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Divergent effects of vitamin C on relaxations of rabbit aortic rings to acetylcholine and NO-donors

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1 Vitamin C may influence NO-dependent relaxation independently of effects on oxidant stress.

2 We investigated effects of vitamin C (0.1–10 mmol l^{-1}) on relaxation of pre-constricted rabbit aortic rings to acetylcholine (ACh), authentic NO and the NO-donors glyceryl trinitrate (GTN), nitroprusside (NP) and *S*-nitroso-*N*-acetyl-penicillamine (SNAP). DETCA (2–6 mmol l^{-1}), a cell permeable inhibitor of endogenous Cu-Zn superoxide dismutase (SOD) was used to increase intracellular superoxide anion (O_2^-).

3 Vitamin C reduced the response to ACh (71 ± 7% inhibition of maximum relaxation at 10 mmol l^{-1}) and inhibited relaxation to authentic NO. Vitamin C inhibited relaxation to GTN but potentiated relaxations to NP and SNAP, causing a parallel shift to a lower concentration range of the log dose-response curve by approximately one log unit at the highest dose.

4 Vitamin C increased the concentration of NO in bath solution (plus EDTA, 1.0 mmol l^{-1}) following the addition of SNAP from 53 ± 14 to 771 ± 101 nmol l^{-1} over the range 0.1–3.0 mmol l^{-1}.

5 DETCA inhibited relaxation to ACh (71 ± 9% inhibition of maximum relaxation). This inhibition was abolished by a cell permeable SOD mimetic, but not by vitamin C. DETCA inhibited relaxation to SNAP but not that to NP nor to GTN.

6 Vitamin C inhibits endothelium-dependent relaxations of rabbit aortic rings to ACh and authentic NO and does not reverse impaired relaxation resulting from increased intracellular oxidant stress. Vitamin C potentiates relaxation to the NO-donors NP and SNAP by a mechanism that could involve release of NO from nitrosothiols.

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Abbreviations: ACh, acetylcholine; cyclic GMP, guanosine 3′5′ cyclic monophosphate; DETCA, diethyldithiocarbamate; EC_{50} effective concentration giving 50% maximal relaxation; EDNO, endothelium-derived nitric oxide; EDTA, ethylenediaminetetraacetic acid; Emax, maximal relaxation; GTN, glyceryl trinitrate; MnTMPyP, manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin; NE, norepinephrine; NO, nitric oxide; NP, nitroprusside; O_2^-, superoxide anion; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PE, phenylephrine; RSNO, nitrosothiol; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; SOD, superoxide dismutase

Introduction

Acute administration of vitamin C into the brachial artery producing local plasma concentrations in the order of 1–10 mmol l^{-1} improves endothelium-dependent vasodilation in the forearm vasculature of smokers (Heitzer *et al*., 1996), subjects with hypertension (Taddei *et al*., 1998), hypercholesterolaemia (Ting *et al*., 1996a), type 2 diabetes (Ting *et al*., 1996b) and heart failure (Hornig *et al*., 1998). Acute oral administration of vitamin C in a dose estimated to produce ascorbate concentrations of around 100 μmol l^{-1} also improves endothelium-dependent flow mediated dilation in patients with established coronary artery disease (Levine *et al*., 1996) and in experimentally induced hyperhomocysteinaemia (Chambers *et al*., 1999). Short term treatment with oral vitamin C may lower blood pressure in hypertensive subjects (Duffy *et al*., 1999). Inactivation of endothelium-derived nitric oxide (EDNO) by reactive oxygen species such as superoxide anion (O_2^-) may contribute to endothelial dysfunction in the above conditions (Cai & Harrison, 2000) and increase blood pressure through inactivating NO. The source of the O_2^- is assumed to be mainly intracellular since Cu-Zn superoxide dismutase, which does not penetrate cell membranes, does not reverse endothelial dysfunction in conditions where vitamin C is effective (Garcia *et al*., 1995a,b). Beneficial effects of vitamin C have been attributed to intracellular scavenging of O_2^-. However, the rate constant for the reaction of NO with O_2^- greatly exceeds that for the reaction of ascorbate with O_2^- making this mechanism unlikely except possibly at ascorbate concentrations achieved during intra-arterial delivery of vitamin C (Jackson *et al*., 1998). Another possible mechanism through which vitamin C might potentiate NO-dependent relaxation is by increasing the release of NO from nitrosothiols (RSNO). This may occur *via* direct nucleophilic attack by ascorbate on the nitroso group of RSNO (Holmes & Williams, 1998) or through the reduction of transition metal

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ions, particularly copper, by vitamin C increasing the catalysis by these ions of the RSNO to NO reaction (Singh *et al*., 1996; Holmes & Williams, 1998). To investigate these possibilities we examined effects of vitamin C on endothelium-dependent relaxations to acetylcholine (ACh), relaxations to authentic NO and to three chemically distinct classes of NO-donors thought to act through nitrosothiols as an intermediate (Feelisch, 1998). We did this under control conditions and under conditions of increased oxidant stress using diethyldithiocarbamate (DETCA), a cell permeable inhibitor of endogenous Cu-Zn superoxide dismutase (SOD) to increase intracellular O_2^-(Heikkila *et al*., 1976). We obtained the surprising result that, in this preparation, vitamin C inhibits relaxation to ACh and to authentic NO but potentiates some NO-donors. Measurement of NO in solution following the addition of SNAP suggested that vitamin C enhances release of NO from nitrosothiols.

Methods

Preparation of aortic rings

New Zealand white male rabbits (2–2.5 kg) were killed by an injection of sodium pentobarbitone (200 mg animal^{-1}) *via* the lateral ear vein and exsanguinated. Descending thoracic aortas were removed and trimmed of adhering connective tissue and fat. Transverse 2 mm wide rings were cut and mounted in 3 ml organ baths containing Krebs' solution. Organ bath solutions were maintained at 37°C and continuously oxygenated with 95% O_2–5% CO_2. Tissues were placed under 2 g resting tension for 60 min and re-tensioned to 2 g for a further 30 min. Isometric measurements were recorded *via* force transducers (Grass FT03, West Warwick, NY, U.S.A.). Tissues were contracted with increasing doses of norepinephrine (NE, 10^{-8}–10^{-6} mol l^{-1}) or phenylephrine (PE, 10^{-8}–10^{-6} mol l^{-1}) to determine a concentration which gave 80% maximum contraction. Relaxation dose-response curves to cumulative doses of ACh (10^{-8}–10^{-5} mol l^{-1}) were obtained to establish the integrity of the endothelium. Relaxation to ACh, NO and NO-donors in the absence and presence of vitamin C and/or other drugs was then determined according to the protocols described below. Control responses to acetylcholine were determined between applications of vitamin C and other drugs to ensure consistent responses throughout the time-course of an experiment. In all cases there was no significant change in relaxation to ACh between control curves obtained at the beginning of an experiment, and recovery curves obtained following drug administration.

Preparation of authentic (aqueous) nitric oxide

Authentic nitric oxide (NO) solutions (2 mmol l^{-1} stock) were prepared in air-tight bags by dissolving NO gas in deoxygenated distilled water containing ion exchange resin to bind nitrate and nitrite. Serial dilutions were prepared in sealed vacutainer tubes. Each tube (kept on ice) was filled with 9 ml of distilled water and bubbled vigorously with nitrogen *via* long catheter needles. After at least 40 min the needles were carefully removed with the gas still on to prevent air leaking into the tubes. Then using a 1 ml syringe flushed with nitrogen gas a 1 : 10 serial dilution of the 2 mmol l^{-1} stock was performed. Addition of aqueous NO at the required concentrations to the organ bath led to rapid and transient relaxations of the aortic rings.

Effects of vitamin C on relaxation to ACh, authentic NO and NO-donors

Control responses to ACh, NP (10^{-8}–10^{-4} mol l^{-1}) and authentic NO (10^{-9}–10^{-4} mol l^{-1}) were obtained in the absence of vitamin C. Rings were then washed out and incubated (15 min) with vitamin C (0.1, 1.0, 3.0 or 10.0 mmol l^{-1} or vehicle control) and contraction and relaxation to ACh, NP or NO repeated. The effect of vitamin C on relaxations to SNAP (10^{-8}–10^{-5} mol l^{-1}) and GTN (10^{-8}–10^{-5} mol l^{-1}) was examined using the same protocol.

Nitric oxide release from SNAP: NO detection with an electrochemical sensor

NO release from SNAP (250 μmol l^{-1}) was measured using the ISO-NO Mark II meter (World Precision Instruments, Stevenage, Herts, U.K.) using an ISO-NOP (2.0 mm) combination electrode. This electrode is shielded with a selective gas-permeable membrane separating the internal electrode from the sample medium enabling the amperometric measurement of NO in solution (Mayer *et al*., 1995). Oxidation of NO at the working electrode results in an electrical current output proportional to the concentration of NO in the sample. Calibration of the probe was achieved by the generation of known concentrations of NO by successive addition of KNO_2 solution (100 μmol l^{-1}) to 50 ml KI/H_2SO_4 solution (0.1 mol l^{-1}). This results in rapid NO production in accordance with the following reaction:

$$2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + 2K_2SO_4$$

Calibration was repeated both at the beginning and end of each experiment and cross checked using solutions of authentic NO described above. Concentration of NO in bath solution 100 s after the addition of SNAP (250 μmol l^{-1}), when this was at an approximate plateau, was recorded and this was repeated in the presence of EDTA (1 mmol l^{-1}) and in the presence of EDTA plus vitamin C (0.1–3.0 mmol l^{-1}). Addition of EDTA or vitamin C had no effect on basal concentrations of NO.

Effects of $CuSO_4$ and neocuproine on relaxation to NP

Relaxation responses to NP were examined before and after/during incubation with $CuSO_4$ (50–200 μmol l^{-1}) for 15 min. In further experiments effects of neocuproine (60 μmol l^{-1}; 15 min), a specific Cu^+ chelator (Al Sa'doni *et al*., 1997), on relaxation to NP were examined. The effect of co-incubation of neocuproine (60 μmol l^{-1}) and vitamin C (3 mmol l^{-1}) each for 15 min on relaxation to NP was also examined.

Effects of ODQ on ACh and NO donors

The effect of a selective inhibitor of soluble guanylyl cyclase 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) on relaxation to ACh, NP, GTN and SNAP was measured, in

order to investigate the mechanism of the divergent effects of vitamin C on these agonists. Responses were examined before and after/during incubation with ODQ (10 μmol l^{-1}, 15 min). 8-bromo-cyclic GMP, a cyclic GMP analogue, was used as a positive control.

Effects of DETCA on ACh

Pilot studies were performed to investigate effects of DETCA on relaxations to ACh. Rings were pre-treated with DETCA for 15 min followed by a 15 min washout period. The inhibitory effect of DETCA varied in rings obtained from different animals and in subsequent experiments the dose of DETCA was adjusted over the range 2–6 mmol l^{-1} to obtain at least 50% inhibition (estimated by visual inspection of the relaxation curve) of the response to ACh. Effects of manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP, a cell permeable SOD-mimetic, 100 μmol l^{-1}) and vitamin C (3 mmol l^{-1}) on inhibition by DETCA of relaxations to ACh were examined. Relaxation to ACh of pre-contracted rings was determined after incubation with DETCA (15 min, plus 15 min washout) in the presence and absence of MnTMPyP or vitamin C (added at the start of the 15 min washout period following incubation with DETCA).

Effects of DETCA on NO-donors

Pre-contracted rings were treated with increasing concentrations of an NO-donor: NP (10^{-7}–10^{-4} mol l^{-1}), GTN (10^{-8}–10^{-5} mol l^{-1}) and SNAP (10^{-8}–10^{-5} mol l^{-1}), each agonist being studied in a separate experiment. Rings were then treated as above with DETCA followed by 15 min recovery, re-constricted by contractile agonists as above, and addition of NP, GTN or SNAP repeated. After washout and a further 15 min recovery rings were re-constricted and ACh added as above to confirm that inhibitory effects of DETCA on endothelium-dependent relaxation were still evident. In some experiments relaxation to NP of pre-contracted rings was determined following incubation with DETCA as above in the presence of vitamin C (3 mmol l^{-1}, added at the start of the 15 min washout period of DETCA).

Drugs

NE, PE, ACh, SNAP, ODQ, neocuproine and 8-bromo-cyclic GMP were obtained from Sigma Chemical Co. (Poole, Dorset, U.K). $CuSO_4$ was from BDH Laboratory Supplies, (Poole, Dorset, U.K.). MnTMPyP and DETCA were from Alexis Chemicals (Nottingham, U.K.). NP was from David Bull Laboratories (Warwickshire, U.K.), GTN from Faulding Pharmaceuticals (Warwickshire, U.K.) and vitamin C (ascorbic acid 100 mg/ml) from South Devon Healthcare (Devon, U.K.).

Data analysis and statistical methods

All vascular responses to vasodilator agonists are reported throughout as the percentage reduction in tension (per cent relaxation) as compared with the level of tone induced by contraction with phenylephrine or norepinephrine. Results from experiments performed on multiple rings (usually 2 to 4) from one animal were averaged and used in subsequent analysis. Numbers (n) refer to numbers of animals used for each protocol. Results are expressed as means $\pm$ s.e.mean. Analysis of variance for repeated measures was used to compare effects of vitamin C, DETCA and other agents on relaxation to the agonists. Dose response curves were fitted to a sigmoidal curve using GraphPad version 2 software (San Diego, U.S.A). The effective dose producing 50% maximal relaxation (EC_{50}) was used to assess a parallel shift in the log dose-response curve. Where inhibitors decreased maximum relaxation (E_{max}) to agonists, per cent inhibition of the response was calculated as: $[(E_{max\ control} - E_{max\ inhibitor})/\ E_{max\ control}] \times 100$.

Results

Effects of vitamin C on ACh, authentic NO and NO-donors

Vitamin C (0.1–10.0 mmol l^{-1}) produced a concentration-dependent attenuation of the response to ACh (Figure 1a, $P<0.001$ for all doses by ANOVA for repeated measures, $n=4$). For individual doses this reached significance at 10 mmol l^{-1} with a decrease in E_{max} of 71 $\pm$ 7%. Vitamin C also produced a concentration-dependent attenuation of relaxation to authentic NO (Figure 1b, $P<0.001$ for 3 and 10 mmol l^{-1} vitamin C, $n=5$). A parallel shift to a higher concentration range of the log dose-response curve to NO was observed (EC_{50} -7.7 ± 0.22 and -5.9 ± 0.14 log units for control curves and for vitamin C 10 mmol l^{-1} respectively). By contrast vitamin C caused a concentration-dependent potentiation of relaxation to NP (Figure 2a, $P<0.001$, $n=5$). A parallel shift to a lower concentration range of the log dose-response curve was observed (EC_{50} -6.0 ± 0.05 and -6.9 ± 0.09 log units for control curves and for vitamin C 10 mmol l^{-1} respectively). Vitamin C also potentiated relaxation to SNAP (Figure 2b, $P<0.01$, $n=6$). Concentrations of vitamin C up to 3 mmol l^{-1} had no significant effect on relaxation to GTN (Figure 2b). However vitamin C (10 mmol l^{-1}) caused a small but significant attenuation of the response to GTN, (EC_{50} -7.4 ± 0.1 and -6.9 ± 0.1 log units for control *vs* vitamin C 10 mmol l^{-1} respectively, $n=6$, $P<0.05$).

Effects of vitamin C on NO released from SNAP measured using a NO electrode

Vitamin C (0.1–3.0 mmol l^{-1}) produced concentration-dependent potentiation of NO release from SNAP in the presence of EDTA (1 mmol l^{-1}). Vitamin C increased the concentration of NO in bath solution following the addition of SNAP (250 μmol l^{-1}) from 53.4 ± 14.1 to 771.5 ± 101.3 nmol l^{-1} over the range 0.1–3.0 mmol l^{-1}. (Figure 3, $P<0.01$ for 0.6, 1.0 and 3.0 mmol l^{-1} vitamin C, $n=5$). NO in bath solution was not detected following addition of NP with or without vitamin C (data not shown).

Effects of $CuSO_4$ and neocuproine on NP

$CuSO_4$ (50–200 μmol l^{-1}; 15 min) had no significant effect on relaxation to NP (EC_{50} -5.9 ± 0.06 *vs* -5.8 ± 0.03, $n=4$).

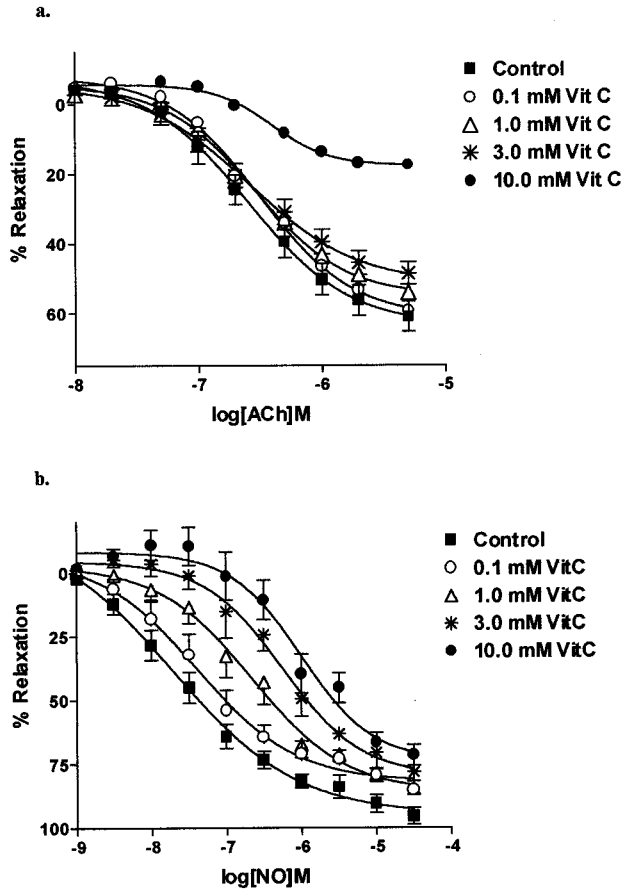

Figure 1 (a) Effects of vitamin C on relaxation of pre-constricted rabbit aortic rings to acetylcholine (ACh). Per cent relaxation refers to the percentage reduction in isometric tension as compared with the level of vascular tone induced by the contractile agonist. Vitamin C ($0.1-10.0$ mmol l^{-1}) produced a concentration-dependent attenuation of the response to ACh (Figure 1a, $P<0.001$ for all doses by ANOVA for repeated measures, $n=4$). For individual doses this reached significance at 10 mmol l^{-1} with a decrease in Emax of $71\pm7\%$. (b) Effects of vitamin C on relaxation of pre-constricted rabbit aortic rings to authentic nitric oxide (NO). Vitamin C produced a shift to a higher concentration range of the dose-response curve to NO ($P<0.001$ for vitamin C 3 and 10 mmol l^{-1} *vs* control, $n=5$).

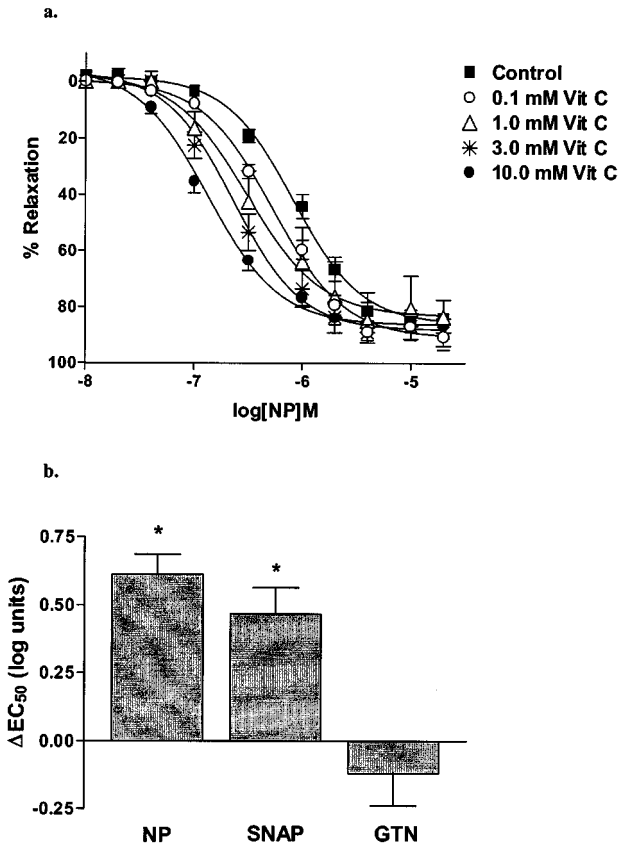

Figure 2 (a) Effects of vitamin C on relaxation of pre-constricted rabbit aortic rings to nitroprusside (NP). Per cent relaxation refers to the percentage reduction in isometric tension as compared with the level of vascular tone induced by the contractile agonist. Vitamin C produced a shift to a lower concentration range of the dose-response curve to NP ($P<0.001$ by ANOVA, $n=5$). (b) Change in the dose producing 50% relaxation (ΔEC_{50}, log units) of pre-constricted rabbit aortic rings following vitamin C (3 mmol l^{-1}) for nitroprusside (NP, $n=5$), *S*-nitroso-*N*-acetyl-penicillamine (SNAP, $n=6$) and glyceryl trinitrate (GTN, $n=5$). $^*P<0.01$.

Neocuproine (60 μmol l^{-1}, 15 min), a selective Cu^+ chelator had no significant effect on relaxation to NP when given alone and co-incubation with neocuproine (60 μmol l^{-1}) and vitamin C (3 mmol l^{-1}) for 15 min had no significant effect on the relaxation to NP in the presence of vitamin C alone (EC_{50} -6.7 ± 0.16 *vs* -6.7 ± 0.12).

Effects of ODQ on ACh and NO-donors

ODQ (10 μmol l^{-1}) a selective inhibitor of soluble guanylyl cyclase, produced almost complete inhibition of relaxation to ACh and each of the NO-donors ($89.8\pm7.8\%$, $98.4\pm1.6\%$, $85.8\pm8.1\%$ and $92.2\pm5.0\%$ change in response to the concentration giving E_{max} in the absence of ODQ for ACh, NP, SNAP and GTN respectively ($n=7$ for ACh, $n=4$ for each NO-donor, all $P<0.01$, Figure 4), while having no significant effect on relaxations to 8-bromo-cGMP, a cell permeable cyclic GMP analogue ($2.6\pm14.1\%$ change in E_{max}, $n=3$).

Effects of DETCA on ACh

Relaxation to ACh was markedly attenuated following incubation with DETCA with a $71\pm9\%$ reduction in E_{max} (Figure 5). Inhibitory effects of DETCA were abolished by MnTMPyP (100 μmol l^{-1}, $n=7$, Figure 5). Vitamin C (3 mmol l^{-1}), however, had no significant effect in reversing effects of DETCA, rather tending to impair relaxation further (Figure 5).

Effects of DETCA on relaxation to NO-donors

DETCA had no significant effect on relaxation to NP (EC_{50} -5.86 ± 0.01 and -5.80 ± 0.03 log units for control curves and DETCA respectively, $n=6$), nor to GTN (EC_{50} -6.93 ± 0.2 and -6.84 ± 0.2 log units for control curves and DETCA respectively, $n=4$). The potentiation of NP by vitamin C was not affected by DETCA. In the presence of DETCA vitamin C (3 mmol l^{-1}) produced a

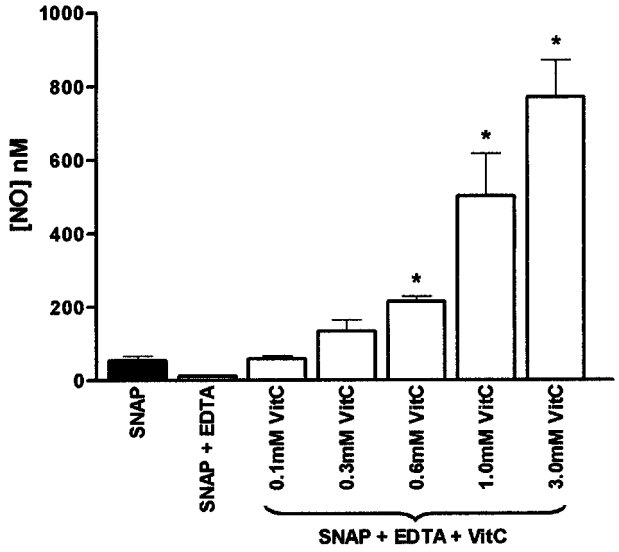

Figure 3 Concentration-dependent potentiation of nitric oxide (NO) release from *S*-nitroso-*N*-acetyl-penicillamine (SNAP, 250 μmol l^{-1}) by vitamin C in the presence of ethylenediaminetetra-acetic acid (EDTA, 1 mmol l^{-1}, $n=5$). NO was measured using a Clark-type NO electrode 100 seconds after addition of SNAP. *$P<0.01$.

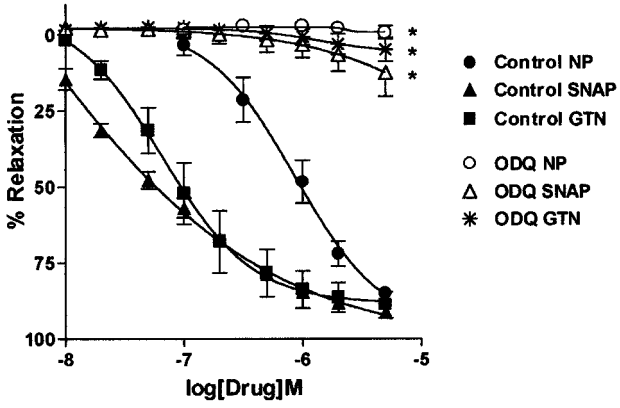

Figure 4 Effects of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μmol l^{-1}) on relaxation of pre-constricted rabbit aortic rings to nitroprusside (NP), *S*-nitroso-*N*-acetyl-penicillamine (SNAP) and glyceryl trinitrate (GTN). Per cent relaxation refers to the percentage reduction in isometric tension as compared with the level of vascular tone induced by the contractile agonist. ODQ inhibited each NO-donor $P<0.01$. Each $n=4$.

parallel shift to a lower concentration range of the log dose-response curve to NP of 0.7 ± 0.3 log units ($n=7$), similar to the potentiation observed in the absence of DETCA. DETCA caused a small but significant attenuation of relaxation to SNAP ($P<0.01$, $n=4$), with a shift to a higher concentration range in the log dose-response curve (EC_{50} -6.65 ± 0.09 and -6.17 ± 0.06 log units for control curves and DETCA respectively). Relaxation to ACh remained inhibited by DETCA to a similar degree after washout of the NO-donors as immediately after DETCA washout (i.e. before administering NO-donor, data not shown).

Discussion

The major finding of the present study is that vitamin C inhibits relaxation of rabbit aortic rings to ACh whereas it potentiates some NO-donors (NP and SNAP). Inhibition of ACh occurs at high pharmacological concentrations of vitamin C (>1 mmol l^{-1}) whereas effects on NP are significant at physiological concentrations (100 μmol l^{-1}) and increase with increasing concentrations into the pharmacological range. Relaxation to ACh in this preparation is mediated mainly by endothelium-derived NO and the effect of vitamin C on ACh is likely to result from the inactivation of NO. Indeed vitamin C produced marked inhibition of relaxation to authentic NO. The characteristics of the inhibition of NO (shift of the dose-response curve to higher concentrations) differed from that for ACh (reduction in E_{max}). However, this may be explained by differing sites and kinetics of NO delivery to the vascular smooth muscle. The mechanism by which vitamin C inactivates NO was not addressed in the present study but could involve a reaction between the ascorbyl radical and NO.

Potentiation of the NO-donors NP and SNAP by vitamin C is unlikely to be due to an effect of vitamin C in scavenging intracellular O_2^- since inhibition of intracellular SOD by DETCA had little effect on relaxation to NO-donors and did not affect potentiation of NP by vitamin C. A direct action of vitamin C on NO-donors independent of intracellular O_2^- is therefore likely. The final step in the pathway through which NO-donors release NO is thought to involve a reaction whereby NO is released from a nitrosothiol (Feelisch, 1998). This reaction is catalysed by transition metal ions such as copper. Vitamin C reduces Cu^{++} to Cu^{+}, which is thought to be more potent in catalysing the reaction than Cu^{++} (Singh *et al*., 1996). In addition vitamin C may directly catalyse the release of NO from nitrosothiols (Holmes & Williams, 1998). In the present study addition of vitamin C to SNAP (which does not require tissue activation) in bath solution led to an increase in the release of NO into solution. This was observed in the presence of a concentration of EDTA (1 mmol l^{-1}) sufficient to chelate trace concentrations of Cu^{++}. However no NO generation was measurable in bath solution for NP due to the requirement of tissue activation and the absence of tissue in the solution. Furthermore, relaxation to NP was not affected by $CuSO_4$ ($\leqslant 200$ μmol l^{-1}) and potentiation of NP by vitamin C was not affected by the specific Cu^{+} chelator neocuproine (Al Sa'doni *et al*., 1997). Thus, it is likely that vitamin C potentiates these NO-donors by enhancing the release of NO from a nitrosothiol through a copper ion independent mechanism.

The effects of vitamin C in potentiating NO-donors varied with a greater effect on NP than on SNAP and no effect on GTN. In some preparations NO-donors have been shown to act through different downstream signalling pathways (Tseng *et al*., 2001). However, in this preparation, relaxations to NP, SNAP and GTN as well as ACh are all almost completely abolished by inhibition of soluble gaunylyl cyclase by ODQ (10 μmol l^{-1}). It is, therefore, unlikely that different downstream signalling pathways account for the different effects of vitamin C on the NO-donors. Since vitamin C may both potentiate release of NO from NO-donors and inhibit actions of NO, it could potentially either enhance or inhibit relaxant responses to NO-donors. Thus its effects are likely to depend

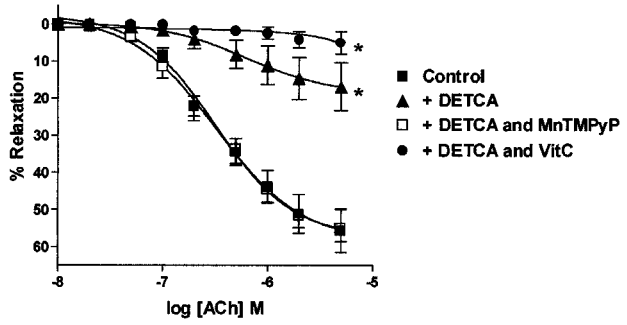

Figure 5 Effects of diethyldithiocarbamate (DETCA 2–6 mmol l^{-1}), and manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP, 100 μmol l^{-1}), a cell permeable SOD mimetic on relaxation to acetylcholine (ACh) of pre-constricted rabbit aortic rings. Per cent relaxation refers to the percentage reduction in isometric tension as compared with the level of vascular tone induced by the contractile agonist. *$P<0.002$ compared with control and DETCA plus MnTMPyP, $n=7$.

upon the mechanism and kinetics of the release of NO from nitrosothiols and the mechanism and site of production of the nitrosothiol intermediates.

Effects of vitamin C on NO-dependent vasodilation in this preparation differ from those seen in human forearm conduit and resistance vessels *in vivo*. The most widely reported action of vitamin C when given in high concentrations *in vivo* is to normalize impaired vasodilator responses to cholinergic agonists in conditions associated with increased oxidative stress, restoring values to those seen in healthy control subjects (Heitzer *et al*., 1996; Ting *et al*., 1996a, b; Chambers *et al*., 1999; Hornig *et al*., 1998). No effect of vitamin C on responses to NO-donors including NP has been observed in these studies. The findings of the present study that vitamin C inhibits relaxation to ACh and potentiates relaxation to NP are therefore in direct contrast to those in these *in vivo* human studies. The disparity is unlikely to be related simply to dose, since the *in vitro* findings in the present study extended over a range of concentrations encompassing plasma concentrations achieved *in vivo*. Furthermore, when intracellular oxidant stress was increased to produce a selective impairment of endothelium-dependent relaxation, effects of vitamin C were unaltered.

It is possible that nitrosothiols are formed by different mechanisms *in vivo* and *in vitro*. Alternatively vitamin C may promote the release of NO from carriers such as nitrosoalbumin or S-nitrosohaemoglobin that are present *in vivo* (Scorza *et al*., 1997; Shivasaki *et al*., 1988) and which may act as a reservoir for NO released from the luminal surface of the endothelium. Irrespective of the differences between the actions of vitamin C *in vitro* and *in vivo* the present study indicates that enhancement of NO-dependent relaxation by vitamin C is not necessarily related to its antioxidant actions.

A further notable finding of the present study is that concentrations of DETCA sufficient to inhibit relaxation to ACh had no effect on relaxation to GTN and NP. DETCA did inhibit SNAP but to a lesser extent than its effect on ACh. The mechanism underlying the differing sensitivity to DETCA of relaxations to ACh and NO-donors is unknown, but could relate to the sites of O_2^-/NO release, to differing kinetics of release of free NO from an intermediary species or to a direct action of such intermediary or other species. Inactivation of NO in the extracellular compartment is also unlikely to account for effects of DETCA on endothelium-derived NO since it is not reversed by Cu-Zn SOD (Mackenzie & Martin, 1998) which enters the extracellular space but does not penetrate cell membranes. NO derived from NO-donors may be released in close proximity to guanylyl cyclase rendering it less susceptible to inactivation by O_2^- . This may be true for NP and GTN which are thought to undergo transformation to NO within vascular smooth muscle rather than SNAP which does not require tissue activation (Feelisch, 1998). Alternatively, nitrosothiols formed from NO-donors may protect NO from O_2^- by reacting with O_2^- to release NO (Singh *et al*., 1999) or directly activate guanylyl cyclase or possibly other relaxant mechanisms (Kowaluk & Fung, 1990). The relative lack of effect of DETCA on NO-donors is consistent with observations that responses to NO-donors are usually preserved in conditions associated with increased oxidative stress (Anderson, 1999). The divergent effects of DETCA on ACh and NO-donor responses call into question the assumption that blunted responses to ACh with preserved response to NO-donors necessarily implies a defect in NO biosynthesis.

In conclusion, vitamin C inhibits endothelium-dependent relaxations of rabbit aortic rings to ACh and authentic NO and does not reverse impaired relaxation resulting from increased intracellular oxidant stress. Vitamin C potentiates relaxation to the NO-donors NP and SNAP by a mechanism likely to involve release of NO from nitrosothiols. Effects of vitamin C in potentiating NO-dependent relaxation can not necessarily be attributed to an antioxidant effect.

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