vascular smooth muscle. This "endothelium-dependent hyperpolarizing factor (EDHF)" pathway is inhibited by blockers of calcium-activated potassium channels (K_{Ca}, for review: Busse et al., 2002). Vascular cells, including endothelial cells, produce hydrogen peroxide which, depending on the vascular bed, can evoke hyperpolarization and relaxation of smooth muscle. The possibility that hydrogen peroxide could act as an EDHF has been recently proposed although this hypothesis remains controversial.

Most of the evidence in favour of hydrogen peroxide being an EDHF came from the observations that, in some blood vessels such as the murine, human and rat mesenteric arteries (Matoba et al., 2000, 2002; Kimura et al., 2002; Morikawa et al., 2003) or the canine, porcine and human coronary arteries (Matoba et al., 2003; Miura et al., 2003; Yada et al., 2003), the agonist- and flow-induced EDHF-mediated responses were partially or totally inhibited by catalase. In these blood vessels, exogenously added hydrogen peroxide appeared to mimic the effects of EDHF, since it produced endothelium-independent relaxation and/or hyperpolarization of the vascular smooth muscle cells by a mechanism involving K_{Ca} activation (Hayabuchi et al., 1998). Furthermore, in murine mesenteric (Matoba et al., 2000;

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doi:10.1016/j.ejphar.2005.02.042

Morikawa et al., 2003) and porcine coronary arteries (Matoba et al., 2003), the agonist-induced EDHF-mediated response was associated with a catalase-sensitive production of hydrogen peroxide by the endothelium. Additionally, hydrogen peroxide can activate endothelial K_{Ca} (Bychkov et al., 1999). As the hyperpolarization of the endothelial cells can be transmitted to the adjacent myocytes, hydrogen peroxide could contribute to EDHF-mediated responses not only as a diffusible factor that activates smooth muscle K_{Ca}, but also as an intracellular endothelial cell messenger that modulates the activity of endothelial K_{Ca}.

However, reports from other groups refute the hypothesis that EDHF-mediated responses could be attributed to hydrogen peroxide generation (Ellis and Triggle, 2003). Indeed, catalase does not inhibit those responses in the porcine canine and rat coronary arteries (Beny and Von Der Weid, 1991; Fulton et al., 1997; Pomposiello et al., 1999; Tanaka et al., 2003), human radial arteries (Hamilton et al., 2001), murine aorta and mesenteric artery (Ellis et al., 2003) and in rabbit ileo-femoral and mesenteric arteries (Chaytor et al., 2003; Itoh et al., 2003). Furthermore, in the rabbit ileo-femoral arteries, although the calcium ionophore A23187, and to a much lesser extent acetylcholine, induced the endothelial production of hydrogen peroxide which caused smooth muscle relaxation, there was virtually no associated hyperpolarization (Chaytor et al., 2003).

In the guinea-pig isolated carotid artery, one of the reference arteries used for studying EDHF (Busse et al., 2002), the contribution of hydrogen peroxide to endothelium-dependent hyperpolarizations is unknown. Thus the purpose of the present study was to evaluate the role of hydrogen peroxide (if any) in the endothelium-dependent hyperpolarization evoked by acetylcholine in this blood vessel.

2. Methods

The protocols described in this manuscript comply with the guidelines of the Declaration of Helsinki and Tokyo, for humans, and the European Community guidelines for the use of experimental animal and have been approved by our Institutional Ethical committee.

2.1. Membrane potential recording

Male Hartley guinea pigs (250–300 g) were killed with an overdose of pentobarbitone (200 mg/kg, i.p.) and the internal carotid arteries with their branches were dissected free. Segments of artery (1 cm in length) were cleaned of adherent connective tissues and pinned down to the bottom of an organ chamber (0.5 ml in volume) superfused at a constant flow (2 ml/min and 37 °C) with modified Krebs-Ringer bicarbonate solution of the following composition (in mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1 and ethylenediamine tetra-acetic acid (EDTA) 0.026 (buffered with 95% O_2 and 5% CO_2, pH 7.4). Transmembrane potentials were recorded from the adventitial side of the internal carotid arteries with glass capillary microelectrodes (tip resistance of 30–90 MΩ) filled with KCl (3 M) and connected to the headstage of a recording amplifier (World Precision Instrument, intra 767, New Haven, CT); an Ag/AgCl pellet, in contact with the bathing solution and directly connected to the amplifier, served as the reference electrode. The signal was continuously monitored on an oscilloscope (Gould DSO 405, Valley View, OH, USA) and recorded using pClamp software (Axon instrument, Foster City, CA, USA). Successful impalements were signalled by a sudden negative drop in potential from the baseline (zero potential reference) followed by a stable negative potential for at least 3 min. The acetylcholine-induced hyperpolarizations, which in this artery are fully endothelium-dependent (Corriu et al., 1996a,b), were analysed using pClampfit (Axon instrument, Foster City, CA, USA) and hyperpolarization values are expressed as the maximal amplitude between resting membrane potential and the membrane potential in the presence of the hyperpolarizing drugs (peak amplitude). Drugs were added by continuous superfusion via the Krebs solution reservoir. The incubation time was at least 45 min with the various inhibitors studied.

2.2. Drugs

Acetylcholine, hydrogen peroxide, catalase (bovine liver, reference C100), superoxide dismutase (from bovine erythrocytes, reference S-2515), 4,5-dihydroxy-1,3-benzenedisulfonic acid (tiron), *N*-acetyl-L-cysteine Sigma (La Verpillère, France). Cromakalim was synthesised by the Institut de Recherches Servier (Suresnes, France).

2.3. Statistics

Data are shown as mean ± SEM; n indicates the number of different animals from which the blood vessels were taken. Comparisons vs. control were performed statistically using an analysis of variance (ANOVA1 followed by the Dunnett's t-test) or by use of Student's t-test for paired or unpaired observations, as appropriate. Differences were considered to be statistically significant when the P value was less than 0.05.

3. Results

All the experiments were performed in the presence of N^{ω}-nitro-L-arginine (100 μM) and indomethacin (5 μM) in order to inhibit nitric oxide synthase and cyclo-oxygenase, respectively.

Under control conditions, the membrane potential of the smooth muscle cells averaged −51.1 ± 1.3 mV (n = 19 cells

from 9 different guinea-pigs) and was not significantly influenced by the presence of catalase (1200 U/ml), superoxide dismutase (120 U/ml), the combination of catalase plus superoxide dismutase, tiron (1 mM) or *N*-acetyl-cysteine (10 mM).

The endothelium-dependent hyperpolarization of the smooth muscle cells produced by acetylcholine (0.1 and 1 μM) was not significantly influenced by the presence of catalase, superoxide dismutase or their combination (Fig. 1). Tiron (4,5-dihydroxy-1,3-benzene-disulfonic) did not significantly affect the hyperpolarization produced by acetylcholine (0.1 μM) but significantly reduced that evoked by the highest concentration of acetylcholine tested (1 μM, $P<0.05$; Fig. 2). Similarly, *N*-acetyl-cysteine did not influence the effects of acetylcholine (0.1 μM) but significantly reduced that of acetylcholine 1 μM (hyperpolarization: 21.3 ± 1.8 mV, $n=6$ and 12.2 ± 2.2 mV, $n=3$, in control and the presence of *N*-acetyl-L-cysteine, respectively). The cromakalim-induced hyperpolarizations were not affected by either tiron or *N*-acetyl-L-cysteine (data not shown).

Hydrogen peroxide (10 and 100 μM) did not produce any significant changes in the resting membrane potential of the vascular smooth muscle cells. The hyperpolarization evoked by acetylcholine (1 μM) was not affected significantly by the presence of hydrogen peroxide (100 μM; Fig. 3).

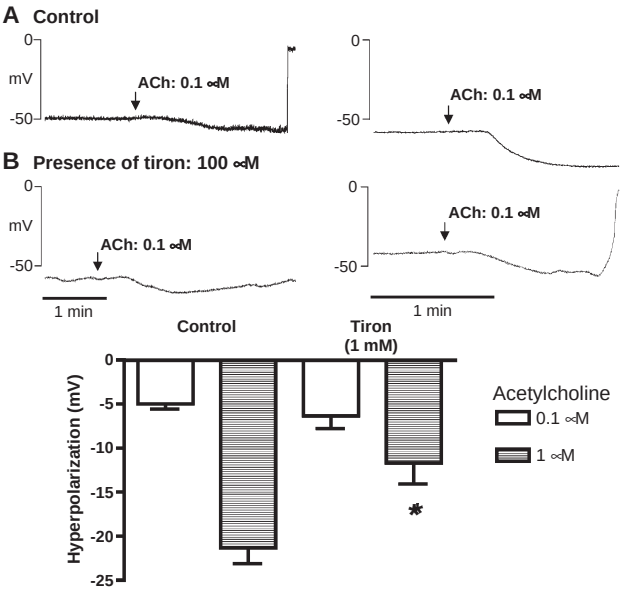

Fig. 2. Acetylcholine (0.1 and 1 μM)-induced endothelium-dependent hyperpolarization in the guinea-pig isolated carotid artery (in the presence of N^{ω}-nitro-L-arginine, 100 μM, and indomethacin, 5 μM). Top panels: original traces showing the endothelium-dependent hyperpolarizations elicited by acetylcholine 0.1 μM (left) and 1 μM (right), under control conditions (A) and in the presence of tiron (1 mM, B). Lower panel: summary bar graph showing the effects of tiron (1 mM). Data are shown as mean ± S.E.M. ($n=3$ and indicates the number of different animals from which the blood vessels were taken). The asterisk indicates a statistically significant difference versus the control values ($P<0.05$).

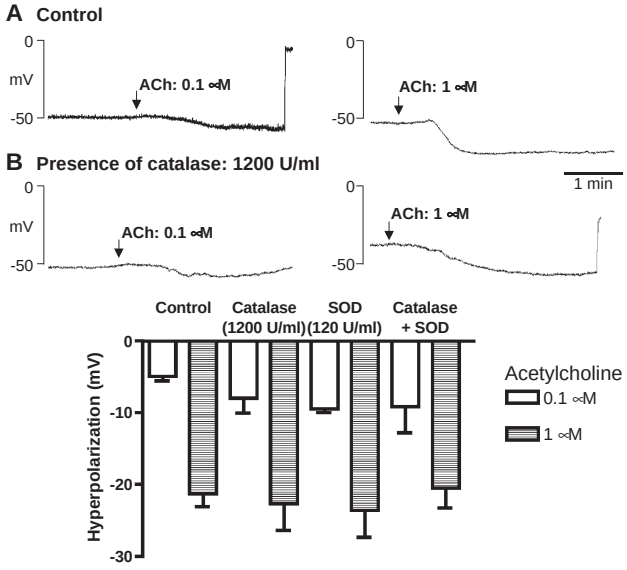

Fig. 1. Acetylcholine (0.1 and 1 μM)-induced endothelium-dependent hyperpolarization of the smooth muscle cells in the guinea-pig isolated carotid artery (in the presence of N^{ω}-nitro-L-arginine, 100 μM, and indomethacin, 5 μM). Top panels: original traces showing the endothelium-dependent hyperpolarizations elicited by acetylcholine 0.1 μM (left) and 1 μM (right), under control conditions (A) and in the presence of catalase (1200 U/ml, B). Lower panel: summary bar graph showing the effects of catalase (1200 U/ml), superoxide dismutase (SOD, 120 U/ml) and their combination. Data are shown as mean ± S.E.M. ($n=3$–4 and indicates the number of different animals from which the blood vessels were taken).

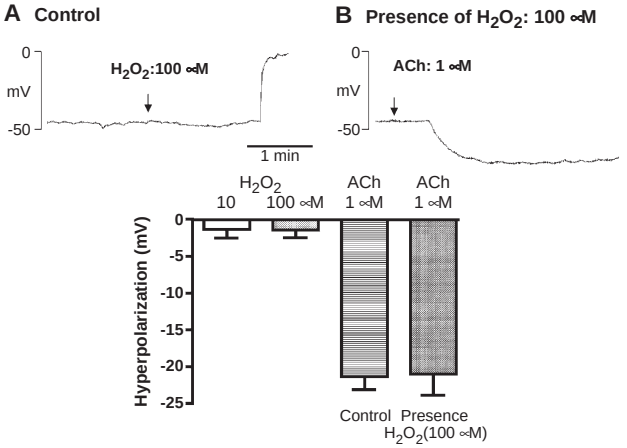

Fig. 3. Effects of hydrogen peroxide (H_2O_2: 10 and 100 μM), acetylcholine (ACh: 1 μM) and the combination of hydrogen peroxide (100 μM) plus acetylcholine (1 μM) on the membrane potential of the smooth muscle cells of the guinea-pig isolated carotid artery with endothelium (in the presence of N^{ω}-nitro-L-arginine, 100 μM, and indomethacin, 5 μM). Top panel: original traces showing the changes in membrane potential elicited by H_2O_2 (100 μM, A) and the endothelium-dependent hyperpolarizations elicited by acetylcholine (1 μM) in the presence of H_2O_2 (100 μM, B). Lower panel: summary bar graph showing the changes in membrane potential produced by hydrogen peroxide (H_2O_2: 10 and 100 μM), and the endothelium-dependent hyperpolarizations elicited by acetylcholine (ACh: 1 μM) under control conditions or in the presence of hydrogen peroxide (100 μM). Data are shown as mean ± S.E.M. ($n=3$–6 and indicates the number of different animals from which the blood vessels were taken).

4. Discussion

The present study suggests that hydrogen peroxide does not contribute to the endothelium-dependent hyperpolarization elicited by acetylcholine in the isolated guinea-pig carotid artery.

In this blood vessel, acetylcholine induced a concentration-and endothelium dependent hyperpolarization (Corriu et al., 1996a,b; Gluais et al., 2005). Catalase, the enzyme that converts hydrogen peroxide into water and oxygen, did not affect the hyperpolarizations evoked by acetylcholine. In in vitro experiments involving isolated arteries, the reported range of effective catalase concentrations varies widely (from 400 to 6250 U/ml). Although the concentration used in the present study (1250 U/ml) is somewhat lower than that used by some workers in studies involving human, porcine and murine arteries (Matoba et al., 2002, 2003; Morikawa et al., 2003), others have shown that this concentration is indeed effective in isolated arteries from various species including the guinea-pig (Feletou and Vanhoutte, 1987; Yang et al., 2002; Fujimoto et al., 2003). Furthermore, 1250 U/ml is within the range used by several groups in various other arteries (Matoba et al., 2000; McNeish et al., 2002; Chaytor et al., 2003; Ellis et al., 2003; Itoh et al., 2003).

The inability of catalase to inhibit EDHF-mediated responses has been reported in many other blood vessels (Beny and Von Der Weid, 1991; Fulton et al., 1997; Pomposiello et al., 1999; Hamilton et al., 2001; Chaytor et al., 2003; Ellis et al., 2003; Itoh et al., 2003; Tanaka et al., 2003). Therefore, in the guinea-pig carotid artery and in the above-mentioned arteries, the absence of an inhibitory effect of catalase is not in favour of hydrogen peroxide being a diffusible EDHF. This is further substantiated in the present study by the inability of hydrogen peroxide up to 100 μM, to elicit any hyperpolarization of the smooth muscle cells or to enhance that elicited by acetylcholine. This observation is also in agreement with other observations performed in rabbit arteries in which hydrogen peroxide elicits hyperpolarization of smooth muscle cells only at concentrations above 100 μM (Chaytor et al., 2003; Itoh et al., 2003).

A pivotal role in hydrogen peroxide-dependent EDHF-mediated responses has been ascribed to the Cu,Zn superoxide dismutase, a so-called "EDHF-synthase" being responsible for the production of hydrogen peroxide from enzymatically-derived superoxide anions (Morikawa et al., 2003, 2004). In murine and human mesenteric arteries as well as in porcine coronary arteries, cell-permeable superoxide dismutase-mimetics such as tempol and tiron, but not polyethyleneglycol-superoxide dismutase, enhance both endothelial hydrogen peroxide generation and EDHF-mediated responses (Matoba et al., 2003; Morikawa et al., 2003, 2004). The ineffectiveness of polyethyleneglycol-superoxide dismutase has been attributed to a poor permeability of this large molecule. In the present study, superoxide dismutase did not enhance the endothelium-dependent hyperpolarization induced by acetylcholine. Whether this lack of effect of superoxide dismutase should indeed be attributed to a failure to gain access to appropriate intracellular compartments is questionable. For instance, polyethyleneglycol-superoxide dismutase and superoxide dismutase enhances the nitric oxide-dependent component of endothelium-dependent relaxations in the human radial and in the rabbit mesenteric arteries. Furthermore, in the latter, the enhancement of the nitric oxide-generated relaxation by superoxide dismutase is dependent on the generation of hydrogen peroxide by the smooth muscle cells, indicating that superoxide dismutase crossed the plasma membrane of the smooth muscle cells (Hamilton et al., 2001; Itoh et al., 2003). However, in these two arteries as well as in the murine mesenteric artery and in the bovine perfused eye preparation, the EDHF-mediated responses were not affected by superoxide dismutase and/or polyethyleneglycol-superoxide dismutase, suggesting again that hydrogen peroxide is not involved in those responses (Hamilton et al., 2001; McNeish et al., 2002; Ellis et al., 2003; Itoh et al., 2003).

In the present study, tiron also failed to enhance the endothelium-dependent hyperpolarization in response to 0.1 μM acetylcholine. Furthermore, this cell-permeable superoxide dismutase mimetic produced a significant *inhibition* of the hyperpolarization elicited by 1 μM acetylcholine. Again, the absence of a tiron-induced potentiation of the endothelium-dependent hyperpolarization is not in favour of a role for hydrogen peroxide in EDHF-mediated responses. The difference between the effects of tiron and superoxide dismutase can be explained either by a difference in their cell permeabilities (see above) or by differences in the properties of the authentic superoxide dismutase and the superoxide dismutase-mimetic (Mackenzie et al., 1999; Itoh et al., 2003). *N*-acetyl-L-cysteine, a thiol-reducing agent, produced a very similar qualitative and quantitative inhibition as that caused by tiron. Inhibition of EDHF-mediated responses by *N*-acetyl-L-cysteine has been reported in the porcine coronary artery and in the bovine perfused eye preparation (McNeish et al., 2002; Ndiaye et al., 2003). Such inhibitory effects of both tiron and *N*-acetyl-L-cysteine appear selective since the hyperpolarization produced by the opener of ATP-sensitive potassium channel, cromakalim, was not affected. This suggests the involvement of a redox-sensitive mechanism in the endothelium-dependent, acetylcholine-induced hyperpolarization of the guinea-pig isolated carotid artery. In this artery, the EDHF-mediated responses involve an increase in $[Ca^{2+}]_i$. This leads to the activation of endothelial intermediate and small conductance calcium-activated potassium channels (IK_{Ca} and SK_{Ca}) followed by the transmission of the endothelial hyperpolarization toward the smooth muscle cells via myo-endothelial gap junctions (Corriu et al., 1996a; Chataigneau et al., 1998, Edwards et al., 1999; Quignard et al., 2000). The mechanism of the inhibition elicited by tiron and *N*-acetyl-L-cysteine is beyond the scope of this study but many proteins can be

regulated by a redox mechanism. For instance, redox modifications of thiol groups could modulate the activity of IK_{Ca} (Cai and Sauve, 1997).

Collectively, these data indicate that, under the current experimental conditions, hydrogen peroxide is unlikely to contribute to the endothelium-dependent hyperpolarization evoked by acetylcholine in the guinea-pig isolated carotid artery.

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