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-benzohydroquinone 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 modulated 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 Vasc Biol. 1998;18:1470-1479.)

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

The generation of free radicals has been proposed to play a crucial role in the development of endothelial dysfunction under certain pathological conditions, including diabetes mellitus, hypercholesterolemia, reoxygenation/reperfusion injury, and septic shock. Among the oxygen free radicals, superoxide anions have been demonstrated to be involved in hyperglycemia-induced impairment of endothelium-dependent relaxation, endothelial cell–mediated LDL oxidation, decreased endothelial cell proliferation initiated by hyperglycemia, and upregulation of adhesion molecules. In addition, superoxide anions neutralize endothelium-derived NO\(_x\) resulting in diminished endothelium-dependent relaxation.

Few studies have examined the effect of superoxide anions on endothelial signal transduction mechanisms. Franceschi et al showed that superoxide anions produced by the xanthine oxidase/hypoxanthine (XO/HX) reaction resulted in short-term membrane hyperpolarization in endothelial cells due to stimulation of Ca\(^{2+}\) entry through a non–voltage-dependent mechanism. In agreement with the findings of increased [Ca\(^{2+}\)]\(_i\), enhanced intracellular prostaglandin I\(_2\) formation after incubation with XO/HX was described in human umbilical vein endothelial cells. In most of these studies, very high concentrations of superoxide anions were used. For example, in coronary artery smooth muscle cells, attenuation of angiotensin II–initiated contraction by such high superoxide anion concentrations (>1 mU/mL XO) did not differ from that induced by H\(_2\)O\(_2\) (>30 μmol/L). We have shown that increased formation of superoxide anion during exposure to a high d-glucose concentration elicits changes in endothelial Ca\(^{2+}\) signaling. However, the superoxide anion levels mediated by high d-glucose treatment, for example, were much lower than those used in the other studies mentioned above while exposure time was prolonged for several hours. Under such conditions, superoxide anion had no short-term effects on resting endothelial [Ca\(^{2+}\)]\(_i\), whereas the mobilization of Ca\(^{2+}\) evoked by bradykinin was enhanced. So far, no detailed studies have been performed to understand how physiological concentrations of superoxide anions affect endothelial Ca\(^{2+}\) signaling. As shown in Figure 1, endothelial cell stimulation with an agonist, such as bradykinin or ATP, results in G protein–mediated activation of phospholipase C...
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$_3$,\textsuperscript{18} cytochrome P450 epoxyxygenase,\textsuperscript{19,20} membrane 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$_4$(5)) and SNARF acetoxymethyl ester (SNARF-1/AM) were from Molecular Probes Inc. Petri dishes were from Corning, and 6- and 24-well plates 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-DMEM containing 3% FCS. Only cells from passage 1 or 2 were used for experiments (~10 to 14 days in culture).

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

Free [Ca$^{2+}$]$_i$ was determined in porcine aortic endothelial cells in suspension or monolayer as indicated by the fura 2 technique as previously described.\textsuperscript{23} In brief, cells were incubated with DMEM containing 2 $\mu$mol/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 $\mu$mol/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{23,24} 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{15} and the general uncertainties of the calibration techniques,\textsuperscript{25} [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 without superoxide anion measurements. The generation of superoxide anions was determined as the difference in the reduction of ferricytochrome c (10 $\mu$mol/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 absorbance 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{26} Production of superoxide anions was controlled for each cell pretreatment procedure in phenol red–free DMEM without XO, 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 XO alone. Likewise, XO up to 1000 $\mu$mol/L did not affect cell viability or size, as determined by trypan blue incorporation (viability only) and the Schäfer cell counter (viability and cell size, Casy-1).

**IP$_3$ Formation**

IP$_3$ was determined by using a customized radioisotope 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 CaCl₂) 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 HClO₄. 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 2000 g, and the resulting supernatant was used for determination of IP₃ content with the radioactive binding assay.

**Microsomal Cytochrome P450 Monoxygenase (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 MgCl₂, and 50 Tris, with pH adjusted to 7.5. 1-Ethoxyxynpye-3,6,8-tris-(dimethylsulfonamide) (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 Ca²⁺ stores with thapsigargin (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 DiBAC₄(5) as previously described. In brief, cultured endothelial cells were suspended in a buffer (≈9.3×10⁵ cells/mL) containing (in mmol/L) choline chloride 145, KCl 5, HEPES-free acid 10, and MgCl₂ 1, adjusted with KOH to pH 7.4. Under constant stirring, DiBAC₄(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 DiBAC₄(5) signal was performed by cumulatively adding KCl (final concentration, 5 to 70 mmol/L) to the solution in the presence of thapsigargin (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.

**TK Activity**

Endothelial TK activity was measured by using a customized spectrophotometric 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, Na₃VO₄ 0.2 , and mercaptoethanol 5; 1 μg/mL leupeptin; and 0.5 μg/mL aprotinin. After sonication of the cell suspension, 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 determined by a horseradish peroxidase–labeled antibody. The time-dependent generation of superoxide anions induced by prolonged exposure of endothelial cells to high concentrations of n-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 superno-
When endothelial cells were stimulated with bradykinin (100 nmol/L) in the presence of 2.5 mmol/L extracellular Ca²⁺, 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 Ca²⁺ 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 Ca²⁺ signaling induced by preincubating the cells with XO/HX. After 30 minutes, a slight increase in Ca²⁺ 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 Ca²⁺ 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-induced Ca²⁺ signaling, according to the protocols shown in Figure 4A and 4B (Figure 4C). Threshold potentiation could be observed with a concentration of XO as low as 100 μU/mL. To demonstrate the effect of superoxide anions on bradykinin-stimulated capacitative Ca²⁺ entry more precisely, Mn²⁺ quench studies were performed (Figure 4D). In agreement with the results on capacitative Ca²⁺ entry, Mn²⁺ 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 Mn²⁺ quench (data not shown).

In contrast to SOD, coinocubation with the TK inhibitor tyrphostin A48 (714 nmol/L) failed to affect XO/HX-mediated alterations in endothelial Ca²⁺ signaling in response to 100 nmol/L bradykinin (Figure 5). In agreement with these findings, coinocubation 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 Ca²⁺ signaling in response to 100 nmol/L bradykinin (data not shown).

Because agonist-induced Ca²⁺ release was shown to be due to the formation of IP₃, the effect of preincubation with the superoxide anion–generating system on endothelial IP₃ formation was studied (Figure 6). A 1-hour preincubation with 150 μU/mL XO in the presence of HX had no effect on basal IP₃ levels, whereas IP₃ formation due to a 30-second stimulation with 100 nmol/L bradykinin was increased significantly (Figure 6).

**IP₃-Independent Capacitative Ca²⁺ Entry**

Endothelial capacitative Ca²⁺ entry depends on the depletion of IP₃-sensitive Ca²⁺ stores. To investigate whether the effect of superoxide anions on endothelial Ca²⁺ entry was due only to increased Ca²⁺ store depletion by IP₃, the effect of XO/HX treatment on endothelial Ca²⁺ 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 Ca²⁺ release and capacitative Ca²⁺ 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 nmol/L bradykinin, 100 μmol/L ATP, or solvent (no ATP) was added, followed by addition of 2.5 mmol/L CaCl₂. Intracellular Ca²⁺ 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 IP$_3$-independent Ca$^{2+}$ 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 Ca$^{2+}$ release induced by either BHQ (Figure 7A) or TG (Figure 7B), Ca$^{2+}$ 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 Ca$^{2+}$ entry and membrane potential has been demonstrated in endothelial cells.$^{19,20}$ 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 IP$_3$-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 N$^\mathrm{\text{-}}$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 Ca$^{2+}$ entry.$^{20,21}$ 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 Ca$^{2+}$ release was not altered by XO/HX (see above), the latter augmented membrane hyperpolarization by 2 μmol/L TG in nominal Ca$^{2+}$-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 Ca$^{2+}$-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). Coincubation with erbrstatin (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±1.06 and 3.33±1.31 U/mg protein; XO/HX, 3.66±0.76 and 4.73±1.27 U/mg protein; n=3).
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 studied. Coincubation with TK inhibitor tyrphostin A48 during preincubation with superoxide anion had no effect on changes in bradykinin-induced Ca2+ signaling. Cells were incubated in DMEM containing 1 mmol/L HX for 1 hour (control), plus 714 nmol/L tyrphostin A48 (control + Tyr.A48), plus 150 μU/mL XO (XO/HX), and plus 714 nmol/L tyrphostin A48 and 150 μU/mL XO (XO/HX + Tyr.A48). After equilibration for 45 minutes in DMEM without both XO/HX and Tyr.A48, cells were resuspended in HEPES buffer in the absence of XO and Tyra.A48. As indicated by horizontal lines, cells were stimulated with 100 nmol/L bradykinin (Bk) followed by 2.5 mmol/L CaCl2. Points represent mean±SEM (n=6).

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Figure 5. Coincubation with TK inhibitor tyrphostin A48 during preincubation with superoxide anion had no effect on changes in bradykinin-induced Ca2+ signaling. Cells were incubated in DMEM containing 1 mmol/L HX for 1 hour (control), plus 714 nmol/L tyrphostin A48 (control + Tyr.A48), plus 150 μU/mL XO (XO/HX), and plus 714 nmol/L tyrphostin A48 and 150 μU/mL XO (XO/HX + Tyr.A48). After equilibration for 45 minutes in DMEM without both XO/HX and Tyr.A48, cells were resuspended in HEPES buffer in the absence of XO and Tyra.A48. As indicated by horizontal lines, cells were stimulated with 100 nmol/L bradykinin (Bk) followed by 2.5 mmol/L CaCl2. Points represent mean±SEM (n=6).

Figure 6. Effect of exposure of endothelial cells to superoxide anion on bradykinin-stimulated formation of IP3. Cultured endothelial cells were exposed for 1 hour in DMEM containing 1 mmol/L HX in the absence (control, open columns) or presence (XO/HX, filled columns) of 150 μU/mL XO. After equilibration for 15 minutes, basal IP3 level and IP3 levels after 30-second stimulation with 100 nmol/L bradykinin were measured by radioactive binding assay. Levels of IP3 are shown as pmol IP3/106 cells, mean±SEM (n=4 to 6). *P<0.05 versus cells not preincubated with XO.

Figure 7. Effect of preincubation of endothelial cells with XO/HX on endothelial Ca2+ signaling initiated by BHQ (A) or TG (B). Cells were exposed for 1 hour in DMEM containing 1 mmol/L HX in the absence (control, open symbols) or presence (XO/HX, filled symbols) of 150 μU/mL XO. After equilibration for 45 minutes in DMEM, suspended cells were stimulated in the nominal absence of extracellular Ca2+ with 15 μmol/L BHQ (A) or 2 μmol/L TG (B), followed by addition of 2.5 mmol/L CaCl2 as indicated by horizontal lines. Endothelial Ca2+ signaling was measured with fura 2 as described in Methods. Each point represent mean±SEM (A, n=16, controls; n=24, HX/XO; B, n=8). Identical results were obtained on single cells (n=12).

Figure 8. Effect of exposure of endothelial cells to XO in the presence of HX on TG-induced microsomal CYP450 MO activity (A) and membrane hyperpolarization (B). Endothelial cells were preincubated with or without N ω-nitro-L-arginine (L-NNA, 300 μmol/L) for 1 hour in DMEM containing 1 mmol/L HX in the absence (control) or presence (XO/HX) of 150 μU/mL XO for 1 hour. After equilibration for 45 minutes in DMEM, effect of 2 μmol/L TG on CYP450 MO activity (A) and membrane potential (B) was measured in cell suspensions. A, CYP450 MO activity was measured in cell suspensions. A, CYP450 MO activity 4 minutes after addition of TG (2 μmol/L) is given as pmol of 1-hydroxypyrene-3,6,8-tris-(dimethylsulfonamide produced per minute by 106 cells, mean±SEM (without L-NNA, n=8; with L-NNA, n=6). *P<0.05 versus cells not preincubated with XO. B, Membrane potential was monitored after 2 minutes of TG stimulation by 1 μmol/L DiBAC4(5) as described in Methods. Changes in membrane potential are expressed in mV, mean±SEM (n=8). *P<0.05 versus cells not preincubated with XO.
Superoxide Anions and Ca\textsuperscript{2+} Signaling

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 measured in cell suspension (B). TK activity was measured as described in Methods and is expressed as U/mg protein. Each column represents mean±SEM (n=4). *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 U/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). *P<0.05 versus cells not preincubated with XO. Similar results were obtained in cell monolayer experiments.

The concentration-response relationship of superoxide anions with respect to amplification of intracellular Ca\textsuperscript{2+} release was identical to that for capacitative Ca\textsuperscript{2+}/Mn\textsuperscript{2+} entry. The augmented intracellular Ca\textsuperscript{2+} release in response to the IP\textsubscript{3}-generating compounds bradykinin and ATP after superoxide anion treatment might be due to enhanced IP\textsubscript{3} formation. However, prolonged incubation with peroxides initiates Ca\textsuperscript{2+} entry per se.

Discussion

Among the oxygen free radicals produced in the vascular wall, only peroxides (H\textsubscript{2}O\textsubscript{2}, 2,5-butyldihydroperoxide) have been intensively studied for their effect on endothelial Ca\textsuperscript{2+} signaling. Thus, peroxides in concentrations >30 μmol/L attenuate agonist-initiated intracellular Ca\textsuperscript{2+} release and capacitative Ca\textsuperscript{2+} entry in a time-dependent manner. However, prolonged incubation with peroxides initiates Ca\textsuperscript{2+} entry per se. In contrast, we demonstrated that hyperglycemia enhances endothelial Ca\textsuperscript{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\textsuperscript{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 adenyl 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\textsuperscript{2+} signaling. These findings do not support the involvement of tyrphostin A48-sensitive TKs in XO/HX-evoked changes in Ca\textsuperscript{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\textsuperscript{2+} signaling cannot be excluded. In spite of this, superoxide anions actually enhanced endothelial TK activity.

The concentration-response relationship of superoxide anions with respect to amplification of intracellular Ca\textsuperscript{2+} release was identical to that for capacitative Ca\textsuperscript{2+}/Mn\textsuperscript{2+} entry. The augmented intracellular Ca\textsuperscript{2+} release in response to the IP\textsubscript{3}-generating compounds bradykinin and ATP after superoxide anion treatment might be due to enhanced IP\textsubscript{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\textsubscript{i} proteins in neurons and for oxynitriles for isoproterenol receptor--.
coupled G, and G\textsubscript{q} proteins in ischemic/reperfused hearts.\textsuperscript{39} Additional studies are needed to clarify whether the reported changes in IP\textsubscript{i} production by superoxide anion preincubation are due to changes in G protein activity/coupling or phospholipase C activity.

Because intracellular Ca\textsuperscript{2+} store depletion regulates the activity of capacitative Ca\textsuperscript{2+} entry pathways in endothelial cells,\textsuperscript{15} one might speculate that the effect of superoxide anions on capacitative Ca\textsuperscript{2+}/Mn\textsuperscript{2+} entry is due to the pronounced depletion of Ca\textsuperscript{2+} pools. Our finding that BHQ- and TG-induced capacitative Ca\textsuperscript{2+} entry was also enhanced in XO/HX-treated cells while intracellular Ca\textsuperscript{2+} release remained unchanged after superoxide anion treatment indicates that in addition to its effect on IP\textsubscript{i} formation, XO/HX directly affects the mechanism(s) involved in capacitative Ca\textsuperscript{2+}/Mn\textsuperscript{2+} entry. The mechanisms of capacitative Ca\textsuperscript{2+} entry regulation in endothelial cells are still poorly understood (Figure 1).\textsuperscript{40,41}

We have provided evidence for the involvement of CYP450 MO–derived arachidonic acid metabolites, the epoxyeicosa trienoic acids (EETs), in autacoid-stimulated capacitative Ca\textsuperscript{2+} entry and membrane hyperpolarization.\textsuperscript{19,20} Although membrane hyperpolarization does not open the capacitative Ca\textsuperscript{2+} entry pathway in endothelial cells, it provides the driving force for Ca\textsuperscript{2+} entry to enter the cell.\textsuperscript{21,42} 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\textsuperscript{2+}/Mn\textsuperscript{2+} entry due to enhanced EET-mediated membrane hyperpolarization. Such enhancement of capacitative Ca\textsuperscript{2+}/Mn\textsuperscript{2+} entry by enhanced membrane hyperpolarization in endothelial cells was described for the K\textsubscript{ATP} channel opener Hoe-234\textsuperscript{43} and for K\textsubscript{Ca} channel activation by cAMP.\textsuperscript{24} In addition, direct effects of EETs on the capacitative Ca\textsuperscript{2+} entry pathway itself are also possible.

In agreement with our findings, in atherosclerotic vessels agonist-induced (ie, Ca\textsuperscript{2+}-mediated) production of NO is augmented,\textsuperscript{45} although reduced NO-mediated relaxation is observed owing to degradation of NO by oxygen free radicals.\textsuperscript{46} 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\textsuperscript{2+}-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\textsuperscript{2+} 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\textsuperscript{2+} 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 fluidity. In agreement with reports from the literature,\textsuperscript{49} 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,\textsuperscript{48} which was associated with a decrease in bradykinin-initiated Ca\textsuperscript{2+} signaling (ie, intracellular Ca\textsuperscript{2+} release and capacitative Ca\textsuperscript{2+} entry) and TG-induced membrane hyperpolarization. In the current work, a negative correlation between membrane viscosity and agonist-stimulated intracellular Ca\textsuperscript{2+} 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\textsuperscript{2+} 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\textsuperscript{49} and in rat ventricular myocytes.\textsuperscript{50} 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.\textsuperscript{51} 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\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} channel activity.\textsuperscript{52,53} 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, K\textsubscript{Ca} 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,\(^4\)\(^5\) whereas on the other hand, superoxide anions attenuate NO bioactivity.\(^4\)\(^5\)\(^4\) 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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