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2,5-Di-*t*-butyl-1,4-benzohydroquinone induces endothelium-dependent relaxation of rat thoracic aorta

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Abstract

The aim of this work was to clarify the mechanism by which 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ) induces relaxation of rat thoracic aorta. In particular, the role of endothelium-derived nitric oxide (NO) was investigated. BHQ concentration dependently (0.1–10 μM) relaxed rat aorta rings precontracted with phenylephrine. This effect was dependent on the intactness of the endothelium, suppressed by preincubation with 100 μM *N*ω-nitro-L-arginine methyl ester and antagonised by 3–30 μM methylene blue. The 10 μM BHQ-induced relaxation, however, was followed by the gradual and slow return to phenylephrine-induced tone. Superoxide dismutase (250 U/ml) increased the BHQ-induced relaxation, while preincubation with 3 mM diethyldithiocarbamate inhibited it in a time-dependent fashion. BHQ gave rise to superoxide anion formation which was markedly inhibited by the addition of superoxide dismutase (250 U/ml), either in the presence or in the absence of aorta rings. The non-specific blocker of Ca^{2+} channels, Ni^{2+}, concentration dependently attenuated the BHQ relaxing effect. BHQ did not modify the relaxation induced by the NO donor 3-morpholino-sydnonimine in endothelium-deprived rings. In conclusion, BHQ induces endothelium-dependent relaxation and gives rise, by auto-oxidation, to the formation of superoxide anion. The former effect results from the enhanced synthesis of NO rather than from its enhanced biological activity; NO synthase is presumed to be stimulated by BHQ-induced activation of Ca^{2+} influx through Ni^{2+}-sensitive Ca^{2+} channels.

Keywords: BHQ (2,5-di-*t*-butyl-1,4-benzohydroquinone); Nitric oxide (NO); Superoxide anion; Ca^{2+} channel, Ni^{2+}-sensitive

1. Introduction

Hindered phenols, either of natural or synthetic origin, represent a class of compounds widely used in food preservation and for the inhibition of lipid peroxidation in living tissues. *tert*-Butyl phenol derivative, as well as other synthetic phenol, have undergone biochemical or pharmacological analysis in order to evaluate their therapeutic potential.

Previous studies from this laboratory (Sgaragli et al., 1993; Gorelli et al., 1995; Fusi et al., 1998b) have shown that a series of phenols related to 2-*t*-butyl-4-methoxyphenol (BHA), in addition to their well-known antioxidant activity, relax smooth muscles by affecting Ca^{2+} homeostasis and that the two properties are linearly correlated. Such compounds, which are characterised by at least one hydroxyl group on the aromatic ring and a highly lipophilic moiety, might be useful for preventing tissue damage caused by ischemia–reperfusion injury (McCall and Panetta, 1992; Sgaragli et al., 1993). In the search for more active compounds, our attention was attracted by 2,5-di-*t*-butyl-1,4-benzohydroquinone (BHQ), a specific inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (Nakamura et al., 1992). The structural requirements for the bifunctional activity described above in fact occur twice in BHQ. Moreover, the plane of symmetry separating the two moieties makes it likely that this molecule acts as a powerful smooth muscle relaxing agent as well as an antioxidant.

Although the biochemical effects of BHQ have been well documented, the full characterisation of its pharmacological activity on vascular smooth muscle has not yet been accomplished. The pleiotypic effects of BHQ on rat vascular smooth muscles have been already described (Fusi et al., 1998a). These consist of a myotonic effect, dependent on the activation of Ca^{2+} influx via a Ni^{2+}-sensitive

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pathway, and a myolytic effect, dependent on either the antagonism of Ca^{2+} entry via L-type Ca^{2+} channels or depletion of intracellular Ca^{2+} stores. The aim of the present work was to investigate the effects of BHQ on vascular smooth muscles in the presence of endothelium, there being little information available to date on its actions on the latter tissue. The vasorelaxant effect of BHQ is described and the underlying mechanism of action is characterised.

2. Materials and methods

2.1. Aortic ring preparation and equilibration period

Male Wistar rats (250–350 g) were anaesthetised with a mixture of Ketavet® (Gellini, Italy) and Rompum® (Bayer, Germany), decapitated and exsanguinated. The thoracic aorta was immediately removed, cleaned of adhering fat and connective tissue and cut into 1.5-mm wide rings with a razor blade slicing device. Care was taken to avoid abrasion of the intimal surface of the rings to maintain the integrity of the endothelial layer. Endothelial cells were removed from some rings by gently rubbing the intimal surface with curved forceps. Each arterial ring was mounted over two rigid parallel stainless steel tubes, one fixed in place and the other attached to an isometric transducer (Basile, Varese, Italy). The preparation was immersed in a water-jacketed organ bath (37°C) containing 5 ml of a modified Krebs–Henseleit physiological salt solution (PSS) (composition mM: NaCl 124, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, KH_2PO_4 0.4, $NaHCO_3$ 25 and glucose 5.5) bubbled with a 95% O_2–5% CO_2 gas mixture to give a pH of 7.4. PSS containing 80 mM KCl (K80 PSS) was prepared by replacing NaCl with equimolar KCl. The vessel segments were allowed to equilibrate for 1 h at a resting tension of 1 g. During the equilibration period, PSS was changed every 15 min. After the equilibration period, the aortic rings were stimulated with K80 PSS until a sustained response was obtained ($\sim$ 15 min), in order to test their contractile capacity. Under these conditions, maximal plateau values of active tension of about 3.33 mN were obtained. Following a 30-min washout, the presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (10 μM) to induce relaxation of rings precontracted with phenylephrine (Furchgott and Zawadzki, 1980). Where not specified, rings possessed a functional endothelium. The rings were then washed and equilibrated for another 30–60 min before testing the different experimental settings (see below). For relaxation studies, the aorta was precontracted with a submaximal (80% of maximum) concentration of phenylephrine (0.1 μM) and cumulative concentration–response curves for BHQ and the NO donor, 3-morpholino-sydnonimine (SIN-1), were made; relaxation was then evaluated as a percentage of the initial tension. Preliminary experiments were conducted to assess whether the absence of a functional endothelium, as well as preincubation with diethyldithiocarbamate, methylene blue, BHQ, *N*ω-nitro-L-arginine methyl ester (L-NAME), Ni^{2+} or superoxide dismutase could affect the phenylephrine-induced contraction. If so, the phenylephrine concentration was adjusted in order to gain the tension achieved with the EC_{80} in vessels with a functional endothelium.

2.2. Characterisation of the relaxant effect of BHQ

Concentration–response curves were made by adding BHQ (or SIN-1) cumulatively to rings (endothelium-intact or endothelium-deprived) precontracted with phenylephrine. When L-NAME or methylene blue was present, addition was made 15 min before phenylephrine. Methylene blue, during the 15-min incubation, increased the resting tone in all but two preparations (0.077 ± 0.019, $n = 9$ and 0.384 ± 0.153 mN, $n = 10$ at 3 and 30 μM, respectively). This effect might be due either to blockade of basal unstimulated soluble guanylyl cyclase and nitric oxide (NO) synthase activity (Moncada et al., 1991; Mayer et al., 1993) or to release of norepinephrine from the intramural nerves (Martin et al., 1985).

2.3. Effect of Ni^{2+} on BHQ-induced relaxation

Increasing concentrations of BHQ were added cumulatively after the rings had been contracted with a sub-maximal concentration of phenylephrine. Different preparations were pretreated with Ni^{2+} (0.3–1 mM) and then similar concentration–response curves for BHQ were made. When Ni^{2+} was present, addition was made 15 min before phenylephrine.

2.4. Effects of diethyldithiocarbamate and superoxide dismutase on BHQ-induced relaxation

Diethyldithiocarbamate, an inhibitor of Cu/Zn superoxide dismutase (Kelner et al., 1989), was added to the organ bath 15, 30 or 45 min before phenylephrine. In view of its irreversible action (Paisley and Martin, 1996), a washout period of 10 min prior to the addition of exogenous superoxide dismutase was used in order to avoid inhibition of the added enzyme. Exogenous superoxide dismutase (250 U/ml) was given 5 min before the addition of phenylephrine.

2.5. Superoxide anion detection

Superoxide anion was detected by using a spectrophotometric assay as described by Goldberg and Stern (1977). In brief, 1 ml PSS containing 192 μM nitroblue tetrazolium was added to polystyrene cuvettes in a spectrophotometer, Shimadzu UV-160, at 37°C. Superoxide anion generation

was assessed by measuring the increase in absorbance at 560 nm associated with the reduction of nitroblue tetrazolium. The increase in absorbance was recorded continuously for 20 min. With 4.5 mM dihydroxyfumaric acid, large and sustained steady-state levels of superoxide anion were attained due to the decomposition of dihydroxyfumaric acid at pH 7.4 (Goldberg and Stern, 1977).

2.6. Effect of BHQ on relaxation produced by SIN-1

The effect of BHQ on the SIN-1-induced relaxation was investigated in endothelium-denuded rings preincubated with superoxide dismutase (250 U/ml). Concentration–response curves for SIN-1 were made by cumulative addition (1 nM–100 μM) to phenylephrine precontracted vessels in the absence or presence of 1 μM BHQ.

2.7. Drugs: commercial sources and solutions

BHQ, superoxide dismutase, $NiCl_2$, nitroblue tetrazolium, diethyldithiocarbamate and phenylephrine were purchased from Sigma (Milan, Italy). Methylene blue was purchased from Carlo Erba (Italy). SIN-1 was a gift from Dr. R. Henning (Cassella, Germany). All other compounds were of analytical grade and used without further purification. Inactivated superoxide dismutase was prepared by heating the solution at 100°C for 30 min. Stock solutions of BHQ (in 100% DMSO, shielded from light with aluminium foil), diethyldithiocarbamate and SIN-1 were prepared freshly each day. Dimethylsulfoxide (DMSO) in the bath did not exceed 0.1% (v/v), at which concentration it had no effect.

2.8. Statistical analysis

All values are expressed as means ± S.E.M.; n is the number of animals (indicated in parentheses). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey's test for multiple comparisons, as appropriate. P values < 0.05 were considered significant. The pharmacological response to each substance, described in terms of pEC_{50} (the negative logarithm to base 10 of EC_{50}), was calculated using the GraphPad Prism 2.01 software computer program (GraphPad Software, USA).

3. Results

3.1. Endothelium-dependent relaxant effect of BHQ

As shown in Fig. 1, the cumulative addition of BHQ relaxed phenylephrine precontracted rings with a pEC_{50} of 6.04 ± 0.06 M ($n = 5$), while that of the endothelium-independent vasodilator SIN-1 was characterised by a slightly higher pEC_{50} (6.54 ± 0.08 M, $n = 6$, $P < 0.001$). The maximal relaxation obtained with 10 μM BHQ, corresponding to 30.8 ± 9.2% of the initial tension ($n = 5$), however, was not sustained, and there was a gradual return to phenylephrine-induced tone. The further addition of 1 mM Ni^{2+} or 10 μM SIN-1 fully relaxed the preparation. In rings deprived of endothelium, BHQ did not induce relaxation (Fig. 2). In contrast, the addition of 10 μM SIN-1 rapidly and fully relaxed these preparations (data not shown). In intact rings, inhibition of NO synthesis by

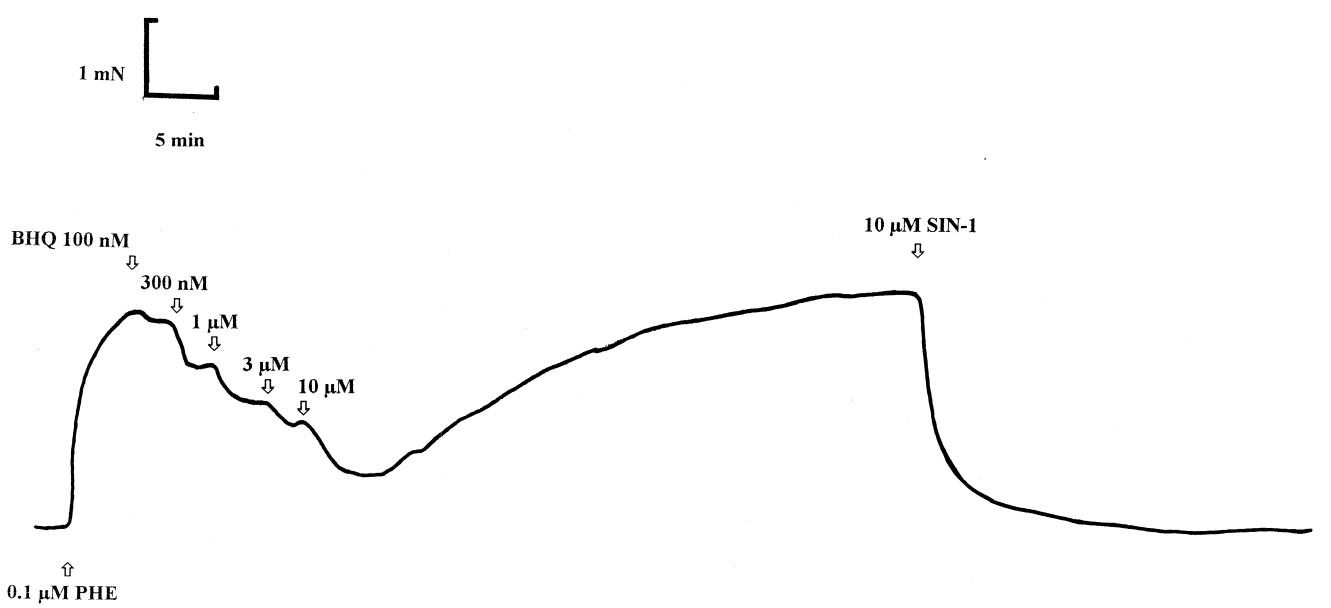

Fig. 1. Biphasic effects of BHQ on phenylephrine-contracted rat aorta rings. After an initial concentration-dependent relaxation, 10 μM BHQ promoted a slow return to the initial tension. When the tension reached the plateau value, 10 μM SIN-1 was added and full relaxation ensued. Trace is representative of recordings for at least five different animals.

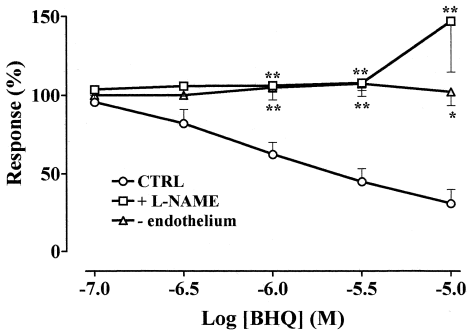

Fig. 2. Concentration–relaxation curves for BHQ, either in the presence (CTRL) or in the absence of functional endothelium, and with functional endothelium preincubated for 15 min with 100 μM L-NAME. Ordinate scale, relaxation is reported as a percentage of the initial tension induced by phenylephrine. Data points are mean values and vertical lines represent S.E.M. ($n = 4–5$). $*\ P < 0.05$, $**\ P < 0.01$, with respect to control, Dunnett's test.

100 μM L-NAME (Moncada et al., 1991) not only suppressed the BHQ-induced relaxation, but also revealed the synergistic effect of BHQ towards phenylephrine (Fusi et al., 1998a). Under these conditions, in fact, phenylephrine-induced tone was increased by about 50%. Once applied and then washed out, BHQ was no longer able to produce relaxation if reapplied. SIN-1 induced relaxation was not inhibited by L-NAME (data not shown).

To assess whether NO synthase and/or soluble guanylyl cyclase activation were involved in BHQ-induced relaxation, rings were incubated with 3 and 30 μM methylene blue and contracted with 10 and 35 nM phenylephrine, respectively. Methylene blue inhibited concentration dependently the BHQ-induced relaxation and the concentration–relaxation curves were shifted to the right (Fig. 3). Furthermore, BHQ was not able to produce relaxation

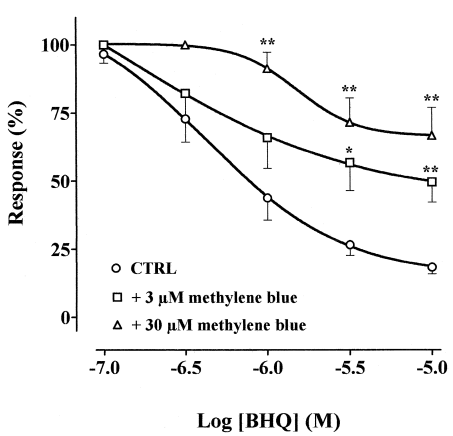

Fig. 3. Effect of methylene blue on the concentration–relaxation curves for BHQ. Responses to BHQ under control conditions (CTRL) and with 3 and 30 μM methylene blue of phenylephrine-contracted rings. Tissues were exposed for 15 min to methylene blue before the addition of phenylephrine. Ordinate scale, relaxation is reported as a percentage of the initial tension induced by phenylephrine. Data points are mean values and vertical lines represent S.E.M. ($n = 4–6$). $*\ P < 0.05$, $**\ P < 0.01$, with respect to control, Dunnett's test.

of the methylene blue-induced tone, but rather tended to increase it.

3.2. Effect of Ni^{2+} on BHQ-induced relaxation

Since Ca^{2+} is required for the activation of calmodulin-dependent NO synthase isozymes, in some experiments Ni^{2+} was added to the incubation solution in order to assess whether the endothelium-dependent BHQ-induced relaxation is mediated by Ca^{2+} influx from the extracellular space. Ni^{2+} (up to 0.6 mM) did not affect phenylephrine-induced contraction; however, when rings were preincubated with 1 mM Ni^{2+}, the phenylephrine concentration had to be increased up to 3 μM in order to obtain a tension comparable to that evoked by 0.1 μM, in the absence of Ni^{2+}. As shown in Fig. 4, 0.6 and 1 mM Ni^{2+} shifted to the right the concentration–relaxation curve for BHQ.

3.3. Effects of diethyldithiocarbamate and superoxide dismutase on BHQ-induced relaxation

To clarify the possible role of vascular smooth muscle Cu/Zn superoxide dismutase in BHQ-induced relaxation, in some experiments the superoxide dismutase inhibitor diethyldithiocarbamate was used. Incubation of the rings with 3 mM diethyldithiocarbamate for 15, 30 or 45 min, followed by a 10-min washout, did not affect significantly the response to phenylephrine (data not shown). After diethyldithiocarbamate treatment, however, the relaxant effect of BHQ was significantly inhibited in a time-dependent fashion and the relaxation curve was shifted to the right (Fig. 5a). Furthermore, in the presence of 250 U/ml superoxide dismutase, the BHQ-induced relaxation was strengthened and the relaxation curve was shifted to the left, leaving the maximal effect unchanged. Exogenous

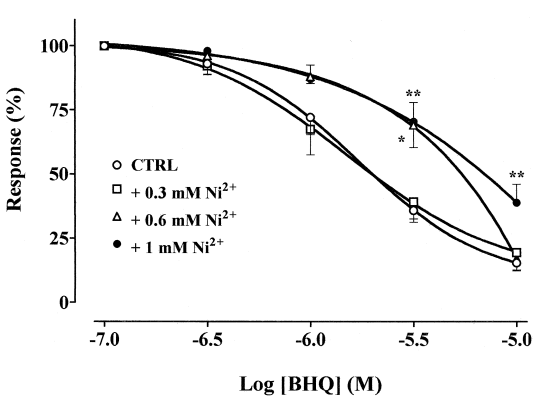

Fig. 4. Inhibitory effect of Ni^{2+} on BHQ-induced relaxation. Responses to BHQ under control conditions (CTRL) and after pretreatment with 0.3, 0.6 and 1 mM Ni^{2+} of phenylephrine-contracted rings. Tissues were exposed for 15 min to Ni^{2+} before the addition of phenylephrine. Ordinate scale, relaxation is reported as a percentage of the initial tension induced by phenylephrine. Data points are mean values and vertical lines represent S.E.M. ($n = 4–11$). $*\ P < 0.05$, $**\ P < 0.01$, with respect to control, Dunnett's test.

superoxide dismutase (250 U/ml) produced a complete reversal of the diethyldithiocarbamate-induced inhibition (Fig. 5b). This reversal was not produced by superoxide dismutase inactivated at 100°C.

3.4. Superoxide anion formation from BHQ

BHQ, either in the absence (data not shown) or in the presence of aorta rings, gave rise to superoxide anion formation, as demonstrated by the reduction of nitroblue tetrazolium (Fig. 6). BHQ-induced superoxide anion formation, at the maximum concentration tested (10 μM), amounted to about one tenth of that formed when 4.5 mM dihydroxyfumaric acid was used. In the presence of 500 U/ml superoxide dismutase, the amounts of both BHQ- or dihydroxyfumaric acid-generated superoxide anion were almost zero. Preincubation of the rings for 30 min with 3 mM diethyldithiocarbamate did not affect the BHQ-induced formation of superoxide anion (data not shown).

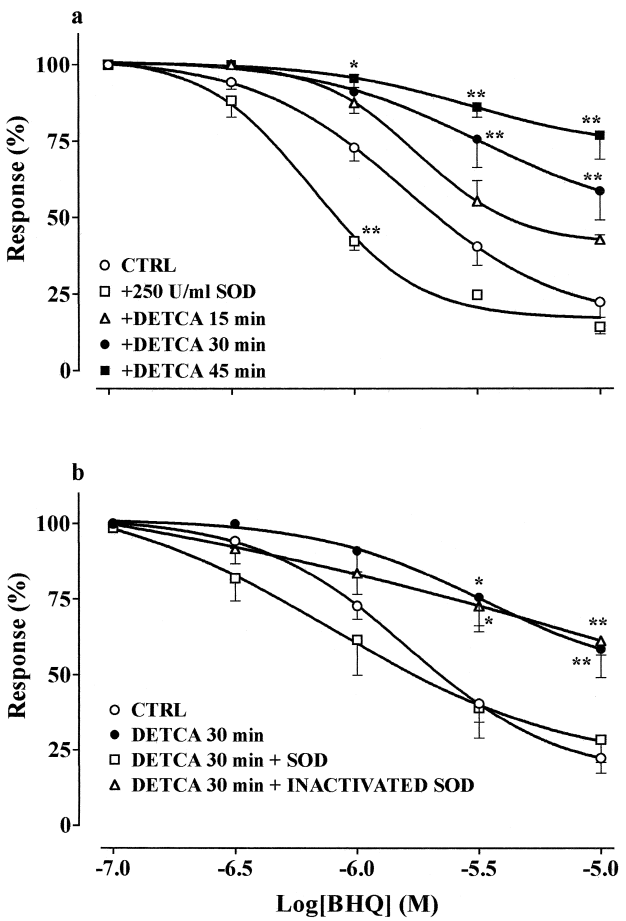

Fig. 5. Concentration–relaxation curves for BHQ: effects of superoxide dismutase (SOD) and diethyldithiocarbamate (DETCA). (a) Time-dependent inhibition by incubation with 3 mM DETCA for 15, 30 and 45 min and SOD (250 U/ml)-induced stimulation as compared to control conditions (CTRL). (b) Inhibition by incubation with 3 mM DETCA for 30 min in the presence of inactivated SOD and its reversal by 250 U/ml SOD as compared to CTRL. Tissues were exposed for 5 min to SOD and for 15–45 min to DETCA before the addition of phenylephrine. Ordinate scale, relaxation is reported as a percentage of the initial tension induced by phenylephrine. Data points are mean values and vertical lines represent S.E.M. ($n = 4$–9). $*\ P < 0.05$, $**\ P < 0.01$, with respect to control, Dunnett's test.

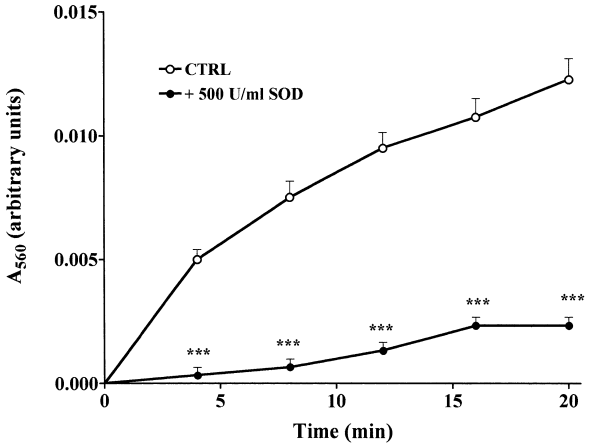

Fig. 6. Superoxide anion formation promoted by BHQ auto-oxidation. The reduction of nitroblue tetrazolium was recorded in the absence (CTRL) and in the presence of superoxide dismutase (SOD) (500 U/ml). Data points are mean values and vertical lines represent S.E.M. ($n = 4$). $***\ P < 0.001$, with respect to BHQ alone, Tukey's test.

3.5. Effect of BHQ on the relaxation promoted by SIN-1

To test whether BHQ enhances NO biological activity, concentration–relaxation curves for SIN-1 were made with endothelium-denuded rings precontracted with phenylephrine and preincubated with BHQ (1 μM) in the presence of superoxide dismutase (250 U/ml). In rings preincubated with 1 μM BHQ, the phenylephrine concentration had to be raised up to 0.5 μM in order to obtain a tension similar to that evoked by 0.1 μM BHQ. When SIN-1 was added cumulatively, the concentration–relaxation curve was not significantly modified by BHQ (SIN-1 pEC_{50} 7.14 ± 0.05 and 7.20 ± 0.27 M, $n = 5$, respectively).

4. Discussion

The present results show that BHQ induces relaxation via an endothelium-dependent mechanism in rat aorta contracted with phenylephrine. BHQ is a selective endoplasmic reticulum Ca^{2+}-ATPase inhibitor (Nakamura et al., 1992) and its relaxing effect appears to be mediated by NO, as already observed for cyclopiazonic acid and thapsigargin (Mikkelsen et al., 1988; Moritoki et al., 1994; Zheng et al., 1994), two structurally unrelated inhibitors of the endoplasmic reticulum Ca^{2+}-ATPase (Seidler et al., 1989; Thastrup et al., 1990). Considerable experimental evidence supports this concept. BHQ-induced relaxation, in fact, disappears when the endothelium is scraped off or is functionally impaired by L-NAME, an inhibitor of NO synthase (Moncada et al., 1991). The production of an endothelium-dependent hyperpolarizing factor can be ex-

cluded, because its relaxing effect is not modified by NO synthase inhibitors (Fukao et al., 1995). The BHQ-induced relaxation does not seem to depend on the production of prostanoids, which indeed is depressed by BHQ through inhibition of cyclo-oxygenase activity (Leis et al., 1996). Furthermore, it can be excluded that an enhanced sensitivity of vascular smooth muscles to NO may be involved, as BHQ did not modify the pEC_{50} of SIN-1. Finally, methylene blue, an inhibitor of both NO synthase (Mayer et al., 1993) and soluble guanylyl cyclase (Moncada et al., 1991), suppressed the relaxing effect of BHQ.

There seems to be some variability in the relaxant potency of BHQ under control conditions. This can be ascribed to several factors: (i) BHQ-induced generation of superoxide anion (see below); (ii) minor differences in the integrity of the endothelium; (iii) access of BHQ into endothelial cells; and (iv) partitioning of BHQ between muscle and endothelial cells.

In a previous study, the contracting effects of BHQ on endothelium-denuded rat aorta rings were described (Fusi et al., 1998a). It is conceivable that the final effect observed on intact aorta rings results from two opposite responses, i.e., a contracting effect and a relaxing effect. Thus, the final pharmacological action of BHQ on rat thoracic aorta results hierarchically from its effect on the endothelium. At low concentrations (< 10 μM) the relaxing effect predominates, while at higher concentrations (≥ 10 μM) its agonistic effect on smooth muscle prevails and a transient relaxation is followed by a slow return to the tension elicited by phenylephrine. The recovery of contractile force over time appeared to depend on the influx of Ca^{2+} through Ni^{2+}-sensitive Ca^{2+} channels, as already observed in this laboratory (Fusi et al., 1998a). Such a dual effect is also typical of thapsigargin (Mikkelsen et al., 1988) and cyclopiazonic acid (Zheng et al., 1994).

The endothelial intracellular Ca^{2+} concentration is a crucial determinant of NO synthesis, as the constitutive NO synthase is Ca^{2+}/calmodulin-dependent (Moncada et al., 1991). Since Ni^{2+} antagonised, in the present study, the BHQ-induced relaxation, it is hypothesised that the latter effect is dependent on the influx of extracellular Ca^{2+} through Ni^{2+}-sensitive channels, which activates NO synthase (Nilius et al., 1997). BHQ, cyclopiazonic acid and thapsigargin have been shown, in vascular endothelial cells, to activate an influx of Ca^{2+} which is sensitive to Ni^{2+}, but not to L-type Ca^{2+} channel blockers (Moritoki et al., 1996; Schilling et al., 1992; Dolor et al., 1992). Since BHQ, thapsigargin and cyclopiazonic acid are structurally unrelated compounds, it might be worth investigating from the conformational point of view which common features underlie the biochemical and pharmacological properties shared by these three drugs. Hoth and Penner (1992) demonstrated that depletion of intracellular stores is the signal which leads to activation of the Ni^{2+}-sensitive Ca^{2+} current, named I_{CRAC} (Ca^{2+}-release-activated Ca^{2+} current) (Berridge, 1995; Karaki et al., 1997). Furthermore, it is conceivable that BHQ-induced Ca^{2+} release from the endoplasmic reticulum (Schilling et al., 1992) might be in part responsible for the Ni^{2+}-resistant portion of the relaxation. Alternatively, transient elevation of cytosolic Ca^{2+} near the cell membrane, subsequent to the BHQ-induced inhibition of the endoplasmic reticulum Ca^{2+} pump, might modulate plasma membrane Ca^{2+} channels and activate the influx of external Ca^{2+} (Pasyk et al., 1995).

NO can react with methyl- or *t*-butyl-substituted phenols to give a phenoxyl radical which subsequently couples reversibly to excess NO. The latter radical may thus function as a NO carrier in biological systems (Janzen et al., 1993). This was the rationale for testing whether BHQ could enhance the biological activity of NO, by using SIN-1, the active metabolite of molsidomine (Reden, 1990), which releases NO and activates soluble guanylyl cyclase in smooth muscle cells. BHQ did not modify the SIN-1-induced relaxation in endothelium-deprived rings that had been preincubated with superoxide dismutase to avoid any interference from the superoxide anion formed from SIN-1 (Reden, 1990) or BHQ (see below). This suggests that BHQ does not act as a NO carrier/amplifier.

Hydroquinones undergo auto-oxidation (Marklund and Marklund, 1974) to form semi-quinone radicals which can donate an electron to molecular oxygen, thereby giving rise to the superoxide anion. The latter free radical can react with NO to form peroxynitrite (Pryor and Squadrito, 1995), thus extinguishing the effects of NO (Gryglewski et al., 1986). It has been suggested (Moncada et al., 1986) that hydroquinones, besides their capacity to form superoxide anion, antagonise NO-mediated relaxation of vascular smooth muscles by acting directly as NO scavengers (Hobbs et al., 1991). From the present findings it is conceivable that BHQ either stimulates endothelial NO synthesis/release or forms the NO inactivator superoxide anion. It was thought that superoxide dismutase, by inactivating BHQ-formed superoxide anion, would block its NO-scavenging effect. In fact, it significantly increased BHQ myorelaxant activity. Inhibition of endogenous Cu/Zn superoxide dismutase activity with diethyldithiocarbamate was expected to enhance the superoxide anion-dependent ability of BHQ to inactivate NO. In fact, a powerful and time-dependent inhibition of the BHQ-induced relaxation was observed. Finally, the diethyldithiocarbamate blockade of the BHQ-induced relaxation was totally reversed by exogenous application of superoxide dismutase, which can metabolise superoxide anion only extracellularly. Thus, superoxide anion formed extracellularly from BHQ appears to have a major role in scavenging NO, as compared to that produced intracellularly. It is important to notice that, once applied and then washed out, BHQ was no longer able to produce relaxation when reapplied. Presumably, the reaction between superoxide anion and NO, produced by BHQ autoxidation and released from the endothelium by BHQ, respectively, gives

rise to peroxynitrite. The latter is a powerful oxidant and is recognized as a potentially important modulator of vascular reactivity (Katusic, 1996).

In conclusion, BHQ induces endothelium-dependent relaxation in spite of its capacity to form, by auto-oxidation, the NO inactivator superoxide anion. This effect results from the enhanced synthesis of NO, via NO synthase stimulation by activation of Ca^{2+} influx through Ni^{2+}-sensitive Ca^{2+} channels, rather than from its enhanced biological activity.

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