Superoxide anion impairs contractility in cultured aortic smooth muscle cells

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OXIDATIVE STRESS is known to cause various vascular diseases, such as hypertension (16), atherosclerosis, coronary disease (5), and diabetic vascular complications (7). Although it has been reported (14) that superoxide anion (O2−) generated by xanthine plus xanthine oxidase (X XO) on the intracellular Ca2+ concentration ([Ca2+]i) and muscle contractility in cultured bovine aortic smooth muscle cells (BASM C). Cells were grown on collagen-coated dish for the measurement of [Ca2+]i. Pretreatment with X XO impaired ATP-induced Ca2+ transient and Ca2+ release-activated Ca2+ entry (CRAC) after thapsigargin-induced store depletion, both of which were reversed by superoxide dismutase (SOD). In contrast, Ca2+ transients induced by high-K+ solution and Ca2+ ionophore A-23187 were not affected by X XO. BASMC-embedded collagen gel lattice, which was pretreated with xanthine alone, showed contraction in response to ATP, thapsigargin, high-K+ solution, and A-23187. Pretreatment of the gel with X XO impaired gel contraction not only by ATP and thapsigargin, but also by high-K+ solution and A-23187. The X XO-treated gel showed normal contraction; however, when SOD was present during the pretreatment period. These results indicate that O2− attenuates smooth muscle contraction by impairing CRAC, ATP-induced Ca2+ transient, and Ca2+ sensitivity in BASMC.

The obtained results indicate that O2− attenuates smooth muscle contraction by impairing CRAC, the ATP-induced Ca2+ transient, and Ca2+ sensitivity of contractile machinery in BASMC.

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MATERIALS AND METHODS

Cell culture. Thoracic aorta from a 1-yr-old calf was obtained from a local slaughterhouse. BASMC were then cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum by the explant method, as previously described (3). Cells grown in confluent were harvested by trypsin digestion and stored at −80°C after a one-step subculture. Smooth muscle α-actin was stained to confirm that the cells retained the nature of smooth muscle cells (not shown). Cells were seeded on a collagen-coated coverslip for the measurement of \([\text{Ca}^{2+}]_i\) and were embedded in collagen gel lattice for the gel contraction assay, as described below.

Measurement of \([\text{Ca}^{2+}]_i\). \([\text{Ca}^{2+}]_i\) was measured by using an Attofluor digital fluorescence microscopy system (Atto Instruments; Rockville, MD). Cells were seeded on coverslip coated with type I collagen (Nitta Gelatin; Osaka, Japan) and cultured for 3–5 days before use. Cells were spread with a fibroblast-like appearance on an uncoated coverslip (Fig. 1A,a), although most cells showed a spindle-like shape on a collagen-coated coverslip (Fig. 1A,b). Cells were loaded with fura 2-acetoxymethyl ester (Dojindo; Kumamoto, Japan) as previously described (22). Fura 2 was excited at two alternative wavelengths (340 and 380 nm) and the fura 2 fluorescence images emitted at 510-nm wavelength were recorded into a rewritable optical disk recorder (LQ-4100A, Panasonic; Osaka, Japan) at a rate of 1 Hz. The fluorescent intensities of these wavelengths (F_340 and F_380, respectively) were obtained from these images to calculate the fluorescence ratio (R), F_340/F_380. R was then converted into the apparent \([\text{Ca}^{2+}]_i\) with the use of in vitro calibration, as described previously (23). Therefore, it should be noted that the calculated \([\text{Ca}^{2+}]_i\) is not the actual in vivo value, and, furthermore, its temporal resolution is limited to 1 Hz due to the sampling rate. Also, we have confirmed by BCECF fluorescence (pH indicator) that pH, which may affect the dissociation constant of fura 2 (33), was not altered significantly by any of the pretreatments and agents used in the present experiments (not shown). All experiments were performed at room temperature (20–25°C).

Chemiluminescent detection of \(O_2^-\) production. \(O_2^-\) was measured by using an \(O_2^-\)-sensitive Luciferin derivative, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo-[1,2-a]-pyrazin-3-one (MCLA; Kasei Kogyo; Tokyo, Japan) (21). Cells were cultured for 4 days on a 96-well culture plate, and the culture medium was replaced with 50 μl of 1 μM MCLA-containing Krebs solutions composed of either xanthine alone or xanthine plus xanthine oxidase (X/XO). The illuminated photons...
were counted for 10 min with a luminescence detection system (Argus-50/2D luminometer, Hamamatsu Photonics; Hamamatsu, Japan).

**Gel contraction assay.** Contractility of cultured smooth muscle cells was examined by gel contraction assay. Harvested BASMC were resuspended in collagen solution containing 0.2% type IA collagen in DMEM at a density of 4 × 10⁴ cells/ml. First, collagen solution (0.3 ml) without BASMC was poured into a 24-well culture plate and allowed to form into gel for 15 min at 37°C, which prevented the cells from spreading on the bottom of the culture well. The BASMC-containing collagen solution (0.5 ml) was then layered and kept at 37°C for another 15 min to form the gel, and 1 ml of DMEM with 10% fetal bovine serum was poured onto the gel. After being cultured for two days at 37°C, the gel was used for the contraction assay. Embedded BASMC showed a spindle-like shape in 24 h, as shown in Fig. 1A,c.

After each pretreatment, the lateral surface of the gel was carefully detached from the culture well with a fine needle. The culture plate was then placed on a hotplate (model MP-10DM; Kitazato Supply; Shizuoka, Japan) and kept at 37°C. The gel images were captured with a digital camera (QV-800SX, Casio; Tokyo, Japan) every minute throughout the experiment (Fig. 1B). Contraction of the gel was then evaluated by measurement of its surface area with the use of image-analysis software (Photoshop, Adobe Systems).

**Drugs and solutions.** The modified Krebs solution used in the present experiment was (in mM) 132.4 NaCl, 5.9 KCl, 1.5 CaCl₂, 1.2 MgCl₂, 11.5 glucose, and 11.5 HEPES (pH was adjusted to 7.4 by NaOH). Ca²⁺-free Krebs solution and high-K⁺ Krebs solution were prepared by replacing CaCl₂ with 1 mM EGTA or 53 mM NaCl with equimolar KCl, respectively. The drugs used in the present experiment were ATP, thapsigargin, A-23187, superoxide dismutase (SOD), respectively. The drugs used in the present experiment were ATP, thapsigargin, A-23187, superoxide dismutase (SOD), H384 SUPEROXIDE AFFECTS CONTRACTION.

**RESULTS**

**Generation of O₂⁻ by X/XO.** In the present study, we used X/XO to generate O₂⁻, xanthine alone as a control, and SOD to scavenge O₂⁻. First, we confirmed by MCLA chemiluminescence the validity for using these agents to generate and scavenge O₂⁻. It has been reported (21) that MCLA chemiluminescence is highly specific for O₂⁻ and singlet molecular O₂ (¹O₂). In a condition where the concentration of xanthine was fixed at 100 μM, XO increased MCLA chemiluminescence in a concentration-dependent manner, whereas 100 μM xanthine alone did not generate chemiluminescence (Fig. 2, open circles). Furthermore, 150 U/ml SOD completely abolished MCLA chemiluminescence even at high concentrations of XO (Fig. 2, solid circles). Therefore, because SOD does not scavenge ¹O₂ (8), these indicate that X/XO but not xanthine alone generates O₂⁻, which is properly scavenged by SOD.

**Effects of O₂⁻ on basal level of [Ca²⁺]ᵢ.** First, we examined the effects of O₂⁻ on the basal level of [Ca²⁺]ᵢ in BASMC grown on a collagen-coated coverslip. Control cells were pretreated with 100 μM xanthine alone, whereas X/XO-treated cells were pretreated with 100 μM xanthine and 10 mM XO, for 30 min at 37°C. Subsequently, [Ca²⁺]ᵢ was measured at room temperature. The value was significantly higher in X/XO-treated cells than in control cells (xanthine alone, 125.1 ± 4.8 nM, n = 157; X/XO, 154.3 ± 4.1 nM, n = 221; P < 0.01).

**Effects of O₂⁻ on ATP-induced increase in [Ca²⁺]ᵢ and gel contraction.** Relatively higher and lower concentrations of ATP (10 and 0.01 μM) induced a steep and gradual increase in [Ca²⁺]ᵢ, respectively, in control BASMC, which were pretreated with xanthine alone (Fig. 3A). In X/XO-treated cells, 10 and 0.01 μM ATP induced similar shapes of Ca²⁺ transients but their amplitudes were smaller than control cells (Fig. 3B). We calculated two parameters to evaluate ATP-induced Ca²⁺ transient, i.e., the peak increment of [Ca²⁺]ᵢ (Fig. 3C,a) and the time integral of the elevated [Ca²⁺]ᵢ for 5 min (Fig. 3C,b). For both parameters, the concentration-response relationships and the threshold were not different between xanthine alone (Fig. 3, open circles) and X/XO (Fig. 3, solid circles), but the amplitudes were smaller in X/XO than xanthine alone in all examined concentrations of ATP. When the pretreatment with X/XO was performed in the presence of 150 U/ml SOD, the amplitude of 10 μM ATP-induced Ca²⁺ transient was restored (Fig. 3C).

ATP (10 μM) induced a rapid contraction of the BASMC-embedded collagen gel, which was pretreated with xanthine (100 μM) alone for 30 min (Fig. 3D, open circles, and Fig. 1B,b). In contrast, when the gel was pretreated with 100 μM xanthine and 10 mM XO, ATP-induced gel contraction was impaired (Fig. 3D, solid circles), and 150 U/ml SOD prevented the impairing effects of X/XO (Fig. 3D, solid squares). Collagen...
gel without BASMC did not show any contraction in response to ATP (Fig. 3D, open squares). Furthermore, BASMC-embedded gel was not contracted at least up to 120 min without application of ATP (not shown). Therefore, it would be rational to speculate that the contraction of BASMC-embedded gel was due to the contraction of BASMC evoked by ATP. These results suggest that O$_2^-$ attenuates ATP-induced Ca$^{2+}$ transient and contraction in BASMC.

**Effects of O$_2^-$ on thapsigargin-induced [Ca$^{2+}$]$_i$ increase and gel contraction.** Thapsigargin, a selective inhibitor of sarcoplasmic Ca$^{2+}$-ATPase (29), induced Ca$^{2+}$ transient in Ca$^{2+}$-free solution in control cells (Fig. 4A). The subsequent application of Ca$^{2+}$-containing Krebs solution induced a further increase in [Ca$^{2+}$]$_i$ (Fig. 4A). Although thapsigargin induced initial Ca$^{2+}$ transient also in X/XO-treated cells (Fig. 4B), its amplitude and time integral were significantly smaller than control cells (Fig. 4C, a and b), suggesting the reduction of stored Ca$^{2+}$. Furthermore, Ca$^{2+}$ reapplication-induced [Ca$^{2+}$]$_i$ increase was also inhibited in X/XO-treated cells (Fig. 4B and C,c). These X/XO-induced alterations in thapsigargin-induced Ca$^{2+}$ mobilization were restored by SOD (Fig. 4C).

Thapsigargin induced a transient contraction of the control gel in Ca$^{2+}$-free solution, and subsequent application of normal Krebs solution induced a further sustained contraction (Fig. 4D, open circles). In contrast, X/XO-treated gel did not contract in response to thapsigargin in Ca$^{2+}$-free solution and also to the subsequent Ca$^{2+}$ reapplication (Fig. 4D, solid circles), both of which were also restored by SOD (Fig. 4D, solid squares).

**Effects of O$_2^-$ on high-K$^+$ solution-induced [Ca$^{2+}$]$_i$ increase and gel contraction.** To investigate the effects of O$_2^-$ on depolarization-induced Ca$^{2+}$ entry, we then
examined the effect of high-K\(^+\) solution on \([\text{Ca}^{2+}]_i\) and gel contraction. \([\text{Ca}^{2+}]_i\) was gradually increased in response to high-K\(^+\) solution both in control and X/XO-treated cells to the same extent, suggesting that voltage-dependent \([\text{Ca}^{2+}]_i\) entry is not affected by \(\text{O}_2\) (Fig. 5, A–C). In contrast, high-K\(^+\)-induced gradual gel contraction was attenuated by X/XO, which was again restored by SOD (Fig. 5D).
Effect of O$_2$ on A-23187-induced [Ca$^{2+}$]i increase and gel contraction. We then examined the effects of Ca$^{2+}$ ionophore A-23187, which mobilizes Ca$^{2+}$ both from intracellular Ca$^{2+}$ stores and extracellular space in a nonspecific manner (17), on [Ca$^{2+}$]i and gel contraction. As expected, 1μM A-23187 induced [Ca$^{2+}$]i increase in Ca$^{2+}$-containing Krebs solution was not different between control and X/XO-treated cells (Fig. 6, A–C). In contrast, A-23187-induced gel contraction was inhibited by the pretreatment with X/XO (Fig. 6D). This was restored by SOD, suggesting that O$_2$ was responsible for the impairment of A-23187-induced gel contraction. Therefore, these indicate that O$_2$ impairs Ca$^{2+}$ sensitivity of BASMC-embedded gel contraction.

DISCUSSION

We used the catalytic reaction between 100μM xanthine and 10mU/ml XO to generate O$_2^-$, which was confirmed by MCLA chemiluminescence (Fig. 2). Control cells were always treated with xanthine alone and SOD reversed all of the effects of X/XO; thus we consider that the present results can be attributed not to the nonspecific action of excess xanthine but to the...
generated O$_2$. We (13, 14) reported that O$_2$ generated in endothelium by hypoxia and glucose overload were equivalent to homogenous XO solution of 0.09 and 0.15 mU/ml, respectively. However, conversion of MCLA chemiluminescence generated by cell-derived O$_2$ into that by homogenous XO solution would lead to the underestimation of the local O$_2$ concentration around the cells. Therefore, we do not consider that the amount of O$_2$ generated by 10 mU/ml XO was much larger than native values exposed to smooth muscle cells in pathological conditions.

We observed that O$_2$ inhibited CRAC in BASMC (Fig. 4B and C,c). The oxidative stress-induced impairment of CRAC has been reported also in the endothelium (10, 12) and therefore, it may be a common property in vascular tissue that CRAC pathway is sensitive to oxidative stress. We have also shown that stored Ca$^{2+}$ was reduced by O$_2$ (Fig. 4C, a and b). Because Ca$^{2+}$ entry through CRAC is supposed to refill the depleted stores (1), we consider that O$_2$-induced reduction of stored Ca$^{2+}$ was partially due to the impairment of CRAC. The basal level of [Ca$^{2+}$]$_i$ was increased in X/XO-treated cells despite the inhibition of Ca$^{2+}$ entry, so the intracellular Ca$^{2+}$ sequestration is probably impaired by O$_2$, and this may also be involved in the reduction of stored Ca$^{2+}$. Furthermore, O$_2$ attenuated the amplitude and the time integral of ATP-induced Ca$^{2+}$ transient without shifting the concentration-response relationships (Fig. 3, B and C). The impairment of CRAC and the reduction of stored Ca$^{2+}$ are probably involved in the reduction of ATP-induced Ca$^{2+}$ transient, and the production and/or action of Ins(1,4,5)P$_3$ may also be impaired by O$_2$ in BASMC. Indeed, it was reported (4) in coronary artery smooth muscle cells that Ca$^{2+}$ release induced by exogenously applied Ins(1,4,5)P$_3$ was inhibited by O$_2$. In contrast, norepinephrine-induced contraction in rabbit mesenteric artery was impaired by O$_2$ free radicals due to the inhibition of Ins(1,4,5)P$_3$ production (30). We have observed that BASMC showed [Ca$^{2+}$]$_i$ increase in response to membrane depolarization with high-K$^+$ solution (Fig. 5A), and this was not affected by O$_2$ (Fig. 5B). Effects of O$_2$ on VDCC have not been reported so far in vascular smooth muscle cells. In previous reports, however, L-type VDCC current measured by whole cell voltage clump was not affected by O$_2$ in glomus cells of the rabbit carotid body (27) and in ferret ventricular myocytes, where L-type VDCC current was...
augmented by O₂, but through the generation of peroxynitrite (ONOO⁻) (2). Therefore, it would be acceptable to consider that VDCC, especially the L-type, is rather resistant to O₂.

Cultured smooth muscle cells normally do not contract because of the phenotypic transition from the contractile form into the proliferative form (25). However, Yamamoto et al. (32) reported that almost no phenotype transition occurred in rabbit arterial smooth muscle cells when they were cultured in three-dimensional collagen gel. In the present experiments, therefore, we tried to establish an in vitro contraction model by using a BASMC-embedded three-dimensional collagen gel lattice (Fig. 1, A, c and B). [Ca²⁺]i was recorded from BASMC grown on collagen-coated coverslip, where the cells showed spindle-like shape (Fig. 1A,b) as in collagen gel lattice (Fig. 1A,c). Therefore, though simultaneous measurement of [Ca²⁺]i and gel contraction is technically not feasible, it would be fair to suppose that cells in collagen gel lattice showed similar Ca²⁺ mobilizations as those on collagen-coated coverslip, and that these separately obtained data are comparable each other. All of the Ca²⁺ mobilizing agents used in the present study, i.e., ATP (Fig. 1B and 3D), thapsigargin (Fig. 4D), high-K⁺ solution (Fig. 5D), and A-23187 (Fig. 6D), successfully induced contraction of the xanthine-treated control gels. The gel even showed relaxation in Ca²⁺-free solution after thapsigargin had induced the initial Ca²⁺ transient (Fig. 4D). Although sustained or gradually developed gel contraction induced by ATP (Fig. 3D), high-K⁺ solution (Fig. 5D), and A-23187 (Fig. 6D) did not follow the time course of [Ca²⁺]i in Ca²⁺-containing solution. This was probably generated by continuous Ca²⁺ entry from the extracellular space and maintained by the characteristic property of smooth muscle contraction known as “latch,” i.e., force maintenance at low-energy cost (31). Furthermore, collagen gel alone without BASMC did not show contraction to any of these agents. Because cells are randomly distributed in three-dimensional collagen gel lattice (Fig. 1A,c), whereas cells are layered in vascular tissues, the BASMC-embedded gel system may not simulate the full characteristics of in vivo vessel. However, these results clearly indicate that the BASMC-embedded collagen gel preserves the two most important functions of smooth muscle cells: contraction and relaxation. Such a culture cell-embedded collagen gel has been used so far as an in vitro model of fibroblast-mediated wound healing, and the contraction of fibroblast-embedded gel is normally developed very gradually in a time course over hours or days (19, 20). In contrast, contraction of the BASMC-embedded collagen gel appeared in a few minutes (Fig. 3D). Therefore, the present results showed for the first time that smooth muscle cell-embedded collagen gel could be a good in vitro model of smooth muscle contraction. Vessel-like muscle fiber constructed from cultured smooth muscle cells and collagen has been reported previously (24). However, in their method, 2 ml of collagen gel solution (type I A and IV) containing 3 × 10⁶ cells/ml was required, and the gel had to be cultured for 7 days in a rectangular well to form a string-shaped reconstructed muscle fiber. The obtained fiber was then mounted in an organ bath to measure isometric force (24). In contrast, our in vitro contraction model requires far fewer cells (0.5 ml of 4 × 10⁴ cells/ml) with a shorter preparation period (2 days) than their method, and a digital camera for general use is sufficient for the measurement. The major advantage of these in vitro contraction models, compared with excised vascular tissues, is that any pretreatments, including drugs and genes, can be applied to smooth muscle cells without being interfered by tight connective tissues or tissue enzymes. Because collagen gel lattice is very porous, the present contraction model would be much more favorable for this purpose than reconstructed muscle fiber (24).

By using this in vitro contraction model, we have found that O₂ impairs smooth muscle contraction induced by ATP, thapsigargin, high-K⁺ solution, and A-23187. At least the inhibition of ATP- and thapsigargin-induced contraction by O₂ (Figs. 3D and 4D) may be attributed to the impairment of Ca²⁺ mobilization. However, gel contractions induced by high-K⁺ and A-23187 were also inhibited by O₂ (Figs. 5D and 6D), whereas [Ca²⁺]i increases by these agents were not affected (Figs. 5B and 6B). This indicates that O₂ impairs muscle contraction even when [Ca²⁺]i was properly elevated, and therefore we suppose that O₂ affects not only Ca²⁺ mobilization pathways but also Ca²⁺ sensitivity in BASMC. Contraction of vascular smooth muscle cells is initiated by the Ca²⁺-calmodulin complex-mediated phosphorylation of myosin light chain kinase, which then phosphorylates the myosin light chain to contract the muscle (26). Therefore, O₂-induced impairment of the contractility of BASMC may be due to the impairment of any of these pathways. However, the detailed mechanism of O₂-induced inhibition of contractile machinery could not be clarified in the present study, and further investigations are definitely warranted.

Attenuation of vascular contractile properties by O₂ free radicals has been attributed mainly to endothelial dysfunction (16, 28). Recently, however, O₂ production inside the smooth muscle layer has been proposed as a possible pathogenesis of atherosclerosis (18). Therefore, the attenuation of muscle contractility shown in the present study may be involved in the initiation and/or deterioration of various vascular diseases. In summary, we showed that O₂ attenuates Ca²⁺ mobilizing pathways and Ca²⁺ sensitivity of contractile machinery, thereby reducing muscle contraction in BASMC.

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