

Nitric Oxide (NO) Scavenging and NO Protecting Effects of Quercetin and Their Biological Significance in Vascular Smooth Muscle

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ABSTRACT

The flavonoid quercetin reduces blood pressure and endothelial dysfunction in animal models of hypertension. However, the results concerning the relationship between quercetin and NO present a complex picture. We have analyzed the mechanisms involved in the NO scavenging effects of quercetin and its repercussion on NO bioactivity in vascular smooth muscle. Quercetin scavenged NO with apparent zero-order kinetics with respect to NO. This effect was strongly dependent on the O_2 concentrations, so that NO decay at pH 7.4 could be fitted to the equation $-d[NO]/dt = k \times [O_2] \times [quercetin]$, where k was $0.15 \text{ M}^{-1} \text{ s}^{-1}$. The NO scavenger effects were prevented by superoxide dismutase (SOD), reduced by lowering pH, accompanied by O_2^- production and correlated with decreased NO bioactivity in rat aortic rings. However, under conditions of increased O_2^- concentrations, quercetin was a better scavenger

of O_2^- than of NO. When NO scavenging by quercetin was prevented by addition of SOD, NO bioactivity was increased. Quercetin also prevented the inhibitory effects of the SOD inhibitor diethyldithiocarbamic acid (DETCA) on NO bioactivity. In the presence of DETCA, quercetin reduced tissue O_2^- as measured by nitro blue tetrazolium staining. In conclusion, quercetin exerts dual effects on O_2^- and NO. At physiological conditions of pH, O_2 concentrations and NO, quercetin effectively scavenged NO in the low micromolar range, and the rate-limiting step was the autooxidation of quercetin and the formation of O_2^- . When the extracellular NO scavenging effect was prevented, quercetin increased the biological activity of NO, an effect related to its O_2^- scavenger properties and/or its inhibitory effect on tissue O_2^- generation.

Flavonoids are polyphenolic compounds widely distributed in dietary fruits, vegetables, and wine. The average daily intake in the occidental diet is ≈ 23 mg, of which quercetin represents 60 to 75% (Hertog et al., 1993; Sampson et al., 2002). Epidemiological studies including more than 100,000 patients have shown an inverse association between dietary flavonoid intake and mortality from coronary heart disease and/or risk of stroke (Hertog et al., 1993; Rimm et al., 1996). Although prospective randomized clinical trials are lacking, several studies using animal models support these potential protective effects of flavonoids in cardiovascular diseases

(Middleton et al., 2000). Interestingly, quercetin exerts systemic and coronary vasodilator effects (Duarte et al., 1993; Perez-Vizcaino et al., 2002); when given orally, it reduces blood pressure, cardiac hypertrophy, and vascular remodeling in spontaneously hypertensive (SHR) and nitric oxide (NO)-deficient rats (Duarte et al., 2001, 2002). It also exerts free radical-scavenging effects (Robak and Gryglewski, 1988; Van Acker et al., 1996), inhibits low-density lipoprotein peroxidation, and reduces the progression of atherosclerosis in vivo (Hayek et al., 1997).

The results concerning the relationship between quercetin and NO yield a confusing picture. Regarding NO synthesis, high concentrations of quercetin ($>100 \mu\text{M}$) inhibit the activity of the endothelial (eNOS), neuronal, and inducible isoforms of NO synthase (Chiesi and Schwaller, 1995). In vivo, high doses of quercetin (above 200 mg/kg/d for 10 days)

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ABBREVIATIONS: SHR, spontaneously hypertensive rat; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; NOS, nitric-oxide synthase; XO, xanthine oxidase; DMSO, dimethyl sulfoxide; HX, hypoxanthine; SOD, superoxide dismutase; cyt c, cytochrome c; NBT, nitro blue tetrazolium; DETCA, diethyldithiocarbamic acid; DPI, diphenylene iodonium.

increased the activity, but not the expression, of vascular eNOS (Benito et al., 2002), whereas at low doses (5 and 10 mg/kg/d for 5 weeks), no changes were observed in either vascular eNOS or inducible NOS expression or total NOS activity (Duarte et al., 2002). Thus, changes in NOS activity seem to be unlikely at doses of quercetin equivalent to those present in the diet. In addition, quercetin directly scavenges the superoxide anion (O_2^-) (Robak and Gryglewski, 1988) and inhibits several O_2^- -generating enzymes such as xanthine oxidase (XO) (Hayashi et al., 1988; Chang et al., 1993) or the neutrophil membrane NADPH oxidase complex (Tauber et al., 1984). In SHR rats, quercetin reduced the oxidative status, as indicated by lower concentrations of markers of such oxidative stress as plasma and hepatic malondialdehyde and urinary isoprostane- $F_{2\alpha}$ (Duarte et al., 2001). By reducing O_2^- concentrations, quercetin is expected to protect NO from O_2^- -driven inactivation. In fact, it improved the endothelial function in SHR rats (Duarte et al., 2001), possibly because of an enhanced NO bioavailability. It also increased the levels of NO determined by electron paramagnetic resonance spectroscopy in rat brain during global ischemia and reperfusion (Shutenko et al., 1999). On the other hand, quercetin and related flavonoids also scavenge NO (Van Acker et al., 1995; Haenen and Bast, 1999). However, the mechanisms of the NO-scavenging effects, the potential NO protecting mechanisms, and their repercussion on NO bioactivity are unclear.

Therefore, in the present study, we have analyzed the mechanisms involved in the NO scavenging effect of quercetin. In addition, we have investigated the mechanisms of the potential protective effects of quercetin on NO bioactivity in vascular smooth muscle.

Materials and Methods

The studies have been carried out in accordance with the Declaration of Helsinki.

Materials. All chemicals and drugs were from Sigma (St. Louis, MO). Quercetin and diphenylene iodonium were dissolved in DMSO daily, hypoxanthine (HX) was dissolved in 0.1% NaOH, and other drugs were dissolved in distilled, deionized water to prepare 1, 10, or 100 mM stock solutions; further dilutions were prepared in the working buffer. To prepare the NO solutions used for biological activity, a vial containing 20 ml of Krebs' solution at 37°C was initially bubbled with N_2 for 15 min and then continuously bubbled with NO (450 ppm from Air Liquide, Paris, France) resulting in a concentration of 0.9 to 1 μ M (as measured by the electrochemical electrode described below; Lopez-Lopez et al., 2001).

NO Scavenging. NO concentrations were monitored with an ISO-NO meter (World Precision Instruments Inc., Sarasota FL) coupled to a data acquisition hardware (PowerLab, AD Instruments Pty Ltd., Castle Hill, Australia) and data recording software (Chart v4.1.2; AD Instruments) in a 20-ml thermostat-equipped, water-jacketed chamber at 37° filled with HEPES-buffered solution (130 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 10 mM glucose, 1.5 mM $CaCl_2$, and 10 mM HEPES; pH 7.4 unless otherwise stated). The chamber was bubbled with N_2 for >15 min then slowly bubbled with NO at 450 ppm until the desired NO concentration (50–70 nM) was achieved (within 3–5 min), and the O_2 concentration was adjusted by addition of O_2 -saturated HEPES solution [bubbled with 100% O_2 (i.e., 1.05 mM)]. At the beginning of the experiment, the system was closed with no headspace, under constant rapid stirring. NO concentration was 50 ± 10 nM, O_2 concentration varied from nominally 0 (< 0.1%) to 5, 10, or 20%, and pH was 7.0 or 7.4. Oxygen concentration was simultaneously measured in the chamber with a Clark O_2 elec-

trode (World Precision Instruments Inc., New Haven, CT) connected to the PowerLab system. Quercetin (20 μ l) was added to the chamber with a microsyringe at a final concentration of 1 to 100 μ M, and NO concentration was followed in the presence of quercetin. In some experiments, superoxide dismutase (SOD; 100 U/ml), XO (5 or 15 mU/ml) plus HX (100 μ M), or pyrogallol (3 or 10 μ M) were added 2 min before quercetin. Corrections were made for the spontaneous degradation of NO under the different O_2 concentrations in the presence of vehicle (20 μ l of DMSO). The electrode was calibrated daily according to the manufacturer by the conversion of known concentrations of $NaNO_2$ into NO in the presence of H_2SO_4 and KI.

Quercetin Auto-Oxidation and O_2^- Released. UV-visible absorption spectra of quercetin in HEPES-buffered solution was recorded in a spectrophotometer (6405; Jenway, Essex, UK) just after dilution and after 48 h of exposure to room air O_2 at 37°C. The oxidation of quercetin at 37°C, room air O_2 concentration, and under constant stirring was followed at 380 nm for 10 h. To measure the release of O_2^- , quercetin (10, 50, or 100 μ M) or vehicle (DMSO) was added to a HEPES-buffered solution at pH 7.4 or 9.0 and incubated at 37°C under room air for 5 min. O_2^- release was determined by measuring luminescence after automatic injection of lucigenin (5 μ M) over 200 s in a scintillation counter (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany) at 5-s intervals and expressed as relative luminescence units/min. In addition, O_2^- release was also determined by measuring SOD-inhibitable cytochrome *c* (cyt *c*) reduction, after changes in absorbance at 550 nm for 5 min, after addition of cyt *c* (final concentration, 20 μ M) in a 1-ml cuvette. The experiments were performed with and without SOD (100 U/ml). O_2^- production was calculated as the fraction of ferricytochrome *c* reduction inhibited by SOD and expressed as nanomolar per minute.

Computer Simulation. The solution technique employed to simulate NO consumption is based on time discretization followed by the solution of the discrete equations using an iterative scheme and updating the concentrations of reagents in each iterative step. The simulation was reproducible when discrete time intervals were of 10 μ s or shorter. Calculations were made using a Turbo Pascal program created by the authors (available on request).

NO Bioactivity in Rat Vascular Smooth Muscle. Preparation of aortic rings from Wistar rats, force measurement under isometric conditions, and endothelial cell removal were performed as described previously (Perez-Vizcaino et al., 2002). After equilibration in Krebs' solution, endothelium-denuded rings were stimulated with 30 mM KCl until they reached a steady-state contractile response and NO (70 nM) was added to the chamber, which induced a transient relaxant response (30–40% of the initial tone). Then, the tissues were washed with Krebs' solution containing 30 mM KCl, incubated for 20 min with the drugs to be tested or vehicle (except HX, which was added in the last minute), and NO was again added. This second relaxant response indicative of NO bioactivity in the presence of the drugs or vehicle was expressed as a percentage of the initial response to NO. In some aortic rings stimulated with 30 mM KCl and exposed to vehicle or quercetin for 20 min, the responses to the vasodilator sodium nitroprusside (3 nM) were recorded.

In Situ Detection of O_2^- by Nitro Blue Tetrazolium Reduction. Tissues were exposed to nitro blue tetrazolium (NBT) to allow O_2^- generated by the tissue to reduce the NBT to blue formazan (Di Wang et al., 1998). Unfixed aortic rings were embedded in OCT (R. A. Lamb Ltd., East Sussex, UK) and frozen in liquid nitrogen. Frozen aortas were cryosectioned at 50 μ m. The sections were then incubated at 37°C in HEPES-buffered solution (composition as above) for 1 h and then in HEPES solution containing NBT (100 μ M) plus DMSO or quercetin (10 μ M) in the presence or absence of DETCA (1 mM) for 90 min, dried, and coverslipped. High contrast images of the sections were obtained in a DM IRB microscope (Leica, Wetzlar, Germany) coupled to a Leica DC300F color digital camera.

Statistical Analysis. Data are expressed as means $\pm$ S.E.M.; *n* indicates the number of experiments. Statistical analysis was performed using Student's *t* test for paired observations or one-way

analysis of variance followed by a Newman Keuls' post hoc test. Differences were considered statistically significant when P was less than 0.05.

Results

NO Scavenging Effects of Quercetin. Under nominally anoxic conditions ($[O_2] < 0.1\%$), NO, at physiological concentrations (50 nM), decayed very slowly (Fig. 1). The NO decay was accelerated by increasing concentrations of quercetin with apparent zero-order kinetics as indicated by the linear relationship of NO concentration and time. However, the values of the observed rate constants (k_{obs}), after correction for the spontaneous degradation of NO in DMSO, were linearly related to the concentrations of quercetin (Fig. 1, inset). Thus, the reaction could be fitted to a kinetic relationship with first-order dependence in quercetin as follows

$$-d[NO]/dt = k_{obs} = k_1 \times [\text{quercetin}] \quad (1)$$

The slope of the plot of k_{obs} versus [quercetin] yields a value of k_1 of $2.86 \times 10^{-7} \text{ s}^{-1}$ with an intercept near zero (Fig. 1 inset). Because the concentration of quercetin was almost constant as measured spectrophotometrically during the 800-s period of study (but see below), a pseudo-zero-order reaction can be expected.

Dependence on O_2 . Fig. 2, A and B, shows the dependence of NO decay on O_2 concentrations in the presence of vehicle or 10 μM quercetin, respectively. These plots also showed pseudo-zero-order kinetics. Because the concentration of O_2 was not apparently modified, as measured by the O_2 electrode during the time of the experiment, the kinetics of this reaction can be approximated to a first-order dependence in O_2 as follows

$$-d[NO]/dt = k_{obs} = k_2 \times [O_2] \quad (2)$$

When k_{obs} values in the presence of 10 μM quercetin, after correction for the spontaneous degradation of NO in DMSO at the different O_2 concentrations, were plotted against O_2 concentrations, the slope of this plot yielded a value of k_2 of $1.51 \times 10^{-6} \text{ s}^{-1}$, with a near-zero intercept (Fig. 2B, inset). Thus, a new equation explaining the kinetics of NO consumption depending on both the concentration of quercetin and O_2 can be proposed

$$-d[NO]/dt = k_3 \times [O_2] \times [\text{quercetin}] \quad (3)$$

where k_3 yields a value of $0.15 \text{ M}^{-1} \text{ s}^{-1}$. The dashed lines in Fig. 2B show that this equation predicts very accurately the NO decay at different concentrations of O_2 .

Role of O_2^- and pH on Quercetin-Induced NO Scavenging Effects. The possible role of O_2^- produced by quercetin was analyzed by using SOD at 100 U/ml. Figure 3A shows that SOD strongly reduced the rate of decay of NO induced by 10 μM quercetin ($P < 0.01$). However, albumin at a concentration 10 times higher than SOD (2.5 mg/ml) or denatured SOD (boiled for 20 min) had no effect (not shown). Therefore, it can be proposed that the mechanism of the NO scavenging effect of quercetin is related to its oxidation and the generation of O_2^- . In fact, quercetin can undergo auto-oxidation when dissolved in aqueous buffer generating free radicals as indicated by the generation of 5,5-dimethyl-1-pyrroline-*N*-oxide-OH radicals detected by spin resonance spectroscopy (Canada et al., 1990). Likewise, quercetin generated O_2^- at room air as measured by either the luminescence of lucigenin (Fig. 3B) or by SOD-sensitive cyt *c* reduction (Fig. 3C). SOD also strongly inhibited quercetin-induced lucigenin luminescence (e.g., 100 U/ml SOD inhibited the effect of 10 μM quercetin by 78% at pH 9). However, consistent with the strong dependence on pH of quercetin auto-oxidation (Can-

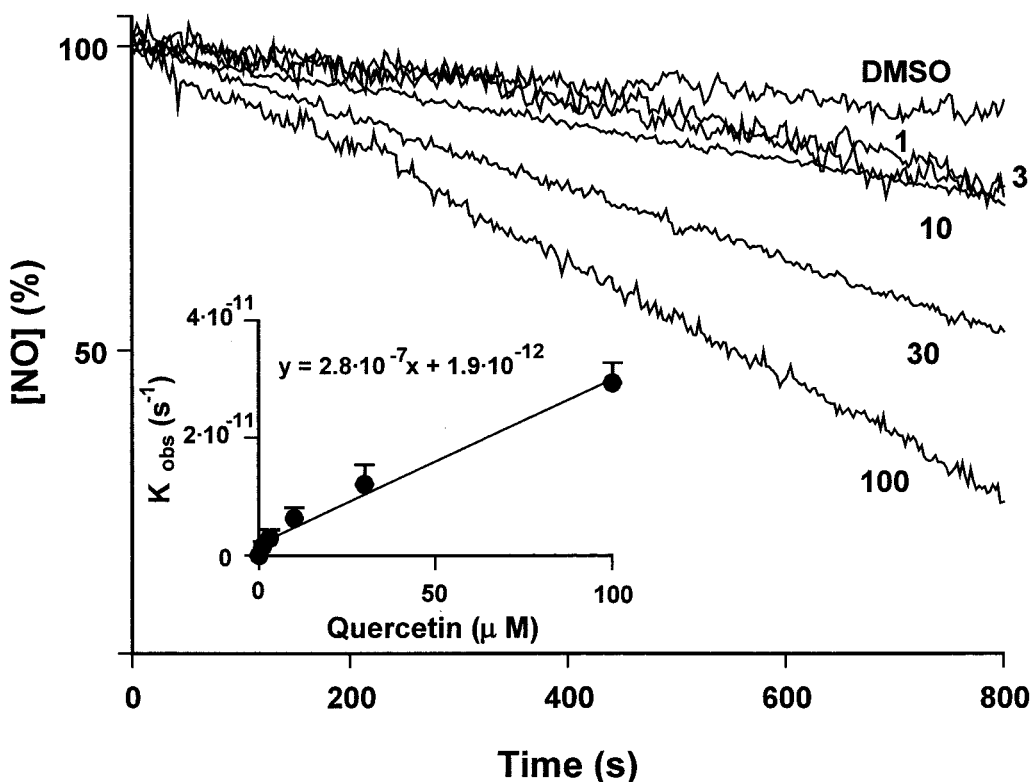


Fig. 1. NO-scavenging effect of quercetin in N_2 -saturated solution. Initial concentration of NO was $50 \pm 10 \text{ nM}$ and $20 \mu\text{l}$ of vehicle (DMSO) or quercetin (final concentrations of 1–100 μM) were added at time 0. The k_{obs} calculated (mean $\pm$ S.E.M., $n = 2$, except DMSO and 10 μM quercetin, where $n = 3$), after correction for the spontaneous degradation of NO in DMSO, was plotted against the concentration of quercetin (inset).

ada et al., 1990), generated O_2^- was clearly observed at pH 9.0, whereas at pH 7.4, the potential O_2^- generation could not be detected by either technique, and only a nonsignificant trend was observed with the cyt *c* reduction at the highest concentration of quercetin. We also analyzed the pH dependence of the NO-scavenging effects of quercetin. Figure 3A shows that small changes in pH result in large changes in NO consumption (e.g., lowering pH from 7.4 to 7.0 halved the rate of NO consumption).

Auto-oxidation of quercetin for 48 h produced a change in the UV-visible spectrum (Fig. 3D) with a reduction in the absorbance of the bands at 270 and 380 nm. These bands are supposed to be associated with the light absorption of the benzoyl (A and B rings) and cinnamoyl systems (B and C rings) in the flavonol structure, respectively (Wolfbeis et al., 1984). The rate of quercetin consumption in HEPES-buffered solution at 37°, pH 7.4, and room air O_2 concentration was measured at 380 nm, and the process could be fitted to a monoexponential decay (i.e., first order in quercetin) with a k_{obs} value of $0.0016 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 3E). In the simplest model, the reaction of O_2^- generation during quercetin auto-oxidation can be assumed as a second-order reaction.

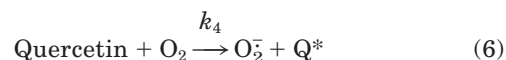
$$d[O_2^-]/dt = k_4 \times [O_2] \times [\text{quercetin}] \quad (4)$$

After this equation, the estimated value for k_4 from the plot in Fig. 3E was $0.12 \text{ M}^{-1} \text{ s}^{-1}$. The theoretical quercetin consumption under these conditions after eq. 4 is shown in Fig. 3F. The estimated values of k_3 and k_4 were very similar,

suggesting the relationship between eqs. 3 and 4. Therefore, it can be proposed that O_2^- generated in eq. 4 reacts rapidly with NO after the equation.

$$-d[\text{NO}]/dt = k_5 \times [O_2^-] \times [\text{NO}] \quad (5)$$

where k_5 is $5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Koppenol, 1998). Therefore, the lower rate of NO decay in the presence of SOD (Fig. 3, A and B) can be explained by competition of SOD and NO for the O_2^- generated by quercetin because SOD dismutates O_2^- with a rate constant of $2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, which is in the same range as k_5 (Koppenol, 1998). Thus, it can be proposed that the NO-scavenging effects of quercetin occurs in two consecutive reactions:



where Q^* is the product of quercetin oxidation. However, it should be noted that because we could not perform a reliable quantitative measurement of O_2^- production at pH 7.4, the stoichiometry of the first reaction and eq. 4 is not guaranteed.

Because O_2 and quercetin concentrations remain fairly constant during the time of the assay for NO consumption (200–400 s) as measured with a Clark electrode and spectro-

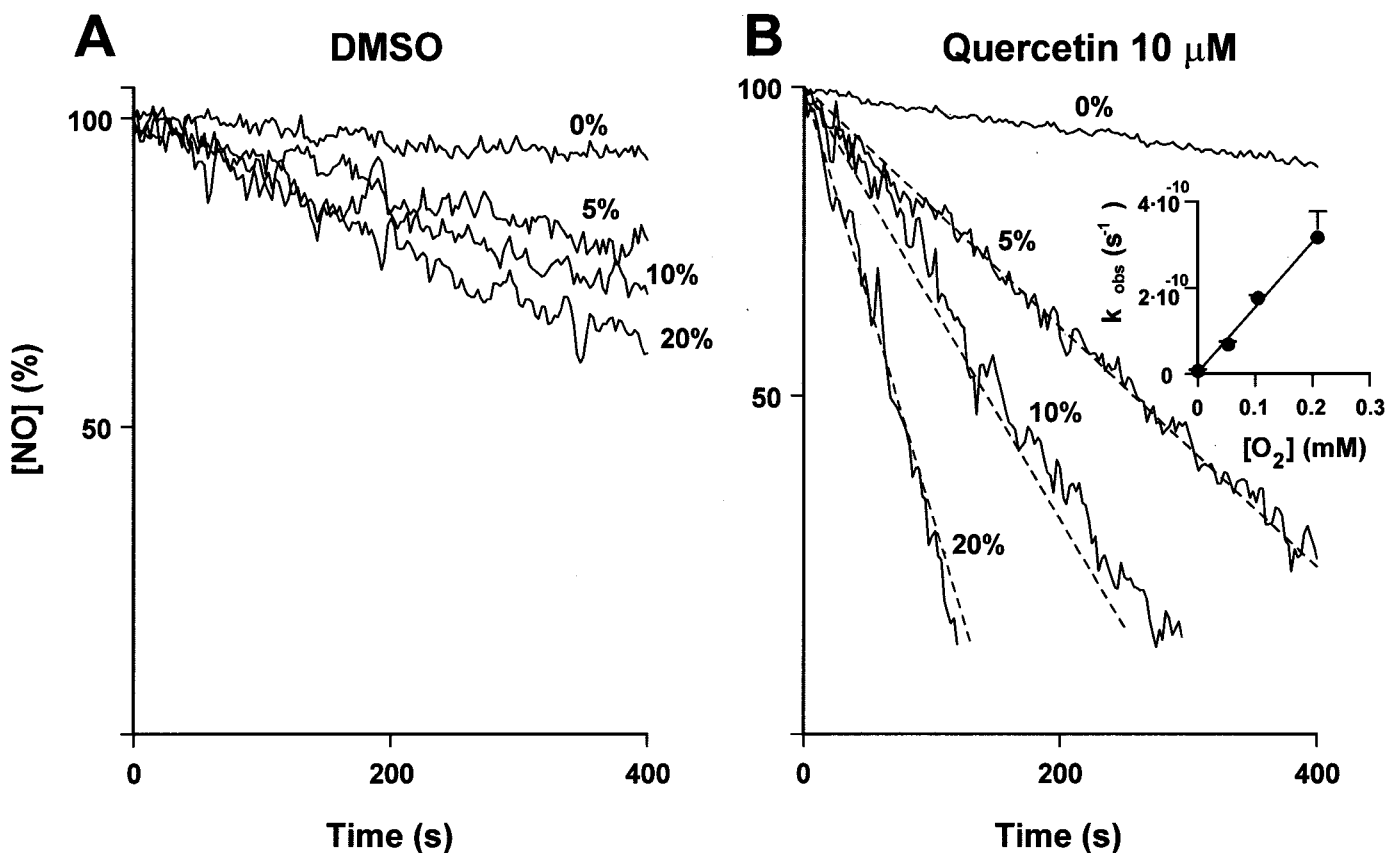


Fig. 2. Kinetics of NO decay in DMSO (A) and in the presence of quercetin (B) as a function of O_2 concentration (0, 5, 10, and 20%). Initial concentration of NO was $50 \pm 10 \text{ nM}$, and 20 μl of vehicle (DMSO) or quercetin (final concentration of 10 μM) was added at time 0. The dashed line shows the predicted NO decay from eq. 1. The k_{obs} calculated from three experiments (mean $\pm$ S.E.M.), after correction for the spontaneous degradation of NO in DMSO, was plotted against the concentration of O_2 (B, inset).

photometrically, respectively, the rate of O_2^- generation by quercetin in eq. 4 can also be assumed to be constant. In addition, because O_2^- consumption in eq. 5 is several orders of magnitude faster than O_2^- generation in eq. 4, the latter is the limiting step in NO consumption; i.e., the NO-scavenging effects of quercetin are entirely dependent on the rate of its auto-oxidation.

Computer Simulation. To simulate the kinetics of NO consumption predicted by eqs. 4 and 5, we employed time discretization and solved the discrete equations using an iterative scheme. The discrete forms of the differential eqs. 4 and 5 are shown in eqs. 8 and 9, respectively.

$$\Delta[O_2] = -\Delta[O_2] = -\Delta[\text{quercetin}] = k_4 \times [O_2] \times [\text{quercetin}] \times \Delta t \quad (8)$$

$$-\Delta[NO] = k_5 \times [O_2] \times [NO] \times \Delta t \quad (9)$$

Then, the values of reagent concentrations after each iteration step k are updated as follows:

$$[O_2]^{k+1} = [O_2]^k + \Delta[O_2] - \Delta[NO] \quad (10)$$

$$[NO]^{k+1} = [NO]^k - \Delta[NO] \quad (11)$$

$$[O_2]^{k+1} = [O_2]^k - \Delta[O_2] \quad (12)$$

$$[\text{quercetin}]^{k+1} = [\text{quercetin}]^k - \Delta[\text{quercetin}] \quad (13)$$

Equations 8 to 13 are solved in each iteration step for as many iterations as required until NO is almost fully consumed. Reproducible results were obtained using discrete time intervals (Δt) of 10 μs or less. An example using 50 nM NO, 20% O_2 , and 10 μM quercetin is shown in Fig. 4. The simulation accurately predicted the apparent zero-order reaction of NO consumption. However, it should be noted that this is not a true zero-order kinetics process; it just looks like zero order because in eq. 5, as the concentration of one of the substrates (NO) is reduced (Fig. 4C), the other (O_2^-) is increased (Fig. 4D), so that the product of both, and hence the

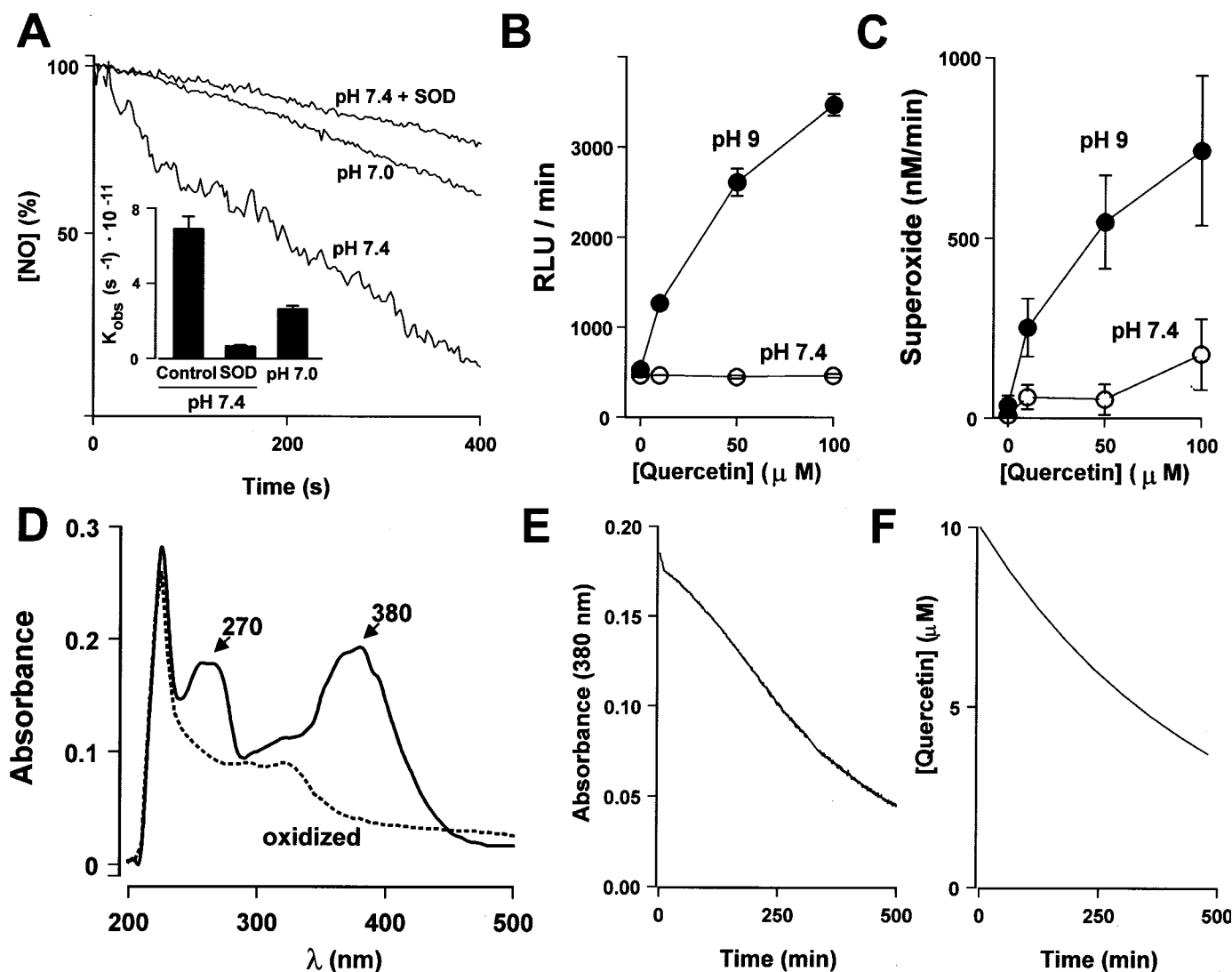


Fig. 3. Role of O_2^- and pH on the NO scavenging effects of quercetin. A, effects of SOD (100 U/ml) and lowering pH from 7.4 to 7.0 on the NO-scavenging effect of 10 μM quercetin at 5% O_2 . The k_{obs} calculated from three experiments (mean $\pm$ S.E.M.) is shown in the inset. B and C, O_2^- generation by quercetin (mean $\pm$ S.E.M.) at pH 7.4 and pH 9 measured by lucigenin luminescence ($n = 3$ and 6 for pH 7.4 and 9, respectively) (B) or by SOD-inhibitable reduction of cyt c ($n = 6$) (C). D, UV spectra of quercetin (10 μM) just after dilution and after 48 h of exposure to room air O_2 at 37°C (oxidized, dotted line). E, kinetics of quercetin (10 μM) oxidation at room air and 100% O_2 concentration measured by its absorbance at 380 nm. The trace is the average of 2 experiments. F, quercetin (10 μM) consumption at room air predicted from eq. 4 where $k_4 = 0.15 M^{-1} s^{-1}$.

rate of NO consumption, remains fairly constant. The model is also consistent with apparently constant concentrations of O_2 and quercetin (Fig. 4, A and B, respectively); e.g., using the above conditions when NO is fully consumed after 160 s, quercetin and O_2 were only decreased by 0.6% and 0.02%, respectively. The model also predicts non-pseudo-zero-order kinetics at concentrations of NO below 1 nM (Fig. 4E).

Effects of Quercetin on O_2^- -Induced NO Scavenging. Quercetin is a well known antioxidant and O_2^- scavenger (Robak and Gryglewski, 1988). Thus, we analyzed the effects of quercetin on NO consumption induced by O_2^- . O_2^- was generated by either XO (5 and 15 mU/ml) using HX as substrate or nonenzymatically with pyrogallol (3 and 10 μ M). Figure 5 shows that XO and pyrogallol increased the rate of NO consumption in a concentration-dependent manner and that these effects were strongly and similarly reduced by 10 μ M quercetin.

Effects on NO Bioactivity in Vascular Smooth Muscle. In rat aortic rings, 30 mM KCl induced a sustained contractile response (984 ± 124 mg, in 11 control arteries). Addition of NO (70 nM) induced a transient vasodilator effect that reached a peak relaxant response of $41 \pm 4\%$ of the previous tone in control arteries. After washout with Krebs' solution containing 30 mM KCl, quercetin (1 and 10 μ M) induced a weak vasodilator effect in precontracted aortas ($10 \pm 2\%$ for 10 μ M quercetin). After 20 min of exposure to quercetin, the peak relaxant effects of NO were inhibited in a concentration-dependent manner (Fig. 6). This inhibitory effect, consistent with the NO scavenging effect described above, was independent of the presence of an intact endothelium (data not shown).

When SOD was included in the bathing media, NO-induced relaxation was only weakly but significantly increased (Fig. 6). As described above, SOD prevented the inhibitory effects of quercetin on NO consumption in the bathing media (Fig. 3A). Furthermore, in the presence of SOD, quercetin not only failed to reduce the biological activity of NO but also increased it in a concentration-dependent manner. Further addition of catalase (500 U/ml) did not modify the results obtained in the presence of SOD (not shown). Because exog-

enously added SOD cannot be taken by the cells, we hypothesized that quercetin scavenges NO in the bathing media, in a SOD-inhibitable manner, but not intracellularly, where it might exert opposite effects (i.e., protection of NO from O_2^- -induced inactivation).

The Cu^{2+} chelator DETCA increases O_2^- by inhibition of

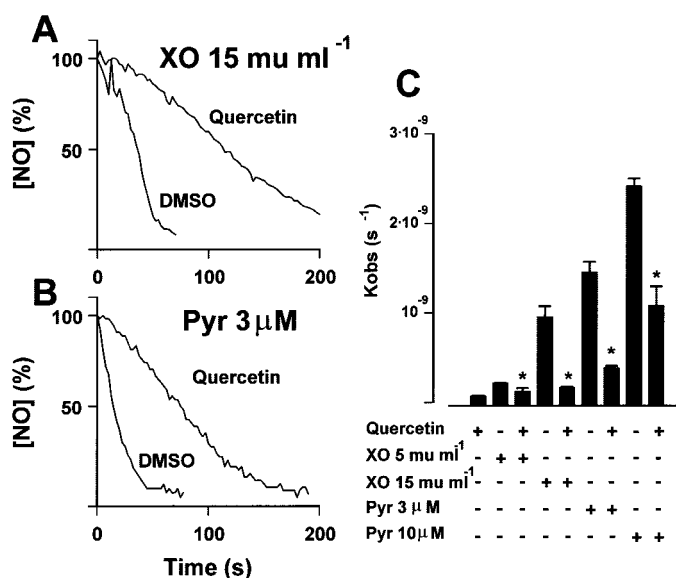


Fig. 5. NO consumption by the O_2^- generating systems XO using HX as substrate (A) and pyrogallol (B) and its prevention by quercetin. Initial concentration of NO was 50 ± 10 nM, O_2 was 5%, and XO (15 mU/ml) plus HX (100 μ M) or pyrogallol (Pyr, 3 μ M) were added at time 0 in the presence of DMSO (control) or quercetin (final concentration of 10 μ M). C, k_{obs} calculated (mean $\pm$ S.E.M.) in the presence of XO with 5 ($n = 4$) or 15 mU/ml of XO plus HX ($n = 3$) or 3 or 10 μ M pyrogallol ($n = 3$) in the presence of DMSO or quercetin, after correction for the spontaneous degradation of NO. *, $P < 0.05$ quercetin versus DMSO.

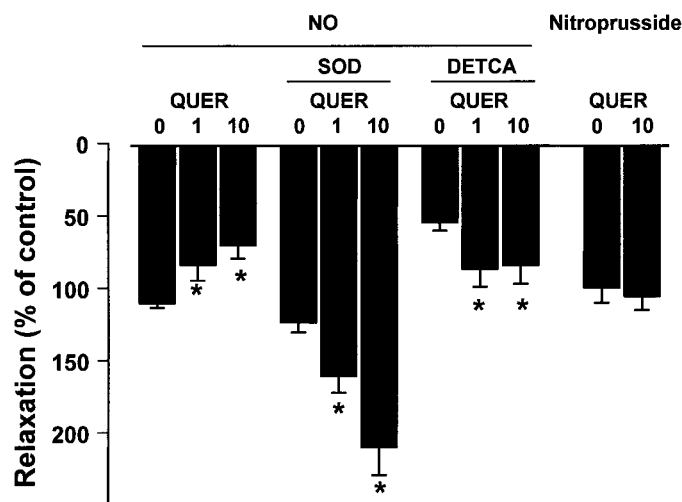


Fig. 6. Effects of quercetin (QUER) on NO and sodium nitroprusside bioactivity in endothelium-denuded aortic rings stimulated by 30 mM KCl. Rings were challenged with 70 nM NO, then exposed to DMSO (0) or quercetin (1 or 10 μ M) in the absence (control) or presence of SOD (100 U/ml) or the SOD inhibitor DETCA (1 mM) for 20 min and the again exposed to 70 nM NO. The vasodilator response to the second exposure to NO was expressed as a percentage of the initial one (mean $\pm$ S.E.M. of 8–11 experiments). The vasodilator response to nitroprusside (3 nM) was recorded in KCl-stimulated rings exposed to DMSO (0) or quercetin (10 μ M) for 20 min. The vasodilator responses to nitroprusside are expressed (mean $\pm$ S.E.M., $n = 7$ and 10 for 0 and 10 μ M quercetin, respectively) as a percentage of the response in DMSO. *, $P < 0.05$ versus DMSO.

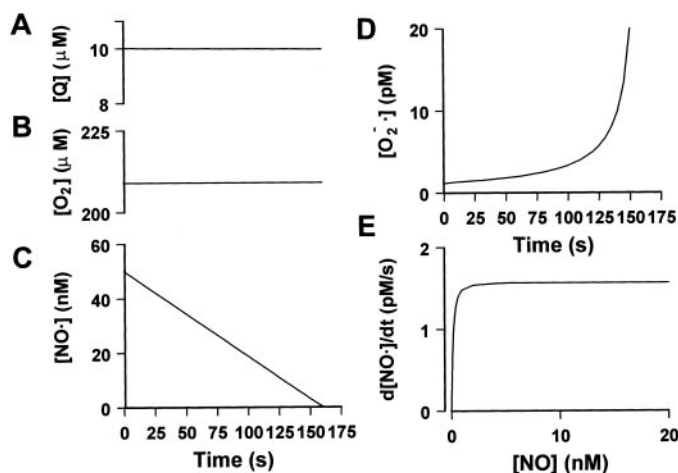


Fig. 4. Computer simulation of the NO scavenging effects of quercetin. Quercetin (A), O_2 (B), and NO (C) consumption, O_2^- concentration (D) and rate of NO consumption as a function of [NO] (E) predicted from a computer simulation as described in the text. Initial concentrations of NO, O_2 , quercetin, and O_2^- were 50 nM, 20%, 10 μ M, and 0, respectively. Values of k_4 and k_5 were $0.15 \text{ M}^{-1} \text{ s}^{-1}$ and $5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, respectively.

endogenous Cu/Zn-SOD activity (Cocco et al., 1981). This drug (at 1 mM) strongly reduced the biological activity of NO in rat aortic rings (Fig. 6) but quercetin (1 or 10 μ M) fully prevented these inhibitory effects of DETCA.

The vasodilator effects of sodium nitroprusside, as well as those of NO, are caused by the activation of soluble guanylate cyclase but, as opposed to those of NO, are unaffected by exogenously or endogenously generated O_2^- (Lopez-Lopez et al., 2001). In contrast to the effects of NO, the vasodilator effects of 3 nM nitroprusside ($31 \pm 3\%$ in control arteries) were unaffected by quercetin (Fig. 6). This suggests that quercetin is not influencing the pathway for NO/cyclic GMP-induced vasodilatation beyond the activation of soluble guanylate cyclase.

Simultaneous Measurements of NO Concentrations and NO Bioactivity. Figure 7 shows the effects of several drugs on NO-induced relaxation in aortic rings simultaneously with NO concentrations measured with the NO electrode introduced in the bathing solution. Quercetin produced a similar inhibition of both effects. SOD did not increase NO concentrations, suggesting that O_2^- concentrations were low in the whole bath, but increased NO bioactivity, which can be explained by the reduction of O_2^- levels within the tissue. As expected from the above results, in the presence of SOD, quercetin-induced NO consumption was strongly reduced. Furthermore, in SOD-treated arteries, not only did quercetin fail to reduce NO-induced relaxation but also the relaxation was even increased. All the above results pointed to different effects of quercetin on NO in the bathing media and within the tissue. To test this hypothesis, we incubated the aortas with quercetin for 20 min and then washed the tissues in quercetin-free media just before the addition of NO, so that presumably only extracellular quercetin was removed. Under

these conditions (QUER+wash; Fig. 7), NO bioactivity increased to an extent similar to that in the presence of SOD, but NO concentrations were only weakly reduced. Therefore, intracellular quercetin increased NO bioactivity even in the absence of exogenously added SOD. The results of exposure to quercetin followed by washing could be mimicked by exposure to DPI, which inhibits flavin-containing enzymes, including the main cellular source of O_2^- [i.e., NAD(P)H oxidase] (Di Wang et al., 1998). Opposite results (i.e., unaffected NO concentration but reduced NO bioactivity) were obtained with DETCA. Finally, when O_2^- was increased in the whole bath by XO plus HX, a parallel reduction in NO concentrations and NO bioactivity was observed.

In Situ Detection of O_2^- by Nitro Blue Tetrazolium Reduction. Reduction of NBT by O_2^- yields the insoluble blue stain nitro blue formazan. The specificity of this reaction in the rat aorta has been reported previously (Di Wang et al., 1998). Incubation of unfixed control (DMSO-treated) aortic ring sections with NBT (100 μ M) resulted in light blue staining (Fig. 8), which was dramatically enhanced by coincubation with DETCA (1 mM). In the absence of DETCA, the intensity of the blue staining was similar in sections incubated with or without quercetin (10 μ M), but a visible quercetin-induced reduction of the staining was observed in DETCA-treated sections.

Discussion

The lifetime of NO in biological systems, in part controlling its steady-state concentration, reflects both the concentrations and compartmentalization of reactive scavengers. In this study, in the presence of O_2 and physiological NO concentrations, NO scavenging by quercetin was faster than previously estimated (Van Acker et al., 1995; Haenen and Bast, 1999). Moreover, the reaction of NO and quercetin was much faster than the reaction of NO with O_2 in aqueous solutions (Ford et al., 1993). However, under physiological conditions, the NO scavenging effect of quercetin occurs at a

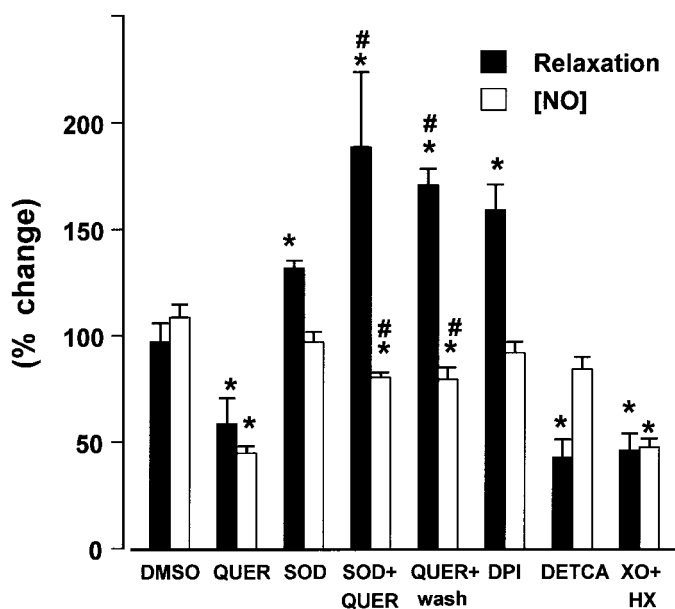


Fig. 7. Effects of several drugs on NO bioactivity (■) in endothelium-denuded aortic rings simultaneously measured with NO concentrations (□) in the tissue bath: DMSO ($n = 6$), quercetin (QUER, 10 μ M, $n = 6$), SOD (100 U/ml, $n = 5$), SOD+QUER ($n = 7$), DPI (10 μ M, $n = 6$), DETCA (1 mM, $n = 6$), XO (5 U/ml) plus HX (100 μ M) (XO+HX, $n = 5$). QUER+wash ($n = 6$) indicate arteries exposed to 10 μ M quercetin and washed just before addition of NO. The responses to the second exposure to NO were expressed as a percentage of the initial ones (mean $\pm$ S.E.M.). *, $P < 0.05$ versus DMSO. # $P < 0.05$ versus QUER.

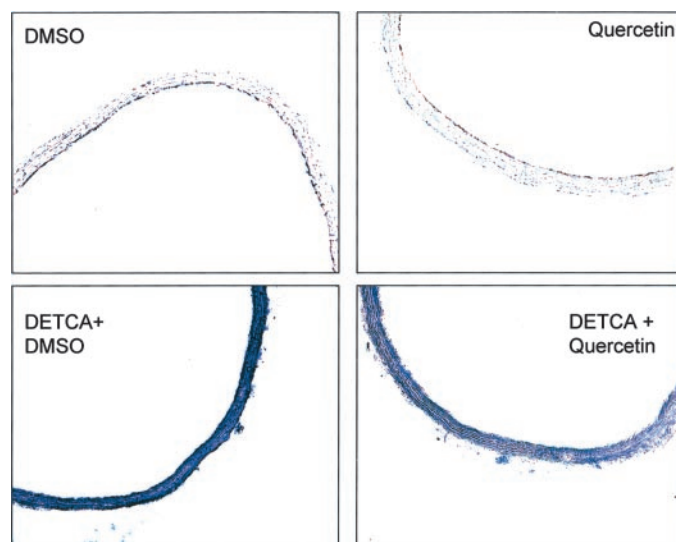


Fig. 8. Effects of quercetin on O_2^- production measured by the reduction of NBT in intact rat aortic sections. Photomicrographs of sections (50 μ m) incubated with NBT (100 μ M) plus DMSO or quercetin (10 μ M) in the presence or absence of DETCA (1 mM) for 90 min. Reduction of NBT by O_2^- yields the blue stain nitro blue formazan. These images are representative of preparations from three rats. Magnification, 10 $\times$.

rate much slower than that of oxyhemoglobin or myoglobin (Gow et al., 1999). The plasma concentrations of quercetin in humans on a normal diet are in the low micromolar range (0.3–2.2 μM ; Scalbert and Williamson, 2000), but quercetin rapidly penetrates the plasma membrane, reaching intracellular concentrations about 10-fold higher than those in the extracellular medium (Fiorani et al., 2002). However, these concentrations are still lower than those of oxyhemoglobin, which are in the high micromolar/low millimolar range. Nevertheless, based on the different distribution of these scavengers (e.g., oxyhemoglobin is restricted to erythrocytes), Haenen and Bast (1999) speculated that the NO-scavenging effect of dietary flavonoids might be relevant. On the other hand, the pseudo-zero-order kinetics on NO indicates that the lower the NO concentration, the shorter its half-life. Therefore, quercetin might contribute to limit NO diffusion in vivo only at low NO concentrations at sites at which other known NO scavengers are also in low concentrations. However, given the involvement of quercetin oxidation in its NO-scavenging effects, this effect in vivo may be limited by antioxidant enzymes and vitamins.

The kinetics of NO consumption by quercetin were best fitted by a straight line, indicating apparent zero-order kinetics. In contrast, Van Acker et al. (1995), using higher NO concentrations and single high (50 μM) concentrations of the flavonoid, described their results as pseudo-first-order kinetics, despite the fact that their plotting of $\ln [\text{NO}]$ versus time did not appear to be a straight line. The present study demonstrates that quercetin does not directly scavenge NO, but the reaction involves the oxidation of quercetin and the production of O_2^- which rapidly reacts with NO at near diffusion rate to produce peroxynitrite (Koppenol, 1998). The strong inhibitory effect of SOD on quercetin-induced NO consumption supports this conclusion even when it cannot be excluded that other radicals, such as those derived from quercetin oxidation, might also react with NO. The proposed reactions could be simulated in a computer, predicting the pseudo-zero-order rate at concentrations of NO above 1 nM (Fig. 4E). The rate of auto-oxidation, and hence O_2^- production, for quercetin has been reported to be highly pH-dependent (Canada et al., 1990). Quercetin and related flavonoids are weak acids. Thus, the lower oxidation at acidic pH seems to reflect the higher stability of the uncharged phenolic groups of quercetin. Quercetin produced a characteristic 5,5-dimethyl-1-pyrroline-*N*-oxide-OH radical, indicating O_2 -derived radical production, that was detectable only at pH values ≥ 9 and auto-oxidation, measured by O_2 consumption, at pH values ≥ 8 (Canada et al., 1990). In the present experiments, quercetin consistently and markedly increased O_2^- production at pH 9, although this increase did not reach statistical significance at pH 7.4. This may reflect the low concentrations of O_2^- produced at pH 7.4. The rates of O_2^- generation ($d[\text{O}_2^-]/dt$) by 10 and 100 μM quercetin predicted by eq. 4 at pH 7.4, 21% O_2 , are as low as 18.8 and 188 nM/min, respectively, and the lower limit of the cyt *c* reduction technique (as used herein) to detect O_2^- in a statistically significant manner is above those values. Therefore, it seems unfeasible to detect such low O_2^- concentrations at pH 7.4 using either the lucigenin or the cyt *c* approach. However, it should be stressed that because of the rapid reaction of NO with O_2^- at low (physiological) NO concentrations as used herein, a low rate of O_2^- generation is sufficient to inactivate NO. In addition, in the absence of NO,

quercetin itself may scavenge its own generated O_2^- and avoid the raise in O_2^- concentrations at neutral pH. Consistent with the pH dependence of O_2^- generation, NO consumption by quercetin was halved when pH was reduced from 7.4 to 7.0 (Fig. 3A).

Quercetin is a well known O_2^- scavenger (Robak and Gryglewski, 1988). Therefore, its expected protection of NO from O_2^- -driven inactivation is opposed to its NO-scavenging effects. Our competition studies (Fig. 5) indicate that, under conditions of increased O_2^- , quercetin is a better scavenger of O_2^- than of NO.

Interestingly, quercetin was much less effective in scavenging NO at pH 7 (i.e., the intracellular pH), suggesting a reduced NO consumption by quercetin intracellularly. On the other hand, quercetin rapidly accumulates intracellularly (Fiorani et al., 2002). In the cytosol, NO binds to the heme of soluble guanylate cyclase with very rapid kinetics ($>1.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$), effectively competing with intracellular scavengers (Zhao et al., 1999). In addition, the cells are endowed with several mechanisms for both generating and metabolizing O_2^- (Wolin et al., 2002), which may modify NO consumption by quercetin. Thus, it can be expected that the NO-scavenging effect of quercetin in vivo would be different from those in cell-free systems. For this reason, we analyzed its effects on the biological activity of NO using rat aortic rings as bioassay systems.

The NO consumption by quercetin correlated with a decreased NO bioactivity (i.e., vasorelaxation). However, when this NO consumption in the bathing solution was prevented by addition of SOD, NO bioactivity was increased. In addition, when NO consumption in the bath was minimized by rapidly washing extracellular quercetin, the remaining intracellular quercetin also increased NO bioactivity. Therefore, under the conditions present in the intracellular medium, the NO consumption by quercetin seems to be decreased, thus favoring other mechanisms protecting NO bioactivity. The lower intracellular NO consumption may exist because quercetin cannot compete with the NO binding to soluble guanylate cyclase or intracellular scavengers or because of the presence of endogenous SOD isoforms or other antioxidant mechanisms. In contrast, quercetin had no effects on the vasodilator responses induced by sodium nitroprusside (present study) or by forskolin (Duarte et al., 1993). Nitroprusside is not a classic spontaneous NO donor, and we are unable to detect NO with the amperometric electrode upon addition of nitroprusside to the bath at concentrations exerting its maximal relaxant response (Lopez-Lopez et al., 2001). The effects of nitroprusside, as well as those of NO, are caused by the activation of soluble guanylate cyclase, indicating that quercetin is not influencing the pathway for NO/cyclic GMP-induced vasodilatation beyond the activation of soluble guanylate cyclase. However, as opposed to NO, the effects of nitroprusside are unaffected by exogenously or endogenously generated O_2^- ; e.g., DETCA inhibits NO- but not nitroprusside-induced relaxation (Lopez-Lopez et al., 2001), suggesting that the differential effects of quercetin on NO- and nitroprusside-induced relaxation are caused by changes in tissue O_2^- . NO bioactivity, but not extracellular NO concentrations, could also be increased by DPI, which inhibits flavin-containing enzymes, including the main cellular source of O_2^- (i.e., NAD(P)H oxidase). Conversely, NO bioactivity was reduced by DETCA, an inhibitor of the main de-

grading enzyme of O_2^- (i.e., Cu/Zn-SOD). Interestingly, quercetin prevented the reduction of NO bioactivity by DETCA. Furthermore, in DETCA-treated arteries, quercetin lowered intracellular O_2^- as measured by the reduction of NBT in rat aorta. Taken together, these results strongly suggest that quercetin protects NO from endogenous O_2^- -driven inactivation and enhances its biological activity. Several mechanisms may account for this effect, including its direct O_2^- scavenger effect (Robak and Gryglewski, 1988). In fact, we have demonstrated that quercetin protects NO from O_2^- generated by XO or pyrogallol in a cell-free system. In addition, quercetin may also inhibit several O_2^- -generating enzymes such as XO (Hayashi et al., 1988; Chang et al., 1993) or the neutrophil membrane NADPH oxidase complex (Tauber et al., 1984). This finding may help to explain the reversal by long-term quercetin treatment of the impaired acetylcholine-induced vasodilatation in spontaneously hypertensive rat (Duarte et al., 2001), an animal model associated to increased oxidative status (Suzuki et al., 1995).

In conclusion, at physiological conditions of pH, O_2 concentrations, and NO, quercetin effectively scavenged NO in the low micromolar range. Analysis of the kinetic data indicated that the rate-limiting step was the autooxidation of quercetin and the formation of O_2^- , which rapidly reacts with NO at a near diffusion rate. The pseudo-zero-order kinetics of the reaction together with the concentrations and rate of NO scavenging of quercetin relative to other physiological NO scavengers suggests that quercetin might contribute to limit NO diffusion in vivo only at low NO concentrations and at sites where other known NO scavengers are in low concentrations. Quercetin was apparently a more effective scavenger of O_2^- than of NO under conditions of increased O_2^- . When the extracellular NO scavenging effect of quercetin was prevented, it increased the biological activity of NO, an effect apparently related to reduced tissue O_2^- .

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