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European Journal of Pharmacology 436 (2002) 159–163

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Quinacrine increases endothelial nitric oxide release: role of superoxide anion

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Received 5 November 2001; received in revised form 14 December 2001; accepted 21 December 2001

Abstract

The effect of acute quinacrine treatment on agonist-induced nitric oxide (NO) release was investigated in cultured human endothelial cells using electrochemical monitoring of the in situ NO concentration. Quinacrine dose-dependently increased NO release with an apparent EC_{50} of 0.2 μM and a maximal effect at 1 μM. Quinacrine did not modify the dependence of NO release on extracellular L-arginine. Acceleration or deceleration of O_2^- dismutation, which altered NO release in control cells, did not modify it in quinacrine-treated cells. Quinacrine did not modify NO amperometric signal or reaction with O_2^- produced by xanthine oxidation. In the presence of quinacrine, agonist-induced NO release became Mg^{2+}-independent and could not be attributed to an inhibition of phospholipase A_2 activity. Quinacrine made NO release insensitive to Cu^{2+} chelation. The present study demonstrates that acute treatment by low quinacrine concentrations increases endothelial NO release, possibly through an inhibition of O_2^- production.

Keywords: Electrode; Endothelial cell; Nitric oxide (NO); Superoxide anion; Quinacrine; Superoxide dismutase

1. Introduction

Quinacrine is an acridine derivative that has been used for its immunological and anti-inflammatory properties as an anti-malarial agent. It is also used as an alternative regimen of birth control in countries without access to more expensive approaches, and in dermatology, especially in treatment of collagen vascular and photosensitivity diseases. A renewed interest came from the very recent observation of its capacity to inhibit prion formation, suggesting that it may constitute an effective treatment for Creutzfel–Jakob disease and other prion diseases (Korth et al., 2001; Doh-Ura et al., 2000). Various mechanisms of action have been reported. They include inhibition of phospholipase A_2, non-competitive inhibition of the nicotinic acetylcholine receptor, lysosomal effects, membrane stabilisation, antioxidant activity and inhibition of superoxide production in neutrophils and macrophages (Struhar et al., 1992; Cutler, 1993; Fox, 1993; Spitzmaul et al., 2001). The mechanisms by which quinacrine inhibits scrapie-associated prion protein formation are unknown. An interesting feature of this inhibitory effect of quinacrine is its IC_{50} of 0.3–0.4 μM (Korth et al., 2001; Doh-Ura et al., 2000), which contrasts with the 0.01–1 mM range required for the other effects described above. We report here that low quinacrine concentrations increase nitric oxide (NO) release in agonist-stimulated cultured human endothelial cells. The influence of quinacrine on NO/O_2^- balance was investigated through the effects of inhibitors of their synthesis and modulators of O_2^- dismutation.

2. Materials and methods

2.1. Chemicals

Bovine copper–zinc superoxide dismutase (4600 units/mg), thrombin (1000 units/mg), diethyldithiocarbamate and *N*-monomethyl-L-arginine (L-NMMA) were obtained from Alexis. Arachidonyl trifluoromethyl ketone ($AACOCF_3$) was from Research Biochemicals International. All the other chemicals were from Sigma-Aldrich. Stock solutions of $AACOCF_3$ (100 mM) were prepared in ethanol.

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0014-2999/02/$ - see front matter
PII: S0014-2999(01)01618-1

2.2. Cell culture and treatments

Endothelial cells were isolated from human umbilical cord vein by collagenase digestion. They were cultured in medium 199 and RPMI 1640 supplemented with 20% foetal calf serum as previously described (Lantoine et al. 1998). Medium was renewed every other day until confluence. Cells were studied at the first passage only, when grown to subconfluence. Medium was renewed 24 h before experiments.

Just before NO measurements, cultured cells were washed and incubated in phosphate buffered saline medium containing 5 mM glucose, 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$. Effects of L-arginine, L-NMMA, Cu^{2+} and cuprizone were assessed after a 5-min pre-incubation at 37 °C. Diethyldithiocarbamate and $AACOCF_3$ were pre-incubated at 37 °C for 10 and 30 min, respectively, then washed. The reduction of the extracellular Ca^{2+} concentration to 3 μM was obtained by omission of Ca^{2+} addition (David-Dufilho et al., 2001).

2.3. Measurements of nitric oxide release

In situ NO concentration was measured by differential pulse amperometry with a three-electrode potentiostatic Biopulse system (Tacussel-Radiometer) and a porphyrinic NO-selective microsensor as previously described (Lantoine et al., 1998). The working electrode was positioned at the cell surface with a micromanipulator attached to the stage of an inverted microscope (Nikon/Narishige), both cells and tip of NO microsensor in the focal plane of microscope.

For each experiment, the electrochemical sensor was calibrated by addition of NO standard solutions as detailed previously (Lantoine et al., 1995). None of the compounds studied interfered with the amperometric signal of NO standard solutions. NO release was expressed as the maximum of the agonist-induced oxidation current.

2.4. Data analysis

Results are expressed as mean±S.E.M. Multiple comparisons and dose-dependent effects were examined by one-way analysis of variance (ANOVA) and Fisher test. Differences reaching a $P<0.05$ were considered as significant.

3. Results

3.1. Effect of quinacrine on thrombin-induced NO release

Acute treatment with quinacrine increased the thrombin-induced NO release (Fig. 1A), without altering its decline rate. This rise was dose-dependent [$F(5,23)=8.2$, $P<0.001$], with an apparent EC_{50} of 0.2 μM and a maximal stimulation reached at 1 μM (Fig. 1B).

To investigate the mechanism(s) by which quinacrine rose NO release, we first examined whether this drug modified its dependence on extracellular L-arginine. L-Arginine dose-dependently increased NO production in quinacrine-treated cells [$F(4,20)=3.8$, $P=0.018$], as it did in untreated cells [$F(5,65)=12.5$, $P<0.001$], with a constant increase in NO release, irrespective of the extracellular L-arginine concentration (Fig. 1C). The apparent EC_{50} were similar (7 and 5 μM), and maximal effects were reached at 100 μM in the presence as in the absence of quinacrine. This demonstrated that quinacrine increased NO release without modifying the dependence of the endothelial NO synthase on extracellular L-arginine. All subsequent experiments were performed in the presence of 100 μM, the optimal L-arginine concentration. As illustrated in Fig. 2A, the presence of quinacrine did not prevent the reduction of NO release due to the additional presence of 500 μM L-NMMA or to the reduction of the extracellular Ca^{2+} concentration to 3 μM.

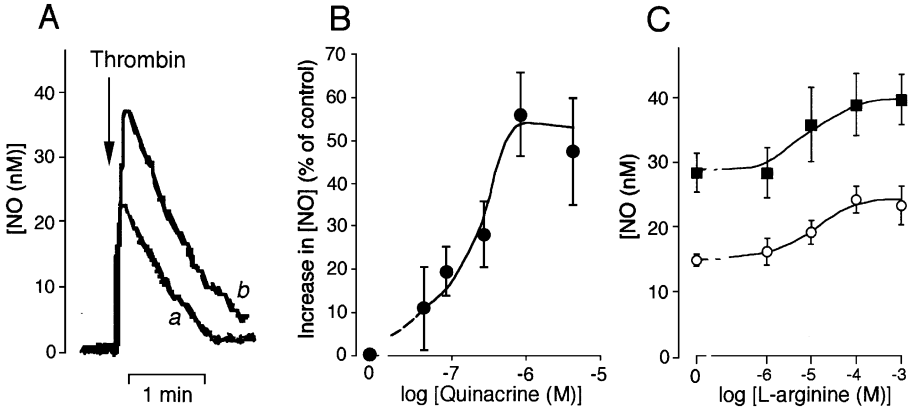

Fig. 1. Effect of quinacrine on NO release from thrombin-stimulated human endothelial cells: In panel A are given representative traces showing NO release from control (trace a) and quinacrine (1 μM) pre-treated cells (trace b). In panels B and C are illustrated the dose-dependent effects of quinacrine (B) and that of extracellular L-arginine in the presence (■) and absence (○) of 1 μM quinacrine. Cells were pre-incubated with quinacrine and/or L-arginine for 5 min at 37 °C and then stimulated by 2 U/ml thrombin ($n=6-21$).

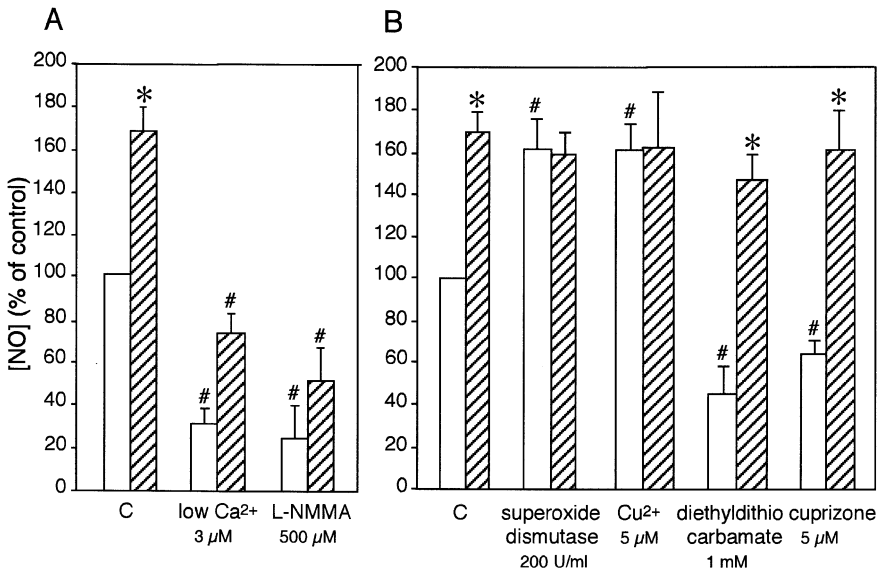

Fig. 2. Effects of quinacrine on the thrombin-activated NO release in HUVECs. Cells were incubated under conditions known to modify NO synthesis (A) or degradation (B), in the presence (hatched bars) or absence (open bars) of 1 μM quinacrine. Cells were pre-incubated as described in methods and then stimulated by 2 U/ml thrombin. Values are expressed as percent of the control value determined in untreated cells. Data are means±S.E.M. from 4 to 25 independent measurements. * indicates a significant effect of quinacrine; # indicates a significant difference from the respective control values.

3.2. Influence of O_2^- dismutation on the stimulation of NO release by quinacrine

As endogenous O_2^- production is the major modulator of local NO concentration, we examined the influence of accelerated or reduced O_2^- dismutation on the quinacrine effects on NO release. The addition of superoxide dismutase (200 U/ml, the maximally effective concentration) or of Cu^{2+}, a superoxide dismutase mimetic (Riley et al., 1991), increased NO release in control cells, bringing it to the level obtained in quinacrine treated cells. Neither superoxide dismutase nor Cu^{2+} addition increased it further in quinacrine-treated cells (Fig. 2B), indicating that the effects of superoxide dismutase or Cu^{2+} and those of quinacrine were not additive. Pretreatment of cells with diethyldithiocarbamate, a superoxide dismutase inhibitor (Cocco et al., 1991), or with cuprizone, a Cu^{2+} chelator, reduced NO release from control cells (Fig. 2B), in agreement with a reduced O_2^- dismutation. The diethyldithiocarbamate and cuprizone effects were lost in quinacrine-treated cells (Fig. 2B).

3.3. Effect of quinacrine on the oxidation signal of authentic NO in the presence of O_2^- produced by xanthine oxidation

To control for the possibility that the quinacrine-induced rise in NO release was a result of artifactual interferences with NO amperometric detection or with the NO/O_2^- reaction, we looked for the effect of quinacrine on the oxidation signal and fate of authentic NO in the presence of O_2^- produced by xanthine oxidation. As illustrated in Fig. 3A, calibration curves of the electrode response were identical in the presence and absence of quinacrine. When xanthine oxidase was added into xanthine and NO containing solution, the NO signal rapidly disappeared. The addition of superoxide dismutase allows the signal of a subsequent NO addition to recover and slowed down its decline (Fig. 3B). The presence of quinacrine (up to 10 μM, the highest concentration tested) did not modify this pattern (Fig. 3C). This indicated that quinacrine interfered neither with NO amperometric signal nor with the NO/O_2^- reaction, and did not exhibit superoxide dismutase-like properties.

3.4. Quinacrine and cellular O_2^- production

We then search for a possible inhibitory effect of quinacrine on cellular O_2^- production. Arachidonate-dependent O_2^- production did not significantly contribute to NO breakdown, since inhibition of the cytosolic phospholipase A_2 by 40 μM $AACOCF_3$, which inhibited arachidonic acid release by more than 70% (Millanvoye Van Brussel et al., 1999), did not modify NO release (25±2 nM in treated cells compared to 22±2 nM in control cells, $n=6$). To determine whether quinacrine altered the activity of membrane NADH/NADPH oxidases that require Mg^{2+} (Cross et al., 1999), the amplitude of quinacrine effects in 0.5 mM Mg^{2+} and Mg^{2+}-free media were compared. In agreement with a reduced O_2^- synthesis, the acute suppression of external Mg^{2+} increased NO release by 66±12% in control cells, reaching values similar to those observed in quinacrine-treated cells. In contrast, the augmentation of NO release

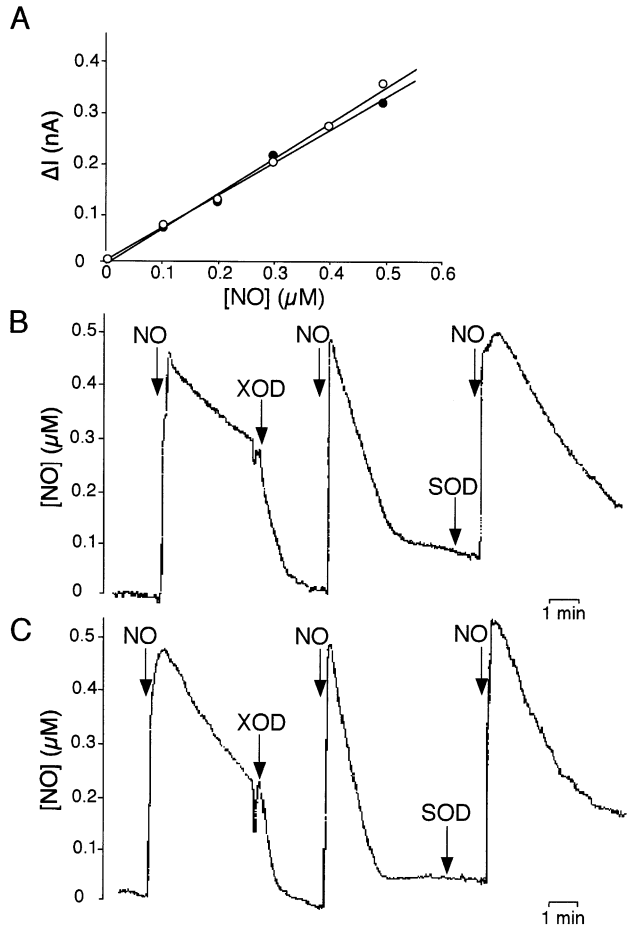

Fig. 3. Effect of quinacrine on the oxidation signal and fate of authentic NO in the presence of O_2^- produced by xanthine oxidation. Calibration curves were performed in the presence (○) and absence (●) of 1 μM quinacrine (A). The amperometric signal of 0.5 μM authentic NO in PBS containing 200 μM xanthine was measured in the absence (B) and presence (C) of 1 μM quinacrine, before and after successive additions of 1 mU/ml xanthine oxidase and 200 U/ml superoxide dismutase. Traces are representative of three independent experiments.

by quinacrine was insensitive to removal of extracellular Mg^{2+} (64±19% in the absence of external Mg^{2+} compared to 66±11% in its presence, $n=7$ for each).

4. Discussion

The NO/O_2^- balance participates in the control of major cell functions including reactivity, proliferation and apoptosis. Inappropriate production of these radicals and their metabolites led to the development of various pathologies. We report here that quinacrine, at sub-micromolar concentrations, increased NO release from human endothelial cells through the reduction of endogenous O_2^-. Chloroquine, a compound structurally related to quinacrine, has been reported to stimulate NO synthesis in endothelial cell lysates (Ghigo et al., 1998). This stimulation required a de novo protein synthesis and was maximal after 24 h with a 20 μM chloroquine concentration. This differs from the acute quinacrine-dependent rise in NO local concentration, reflecting an increased NO/O_2^- balance, observed here in agonist-stimulated intact cells. At the low quinacrine concentration we used, the integrity of the endothelial cell monolayer was preserved (Stuhlmeier, 2000). The quinacrine-dependent NO rise was independent of L-arginine availability. It was similar to that obtained in the presence of optimal exogenous superoxide dismutase or Cu^{2+}, and was insensitive to diethyldithiocarbama and cuprizone. This could have suggested that quinacrine itself exerted a superoxide dismutase-like activity, but this proposal was ruled out by the observation that this compound, even at a 10-μM concentration, did not prolong the half-life of authentic NO in the presence of O_2^- in a cell-free system.

An alternative explanation could be that quinacrine inhibited O_2^- production. In endothelial cells, O_2^- is mainly synthesized by NADH/NADPH oxidases (Mohazzab-H et al., 1994; Meyer et al., 1999). The stimulation of these enzymes by arachidonic acid suggested that the quinacrine stimulatory effect on NO release could reflect phospholipase A_2 inhibition. However, we observed that inhibition of cytosolic phospholipase A_2 by $AACOCF_3$ did not significantly affect NO release, which implies a minor role for arachidonic acid under our experimental conditions. Griendling et al. (1994) have reported an inhibition of the NADH/NADPH-dependent O_2^- synthesis by high quinacrine concentrations in rat vascular smooth muscle cells. Although the maximally effective quinacrine concentration was 6000 times lower in the present study, our observations agree with this proposal that can explain why the presence of quinacrine abolished the modulation of NO release by activators or inhibitors of O_2^- dismutation. This proposal is also supported by the loss of sensitivity to Mg^{2+} removal in quinacrine-treated cells. In agreement with the Mg^{2+} requirement of the NADH/NADPH oxidase complexes for O_2^- synthesis (Cross et al., 1999), NO release from control cells was enhanced in Mg^{2+}-free medium, contrarily to that from quinacrine-treated cells which was not increased further. The present study also demonstrates that the quinacrine effect was copper-independent, as it was unchanged in the presence of cuprizone.

The O_2^- synthesis in sub-confluent endothelial cells is low and difficult to measure (Barbacanne et al., 2000). Our attempts to monitor short term responses to agonist through ferricytochrome c reduction or in situ electrochemical measurement were unsuccessful, although these approaches allowed O_2^- determination in interleukin-1 stimulated endothelial cells (Privat et al., 1999). This probably reflects the predominance of NO release under these conditions, which makes it a better indicator of the NO/O_2^- balance.

Whether the quinacrine effects reported here participate in its beneficial effect as an anti-prion treatment, cannot be established from the present study. It is however interesting to note the similitude of the quinacrine IC_{50} for the rise in

agonist-induced NO release from endothelial cells (0.2 μM), with that for the inhibition of the abnormal prion protein isoform formation (0.3–0.4 μM) (Korth et al., 2001; Doh-Ura et al., 2000). Another interesting feature is that some other inhibitors of the abnormal prion protein formation, such as porphyrins (Caughey et al., 1998) or lovastatin (Taraboulos et al., 1995), also modify the NO/O_2^- balance: for example, the meso-tetrasubstituted porphyrin T(N-Me-4-Py)P-Fe^{3+} accelerates O_2^- dismutation (Riley, 1999), and lovastatin increases basal NO and decreases superoxide (Thakur et al., 2001). Furthermore, normal prion protein has been reported to exhibit a Cu^{2+}-dependent superoxide dismutase-like activity (Brown et al. 1999), while neuronal cells infected by the abnormal prion isoform presented a reduced neuronal NO synthase activity (Ovadia et al., 1996) and an enhanced sensitivity to oxidative stress (Milhavet et al., 2000). It may thus be proposed that quinacrine beneficial effects include a partial correction of an impaired NO/O_2 balance, attenuating its possible consequences on cell functions and on the post-translational cleavage of the prion protein (McMahon et al., 2001).

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