Effects of Superoxide Anions on Endothelial Ca$^{2+}$ Signaling Pathways

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Abstract—Although the involvement of free radicals in the development of endothelial dysfunction under pathological conditions, like diabetes and hypercholesterolemia, has been proposed frequently, there is limited knowledge as to how superoxide anions (O$_2^-$) might affect endothelial signal transduction. In this study, we investigated the effects of preincubation with the O$_2^-$-generating system xanthine oxidase/hypoxanthine (XO/HX) on mechanisms for Ca$^{2+}$ signaling in cultured porcine aortic endothelial cells. Incubation of cells with XO/HX yielded increased intracellular Ca$^{2+}$ release and capacitative Ca$^{2+}$ entry in response to bradykinin and ATP in a time- and concentration-dependent manner. This effect was prevented by superoxide dismutase but not by the tyrosine kinase inhibitor tyrphostin A48. In addition, capacitative Ca$^{2+}$ entry induced by the receptor-independent stimulus 2,5-di-(tert-butyl)-1,4-benzoquinone or thapsigargin was enhanced in O$_2^-$-exposed cells (+38% and +32%, respectively). Increased Ca$^{2+}$ release in response to bradykinin in XO/HX-pretreated cells might be due to enhanced formation of inositol-1,4,5-trisphosphate (+140%). Exposure to XO/HX also affected other signal transduction mechanisms involved in endothelial Ca$^{2+}$ signaling, such as microsomal cytochrome P450 epoxygenase and membrane hyperpolarization to Ca$^{2+}$ store depletion with thapsigargin (+103% and +48%, respectively) and tyrosine kinase activity (+97%). A comparison of bradykinin-initiated intracellular Ca$^{2+}$ release and thapsigargin-induced hyperpolarization with membrane viscosity modulation by XO/HX (decrease in viscosity) or cholesterol (increase in viscosity) reflected a negative correlation between bradykinin-initiated Ca$^{2+}$ release and membrane viscosity. Because intracellular Ca$^{2+}$ is a main regulator of endothelial vascular function, our data suggest that O$_2^-$ anions are involved in regulation of the vascular endothelium. (Arterioscler Thromb Vase Biol. 1998;18:1470-1479.)

Key Words: cytochrome P450 epoxygenase ■ inositol-1,4,5-trisphosphate ■ membrane fluidity ■ membrane potential ■ tyrosine kinase

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and the formation of inositol-1,4,5-trisphosphate (IP$_3$), which in turn releases Ca$^{2+}$ from intracellular Ca$^{2+}$ pools. In addition to the intracellular Ca$^{2+}$ release, an extracellular Ca$^{2+}$ influx is activated due to the depletion of intracellular Ca$^{2+}$ pools, thus representing a so-called “capacitative Ca$^{2+}$ entry” pathway.\textsuperscript{16,17}

In the current study, the mechanism whereby prolonged exposure to small superoxide anion concentrations affects endothelial Ca$^{2+}$ signaling was investigated further. The effect of pretreatment with superoxide anions on endothelial Ca$^{2+}$ signaling (ie, intracellular Ca$^{2+}$ mobilization\textsuperscript{18} and capacitative Ca$^{2+}$ entry\textsuperscript{19}) and mechanisms previously shown to be involved in endothelial Ca$^{2+}$ signaling (Figure 1), such as formation of IP$_3$, cytochrome P450 epoxygenase,\textsuperscript{19,20} mem-

brane hyperpolarization,\textsuperscript{21} tyrosine kinase (TK),\textsuperscript{22} and membrane fluidity, were assessed.

**Methods**

**Materials**

1-Ethoxyxyprene-3,6,8-tris (dimethylsulphonamide), 1-hydroxyxyprene-3,6,8-tris (dimethylsulphonamide), fura 2–acetoxymethyl ester (fura 2-AM), DPH, and 1-(4-trimethylammoniumphenyl)-6-phenyl-DPH (TMA-DPH) were obtained from Lambda Fluorescence Technology. Cell culture materials were from Life Technologies, and FCS was from PAA Laboratories. Bis-(1,3-dibutylbarbituric acid)pentamethine oxonol (DiBAC$_3$(5)) and SNARF acetoxymethyl ester (SNARF-1/AM) were from Molecular Probes Inc. Petri dishes were from Falcon. The protein TK assay kit was purchased from Calbiochem-Novabiochem International. All other chemicals were obtained from Sigma.

**Cell Culture**

Endothelial cells were isolated from porcine aortae by enzymatic digestion with 200 U/mL collagenase (type II) in Dulbecco’s minimal essential medium (DMEM) plus dilutions of (vol/vol) 0.02 amino acids and 0.01 vitamins plus trypsin inhibitor (soybean type I, 1 mg/mL) as described previously.\textsuperscript{15} Cells were cultured in Opti-

**Ca$^{2+}$ Measurement**

Free [Ca$^{2+}$], was determined in porcine aortic endothelial cells in suspension or monolayer as indicated by the fura 2 technique as previously described.\textsuperscript{17} In brief, cells were incubated with DMEM containing 2 µmol/L fura 2-AM in the dark for 45 minutes (suspended cell experiments) or 30 minutes (single-cell experiments) at 37°C. Afterward, the cells were centrifuged and resuspended in DMEM. Just before the experiment, cells were centrifuged and resuspended in nominal Ca$^{2+}$–free (ie, ~10 µmol/L, free extracellular Ca$^{2+}$) HEPES-buffered solution containing (in mmol/L) 145 NaCl, 5 KCl, 1 MgCl$_2$, and 10 HEPES, pH 7.4. [Ca$^{2+}$], was monitored every 0.25 to 2.0 seconds (depending on the instrument used) as the ratio of 340 and 380 nm excitation at 510 nm emission.

To study specifically the effects of superoxide anions on the stimulation of capacitative Ca$^{2+}$ entry, Mn$^{2+}$ quench experiments were performed as previously described.\textsuperscript{19,23,24,25} In brief, this approach is based on use of the surrogate divalent cation Mn$^{2+}$, which enters the cells through the channels involved in capacitative Ca$^{2+}$ entry. However, the entry of Mn$^{2+}$ into fura 2–loaded cells induces a decrease in fluorescence at 360 nm excitation and 510 nm emission (ie, the isosbestic, Ca$^{2+}$–insensitive wavelength of fura 2). The amount of Mn$^{2+}$ entering the cells is proportional to the fractional decrease in fluorescence relative to the initial intensity.

**Data Acquisition**

In view of the reported problems concerning [Ca$^{2+}$], calibration in our system\textsuperscript{26} and the general uncertainties of the calibration techniques,\textsuperscript{27} [Ca$^{2+}$] in each experiment was expressed as the 340- to 380-nm emission ratio. Because of minor differences between the instruments used (Hitachi F2000, Hitachi F4500, and Perkin-Elmer LS-50B/FFA), caution is necessary when comparing given ratio units between different figures. Thus, for each experimental series, results were compared with those obtained in control cells (ie, preincubated in the absence of XO) performed daily and shown in each figure.

**Superoxide Anion Treatment**

Superoxide anions were generated by the reaction of XO with HX in DMEM (containing 1.8 mmol/L Ca$^{2+}$) for incubation with the cells; however, phenol red–free DMEM was used for superoxide anion measurements. The generation of superoxide anions was determined as the difference in the reduction of ferricytochrome c (10 µmol/L, horse heart type III) in the absence or presence of SOD (476 U/mL). The reduction of ferricytochrome c was monitored at 550 nm. The difference in absorption between samples in the absence and presence of SOD directly shows extinction due to superoxide anion–related reduction of ferricytochrome c. Concentrations of superoxide anions were calculated by using the molar extinction coefficient of the reduced form of ferricytochrome c ($e=21 000$).\textsuperscript{14} Production of superoxide anions was controlled for each cell pretreatment procedure in phenol red–free DMEM without addition of XO.

Cells were incubated in DMEM containing 1 mmol/L HX with or without XO at the concentrations indicated for 1 to 3 hours. Experiments were performed in the absence of XO/HX after a 45-minute equilibration (for IP$_3$, measurements, 15 minutes) of the cells in normal DMEM. The percentage of XO-treated cells responding to agonist stimulation was comparable to that of cells treated with HX alone. Likewise, XO up to 1000 µmol/L did not affect cell viability or size, as determined by trypan blue incorporation (viability only) and the Schärfe cell counter (viability and cell size, Casy-1).

**IP$_3$ Formation**

IP$_3$ was determined by using a customized radioactive binding assay (Biotrak, Amersham International) as described previously.\textsuperscript{26} In brief, endothelial cells were cultured to confluence in 6-well plates.
Before experimentation, the cells were washed twice with HEPES-buffered solution (plus 2.5 mmol/L CaCl$_2$) and equilibrated at 37°C in 1 mL of the salt solution. After 15 minutes, the compound to be tested was added at a dilution of 1:100. After a 30-second incubation, the experiment was stopped by the addition of 200 µL of 20% chilled HClO$_4$. After 20 minutes on ice, the pH of the supernatant was adjusted to 7.5 by adding KOH. After 15 minutes at 4°C, the samples were centrifuged for 15 minutes at 2000g, and the resulting supernatant was used for determination of IP$_3$ content with the radioactive binding assay.

**Microsomal Cytochrome P450 Monooxygenase (CYP450 MO)**

Microsomal CYP450 MO was measured as previously described. In brief, cells were suspended in intracellular-like buffer containing (in mmol/L) 150 KCl, 10 MgCl$_2$, and 50 Tris, with pH adjusted to 7.5. 1-Ethoxyxypine-3,6,8-tris(dimethylsulfoxonamide) (25 µmol/L) was added under constant stirring, and cells were permeabilized with 1 mg/mL saponin in the presence of an NADPH-regenerating system (25 IU isocitric dehydrogenase [NADP$^+$], 8 mmol/L Dl-isocitric acid, and 1 mmol/L NADP$^+$). As shown recently, enzyme activity was further enhanced by depletion of intracellular Ca$^{2+}$ stores with thapsigustin (TG; 2 µmol/L). Activity of microsomal CYP450 MO was recorded at 495 nm excitation and 550 nm emission and was calculated by using a standard calibration curve.

**Membrane Potential**

Variations in membrane potential were measured by using DiBAC$_4$(5) as previously described. In brief, cultured endothelial cells were suspended in a buffer (9.3×10$^5$ cells/mL) containing (in mmol/L) choline chloride 145, KCl 5, HEPES-free acid 10, and MgCl$_2$, 1, adjusted with KOH to pH 7.4. Under constant stirring, DiBAC$_4$(5) (1 µmol/L) was added. After an equilibration period, the compound to be tested was added. Membrane potential was monitored at 590 nm excitation and 616 nm emission. Calibration of the DiBAC$_4$(5) signal was performed by cumulatively adding KCl (final concentration, 5 to 70 mmol/L) to the solution in the presence of gramicidin D (800 mmol/mL) as described recently. The membrane potential for each KCl concentration was calculated according Vieira et al and was correlated with the fluorescence readings. Resting potential for each KCl concentration was expected to be −34 mV, owing to the known problems for estimation of absolute membrane potentials with the potentially sensitive fluorescent probes. All data were expressed as changes in millivolts from resting potentials.

**TK Activity**

Endothelial TK activity was measured by using a customized photometric protein TK assay kit from Calbiochem-Novabiochem International as previously described. In brief, cell lysates were obtained by sonication of the cell suspension on ice in a buffer containing (in mmol/L) Tris 20, NaCl 50, EDTA 1, EGTA 1, PMSF 0.2, Na$_2$VO$_3$ 0.2, and mercaptoethanol 5; 1 µg/mL pepstatin; and 0.5 µg/mL leupeptin, with the pH adjusted to 7.4. Mg$^{2+}$ and ATP were added to cell extract aliquots, and the mixture was incubated in the absence or presence of XO/HX as indicated. Phosphorylation of an immobilized substrate was determined by a horseradish peroxidase–labeled phosphotyrosine-specific antibody, and the conversion of tetramethylbenzidine as a substrate of horseradish peroxidase was monitored in a plate-reader photometer at 450 nm.

**Membrane Viscosity**

Membrane viscosity was measured by using a technique based on the depolarization of fluorescence light emitted from TMA-DPH. In brief, cultured endothelial cells were washed twice and harvested by enzymatic digestion (trypsin), centrifuged, resuspended in HEPES buffer (see above), and placed in a thermostatically controlled cuvette in a Hitachi F-2000 spectrofluorometer equipped with polarizers at the excitation site and an analyzer at the emission site. Before the addition of the dye (TMA-DPH, 20 µmol/L), autofluorescence was monitored at each setting of the polarizer and analyzer.

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**Figure 2.** Production of superoxide anions by XO in the presence of HX under experimental conditions. In phenol red-free DMEM-like solution containing 1 mmol/L HX, superoxide anions were produced by 150 µU/mL XO and monitored by reduction of ferricytochrome c (10 µmol/L) at 550 nm in the absence or presence of 476 U/mL SOD. Amount of superoxide anion production was calculated by differences in absorption in the sample with and without SOD as measured by molar extinction coefficient. Linear equation for correlation of time versus superoxide anion production is $y = 0.0352x + 0.08028 (R = 0.91, n = 15)$. After addition of TMA-DPH, cells were equilibrated at 37°C in the dark. Fluorescence intensity was monitored at 355 nm excitation and 450 nm emission at settings $I_a$ (both polarizer and analyzer in the vertical position) and $I_h$ (polarizer in the vertical position and analyzer in the horizontal position). After subtraction of autofluorescence at each setting, membrane viscosity was calculated according to the following equation: $h = [(I_a/I_h) - 1]/[0.73 - 0.27(I_a/I_h)]$, where $h$ is the viscosity expressed in poises.

Additional studies were performed in cell monolayers. Endothelial cells were grown on glass coverslips (1-cm diameter) for ~16 hours. For the experiments, the coverslip was mounted in the cuvette at an angle of 60°. The experimental procedure was identical to that mentioned above.

**Intracellular pH**

Intracellular pH was measured as previously described. In brief, endothelial cells were loaded in DMEM for 45 minutes with 10 µmol/L SNARF-1/AM in the dark. An in situ calibration after each experiment was performed in the presence of 10 µmol/L nigericin for the calculation of pH.

**Statistics**

All data represent the mean±SEM. Experiments were performed in triplicate with at least 3 different cell preparations. Data evaluation was performed by ANOVA. Differences, estimated by Scheffe’s F test, were considered statistically significant at $P<0.05$.

**Results**

**Superoxide Anion Generation**

The time-dependent generation of superoxide anions induced by the XO/HX mixture (150 µU/mL and 1 mmol/L, respectively) is illustrated in Figure 2. Over 60 minutes, the rate of production of superoxide anions by this system was 35.2±0.7 mmol/(L×min) (∼15). By comparison, this rate was 10-fold higher than that for the spontaneous secretion of superoxide anions by cultured endothelial cells, as determined previously. Furthermore, the increased secretion of superoxide anions induced by prolonged exposure of endothelial cells to high concentrations of $\alpha$-glucose was 3-fold less than that generated by the mixture of 150 µU/mL XO and 1 mmol/L HX. However, to adopt the latter conditions for this study, we reasoned that levels of superoxide anions in the superna-
When endothelial cells were stimulated with bradykinin (100 nmol/L) in the presence of 2.5 mmol/L extracellular Ca\(^{2+}\), both the initial spike and the plateau phase, which remained constant for at least 10 minutes, were enhanced in cells pretreated for 1 hour with XO/HX (150 μU/mL and 1 mmol/L, respectively) by 63% and 84%, respectively (data not shown). In agreement with our findings that pretreatment with XO/HX did not affect basal endothelial Ca\(^{2+}\) levels, preincubation with XO/HX (150 μU/mL and 1 mmol/L) for 1 hour did not affect basal intracellular pH (control, 7.53±0.13; with XO/HX, 7.41±0.09; n=7; NS versus control).

Figure 4A and 4B illustrates the time dependence of the enhancement of Ca\(^{2+}\) signaling induced by preincubating the cells with XO/HX. After 30 minutes, a slight increase in Ca\(^{2+}\) signaling was detectable, and a 1-hour preincubation showed the maximal effect, which was not enhanced further by incubation durations up to 3 hours (data not shown). Conversely, endothelial Ca\(^{2+}\) signaling was restored 12 hours after removal of XO/HX after a 1-hour treatment.

The concentration dependence of XO in the presence of 1 mmol/L HX was assessed on bradykinin-stimulated capacitative Ca\(^{2+}\) entry more precisely, Mn\(^{2+}\) quench studies were performed (Figure 4D). In agreement with the results on capacitative Ca\(^{2+}\) entry, Mn\(^{2+}\) quench in response to stimulation with 100 nmol/L bradykinin was significantly enhanced in cells preincubated with 150 μU/mL XO in the presence of 1 mmol/L HX for 1 hour (Figure 4D). Coincubation with SOD completely prevented the effect of the XO/HX treatment on the bradykinin-induced Mn\(^{2+}\) quench (data not shown).

In contrast to SOD, coincubation with the TK inhibitor tyrphostin A48 (714 nmol/L) failed to affect XO/HX-mediated alterations in endothelial Ca\(^{2+}\) signaling in response to 100 nmol/L bradykinin (Figure 5). In agreement with these findings, coincubation with 5 μmol/L erbstatin during preincubation with 150 μU/mL XO in the presence of 1 mmol/L HX also failed to increase endothelial Ca\(^{2+}\) signaling in response to 100 nmol/L bradykinin (data not shown).

Because agonist-induced Ca\(^{2+}\) release was shown to be due to the formation of IP\(_3\), the effect of preincubation with the superoxide anion–generating system on endothelial IP\(_3\) formation was studied (Figure 6). A 1-hour preincubation with 150 μU/mL XO in the presence of HX had no effect on basal IP\(_3\) levels, whereas IP\(_3\) formation due to a 30-second stimulation with 100 nmol/L bradykinin was increased significantly (Figure 6).

**IP\(_3\)-Independent Capacitative Ca\(^{2+}\) Entry**

Endothelial capacitative Ca\(^{2+}\) entry depends on the depletion of IP\(_3\)-sensitive Ca\(^{2+}\) stores. To investigate whether the effect of superoxide anions on endothelial Ca\(^{2+}\) entry was due only to increased Ca\(^{2+}\) store depletion by IP\(_3\), the effect of XO/HX treatment on endothelial Ca\(^{2+}\) signaling induced by

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**Figure 3.** Effect of exposure of porcine aortic endothelial cells to superoxide anion on bradykinin (Bk)- (A) and ATP- (B) stimulated intracellular Ca\(^{2+}\) release and capacitative Ca\(^{2+}\) entry. Cultured cells were preincubated in DMEM with 1 mmol/L HX for 1 hour without (open symbols) or with (closed symbols) 150 μU/mL XO and with 150 μU/mL XO and 250 U/mL SOD (+SOD; B only). Experiments were performed in cell suspension without HX and XO after an equilibration time of 45 minutes. As indicated by horizontal lines, 100 μmol/L bradykinin, 100 μmol/L ATP, or solvent (no ATP) was added, followed by addition of 2.5 mmol/L CaCl\(_2\). Intracellular Ca\(^{2+}\) was expressed as the ratio of fura 2 fluorescence at 340 and 380 nm excitation at 510 nm emission, mean±SEM (A, n=17; B, n=28 for ATP/XO; n=16 for ATP; and n=8 for ATP/XO+SOD, no ATP, and no ATP/XO). The effects of XO/HX pretreatment on the mobilization of Ca\(^{2+}\) induced by the endothelial agonists bradykinin (Figure 3A) and ATP (Figure 3B) were examined. For this series of experiments, the cells were first incubated with or without 150 μU/mL XO for 1 hour in DMEM containing 1 mmol/L HX. The effects of the agonists were initiated in nominally Ca\(^{2+}\)-free solution to assess the release of Ca\(^{2+}\) from intracellular stores. Afterward, in the continuous presence of the agonist (bradykinin or ATP), 2.5 mmol/L Ca\(^{2+}\) was added to the bath to monitor the elevation in cytosolic Ca\(^{2+}\) due to an influx from the extracellular medium. As shown in Figure 3A and 3B, both the release of intracellular Ca\(^{2+}\) and the influx stimulated by bradykinin and ATP were significantly enhanced in cells treated with XO/HX. By contrast, the magnitude of Ca\(^{2+}\) entry obtained after addition of 2.5 mmol/L Ca\(^{2+}\) in the absence of an agonist was not changed significantly by the XO/HX treatment (Figure 3B). Coincubation with SOD (250 U/mL) during exposure to the anion-generating system normalized endothelial Ca\(^{2+}\) signaling to ATP (Figure 3B), whereas SOD did not affect endothelial Ca\(^{2+}\) signaling in control cells (no XO present during preincubation procedure; data not shown). In contrast to SOD, coinocubation with cycloheximide during the XO/HX preincubation period had no effect on the enhancement of endothelial Ca\(^{2+}\) signaling by superoxide anion (data not shown). There was no detectable effect on either ATP-induced Ca\(^{2+}\) release or capacitative Ca\(^{2+}\) entry when the cells were preincubated with 150 μU/mL XO in the absence of HX (data not shown).
the IP₃-independent Ca²⁺ mobilizers 2,5-di-(tert-butyl)-1,4-benzohydroquinone (BHQ; Figure 7A) and thapsigargin (TG; Figure 7B) was studied. Whereas pretreatment with XO/HX had no effect on intracellular Ca²⁺ release induced by either BHQ (Figure 7A) or TG (Figure 7B), Ca²⁺ entry after stimulation with either BHQ (Figure 7A) or TG (Figure 7B) was significantly enhanced in cells pretreated with XO/HX.

**Microsomal CYP450 MO Activity**

The importance of microsomal CYP450 MO activity for endothelial Ca²⁺ entry and membrane potential has been demonstrated in endothelial cells. Thus, the effect of treatment with superoxide anions on CYP450 MO activity in endothelial cells was tested (Figure 8A). Endothelial cells were preincubated for 1 hour with 150 μU/mL XO in the presence of 1 mmol/L HX, and CYP450 MO was activated by depletion of IP₃-sensitive stores with 2 μmol/L TG. In cells preincubated with XO/HX, CYP450 MO activity was augmented by 64% (Figure 8A). Coincubation with SOD (450 U/mL) prevented the effect of XO/HX on endothelial CYP450 MO (data not shown). Inhibition of endothelial NO synthase with N⁵-nitro-L-arginine (300 μmol/L) had no effect on the influence of XO/HX on endothelial CYP450 MO activity (Figure 8A).

**Membrane Hyperpolarization**

In addition to CYP450 MO, membrane hyperpolarization is known to play a crucial role in endothelial capacitative Ca²⁺ entry. Thus, the effect of preincubation with the superoxide anion–generating system XO/HX on TG-induced membrane hyperpolarization was studied (Figure 8B). Although TG-induced intracellular Ca²⁺ release was not altered by XO/HX (see above), the latter augmented membrane hyperpolarization by 2 μmol/L TG in nominal Ca²⁺-free solution by 48% (Figure 8B). Membrane hyperpolarization to TG in untreated as well as XO/HX-pretreated endothelial cells was abolished in the presence of 10 mmol/L tetrabutylammonium chloride (data not shown).

**TK**

Preincubation with XO in the presence of 1 mmol/L HX yielded increased Ca²⁺-activated TK activity in cultured endothelial cells. Use of 150 and 300 μU/mL XO during the preincubation period of 45 minutes increased endothelial TK activity by 97% and 156%, respectively (Figure 9A). The TK inhibitors erbastatin (100 μmol/L) and tyrphostin A48 (714 nmol/L) strongly inhibited all TK activity in lysates of untreated and XO/HX-pretreated cells (control, 4.59±0.60 and 3.33±1.31 U/mg protein; XO/HX, 3.66±0.76 and 4.73±1.27 U/mg protein; n=3).

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**Figure 4.** Effect of exposure to superoxide anion on bradykinin- and ATP-initiated Ca²⁺ signaling in cultured endothelial cells. Cells were preincubated with XO/HX in DMEM as indicated. After a 45-minute equilibration in DMEM without HX and XO, experiments were performed with suspended cells as indicated. A, Time course of exposure to 150 μU/mL XO in the presence of 1 mmol/L HX on bradykinin- (100 nmol/L) initiated signaling (n=33). B, Time course of exposure to 150 μU/mL XO in the presence of 1 mmol/L HX on endothelial Ca²⁺ signaling stimulated by 100 μmol/L ATP (n=12). C, Concentration-response relationship of various XO activities during 1-hour preincubation in DMEM containing 1 mmol/L HX on bradykinin- (100 nmol/L) stimulated intracellular Ca²⁺ release (open symbols) and Ca²⁺ entry (filled symbols) (n=8). D, Effect of 1-hour preincubation with 150 μU/mL XO in the presence of 1 mmol/L HX on bradykinin-stimulated Mn²⁺ entry, expressed as Mn²⁺-mediated quenching of fura 2 fluorescence in response to the addition of 100 nmol/L bradykinin. Each point represents mean±SEM (n=10).
Membrane Viscosity

Exposure of endothelial cells to 150 μU/mL XO in the presence of 1 mmol/L HX significantly reduced membrane viscosity (Figure 9B). This effect was completely prevented when 300 U/mL SOD was present during XO/HX treatment (Figure 9B) or when no HX was present (data not shown).

To investigate whether increased membrane stiffness might result in an effect opposite to increased membrane fluidity, endothelial cells were loaded with cholesterol. As shown in Figure 10A, incubating the cells for 30 or 60 minutes with 250 μmol/L cholesterol (from 25 mmol/L stock in ethanol) yielded increases in membrane viscosity of 113% and 135%, respectively. Moreover, cholesterol loading for 30 or 60 minutes attenuated bradykinin-induced intracellular Ca²⁺ release and capacitative Ca²⁺ entry by 26% and 47% or 63% and 68%, respectively (Figure 10B).

The correlation between membrane viscosity and agonist-induced intracellular Ca²⁺ release (100 nmol/L bradykinin)
was negative (Figure 11A). Endothelial cells were incubated for 20 or 60 minutes in DMEM containing 150 μU/mL XO in the presence of 1 mmol/L HX to decrease cell membrane viscosity. To increase cell membrane viscosity, cells were exposed to 25 or 250 μmol/L cholesterol for 30 or 60 minutes. Similar results were obtained when endothelial capacitative Ca^{2+} entry activity was correlated with membrane viscosity (data not shown). In agreement with these findings on bradykinin-induced Ca^{2+} signaling, alterations of TG-induced hyperpolarization were negatively correlated with changes in membrane viscosity (Figure 11B).

### Discussion

Among the oxygen free radicals produced in the vascular wall, only peroxides (H_{2}O_{2}, 2,5-butyldihydroperoxide) have been intensively studied for their effect on endothelial Ca^{2+} signaling. Thus, peroxides in concentrations >30 μmol/L attenuate agonist-induced intracellular Ca^{2+} release and capacitative Ca^{2+} entry in a time-dependent manner. However, prolonged incubation with peroxides initiates Ca^{2+} entry per se. In contrast, we demonstrated that hyperglycemia enhances endothelial Ca^{2+} signaling owing to the formation of superoxide anions. In agreement with this previous report, the current study shows that preincubation of endothelial cells with a superoxide anion–generating system mimics the effects of hyperglycemia. Because XO had no effect on endothelial Ca^{2+} signaling in the absence of HX, the changes reported herein are due to the formation of superoxide anions. This conclusion is further supported by abolition of the effect of XO/HX in the presence of SOD.

Tan et al showed that oxygen-derived free radicals stimulate adenylyl cyclase via TK activation in A10 cells. In endothelial cells, the TK inhibitor tyrphostin A48 had no effect on superoxide anion–mediated augmentation of agonist-initiated Ca^{2+} signaling. These findings do not support the involvement of tyrphostin A48-sensitive TKs in XO/HX-evoked changes in Ca^{2+} signaling. However, because it is possible that 1 single TK inhibitor is not able to prevent all TK activity, the involvement of TK in XO/HX-mediated changes in Ca^{2+} signaling cannot be excluded. In spite of this, superoxide anions actually enhanced endothelial TK activity. Fleming and coworkers have suggested that TK activity is involved in endothelial capacitative Ca^{2+} entry. On the contrary, Vostal and Shafer found that capacitative Ca^{2+} entry was independent of any TK activity. Although our results show that an increase in tyrphostin A48-sensitive TK activity may not be involved in the enhancement of capacitative Ca^{2+} entry after XO/HX treatment, these findings do not give any indication whether TKs are involved in endothelial Ca^{2+} signaling. Moreover, it needs to be investigated whether the observed increase in TK activity by XO/HX might influence other TK-mediated cellular functions, like shear stress–activated NO formation.

The concentration-response relationship of superoxide anions with respect to amplification of intracellular Ca^{2+} release was identical to that for capacitative Ca^{2+}/Mn^{2+} entry. The augmented intracellular Ca^{2+} release in response to the IP_{3}-generating compounds bradykinin and ATP after superoxide anion treatment might be due to enhanced IP_{3} formation on addition of bradykinin to XO/HX-treated cells. Similar findings were described in airway epithelium. These data indicate that the superoxide anion might affect either phospholipase C activity or receptor–G protein–phospholipase C coupling. Radical-mediated changes in G protein activity/coupling have been reported for NO on G, proteins in neurons and for oxyradicals for isoproterenol receptor–

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**Figure 9.** Effect of exposure of endothelial cells to XO (150 and 300 μU/mL) in the presence of 1 mmol/L HX on TK activity (A) and membrane viscosity (B). A, TK was measured as described in Methods and is expressed as U/mg protein. Each column represents mean±SEM (n=4). \( \ast P<0.05 \) versus control. Differences between 150 and 300 μU/mL were not statistically different. B, Cultured endothelial cells were preincubated for 1 hour in DMEM containing 1 mmol/L HX in the absence of XO (Control), in the presence of 150 μU/mL XO (XO/HX), or in the presence of 150 μU/mL XO and 300 μU/mL SOD (XO/HX+SOD). After a 45-minute equilibration, membrane viscosity was monitored in suspended cells by using 20 μmol/L TMA-DPH as described in Methods. Membrane viscosity is expressed in poises, mean±SEM (n=9). \( \ast P<0.05 \) versus cells not preincubated with XO. Similar results were obtained in cell monolayer experiments.

**Figure 10.** Effect of exposure to cholesterol on endothelial membrane viscosity (A) and bradykinin-initiated Ca^{2+} signaling (B). Cultured endothelial cells were exposed in DMEM with 1% ethanol (solvent, control) or with 250 μU/mL cholesterol (from 25 mmol/L stock solution in ethanol, cholesterol) for 30 and 60 minutes. No difference was found between control cells after 30- and 60-minute exposures. As indicated by horizontal lines, endothelial Ca^{2+} signaling was evoked in suspended cells by 100 mmol/L bradykinin in the nominal absence of extracellular Ca^{2+}, followed by addition of 2.5 mmol/L CaCl_{2}. Columns and points represent mean±SEM (A, n=6; B, n=8), \( \ast P<0.05 \) versus cells not preincubated with cholesterol. Similar results were obtained in cell monolayer experiments (n=7).
coupled G, and G, proteins in ischemic/reperfused hearts. Additional studies are needed to clarify whether the reported changes in IP, production by superoxide anion preincubation are due to changes in G protein activity/coupling or phospholipase C activity.

Because intracellular Ca entry store depletion regulates the activity of capacitative Ca entry pathways in endothelial cells, one might speculate that the effect of superoxide anions on capacitative Ca entry pools. Our finding that BHQ- and TG-induced capacitative Ca entry was also enhanced in XO/HX-treated cells while intracellular Ca release remained unchanged after superoxide anion treatment indicates that in addition to its effect on IP, formation, XO/HX directly affects the mechanism(s) involved in capacitative Ca entry. The mechanisms of capacitative Ca entry regulation in endothelial cells are still poorly understood (Figure 1). Although membrane hyperpolarization does not open the capacitative Ca entry pathway itself, it provides the driving force for Ca entry to the cell. Because CYP450 MO activity was enhanced by preincubation with XO/HX, one might expect that the enhanced formation of EETs results in augmentation of endothelial capacitative Ca entry. Such enhancement of capacitative Ca entry by enhanced membrane hyperpolarization in endothelial cells was described for the KATP channel opener Hoe-234 and for KCa channels activated by cAMP. In addition, direct effects of EETs on the capacitative Ca channel pathway itself are also possible.

In agreement with our findings, in atherosclerotic vessels agonist-induced (ie, Ca-mediated) production of NO is augmented, although reduced NO-mediated relaxation is observed owing to degradation of NO by oxygen free radicals. It needs to be investigated whether superoxide anions may serve as mediators of a compensatory adaptation of the endothelium in states with increased oxygen radical production (eg, hypercholesterolemia or hyperglycemia) to maintain the Ca-mediated release of vasodilator mediators, although the bioactivity of NO is diminished owing to its degradation by free radicals.

Because cycloheximide had no effect on superoxide anion-initiated changes in endothelial Ca signaling, the involvement of nuclear responses via superoxide anion-initiated transcription factors (eg, nuclear factor-kB) followed by changes in gene expression resulting in an altered Ca signaling cascade might be excluded in the short-term model used in this study.

It is possible that each mechanism described might have specific superoxide anion–sensitive elements or that superoxide anions affect 1 parameter common to all mechanisms studied. Such a parameter common to all phenomena studied could be membrane viscosity. In agreement with reports from the literature, incubation with XO/HX decreased membrane viscosity in endothelial cells. This mechanism was prevented by SOD. In contrast, cholesterol loading of endothelial cell membranes yielded an increase in membrane viscosity, which was associated with a decrease in bradykinin-initiated Ca signaling (ie, intracellular Ca release and capacitative Ca entry) and TG-induced membrane hyperpolarization. In the current work, a negative correlation between membrane viscosity and agonist-stimulated intracellular Ca release and TG-induced hyperpolarization could be demonstrated, suggesting that the superoxide anion–mediated decrease in membrane viscosity might have significant impact on endothelial Ca signaling mechanisms. In agreement with our findings, increased phospholipase C activity in response to agonist by decreased membrane viscosity was shown in the mouse brain, heart, and liver and in rat ventricular myocytes. Similar to changes in plasmalemmal enzyme/channel activity by modulations in cell membrane viscosity, microsomal enzyme activities are altered by changes in fluidity of the microsomal membrane. Such increased microsomal membrane fluidity might explain the enhanced microsomal CYP450 MO activity after exposure of endothelial cells to XO/HX presented here. Other than membrane-bound enzymes, changes in membrane viscosity have also been demonstrated to modulate Na+, K+, and Ca channel activity. Additional studies are necessary to clarify whether the observed increase in membrane hyperpolarization in XO/HX-pretreated cells is due to direct modulation of membrane channel activity (eg, KCa channels) or an increase in activity of microsomal CYP450 MOs.

In this study, an additional role for superoxide anions beside their involvement in the development of cellular/vascular dysfunction is proposed. We have demonstrated that superoxide anions modulate the mechanisms involved in
endothelial Ca^{2+} signaling shown in Figure 1 (phospholipase C, membrane hyperpolarization, TK, and CYP450 MO). These effects of superoxide anions might result in enhanced NO biosynthesis during enhanced superoxide formation, whereas on the other hand, superoxide anions attenuate NO bioactivity. Additional studies are necessary to understand under which circumstances the deleterious properties of superoxide anions overcome the beneficial effects reported here.

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