Superoxide Anion Increases Intracellular Free Calcium in Human Myometrial Cells*

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We investigated the effects of superoxide anion on the intracellular free calcium concentration ([Ca"^2+]) in human cultured myometrial cells using a calcium-sensitive fluorescent dye, indo-1, and a digital imaging fluorescence microscopic system. Hypoxanthine (HX) plus xanthine oxidase induced a rise in [Ca"^2+], in a manner dose-dependent on xanthine oxidase. The increase in [Ca"^2+], in the absence of extracellular calcium ([Ca"^2+]), was 10% of that in the presence of [Ca"^2+]. Nifedipine, which blocks voltage-sensitive calcium channels, also reduced the increase in [Ca"^2+], induced by HX-xanthine oxidase. Superoxide dismutase or superoxide dismutase plus catalase, which metabolizes superoxide anion, inhibited the effect of HX-xanthine oxidase on [Ca"^2+]i. The desensitization of the effect of superoxide anion on [Ca"^2+]i was investigated by pulsatile administration of HX and xanthine oxidase. Desensitization was observed on pulsatile administration of HX-xanthine oxidase at 2-min intervals. These data suggest that superoxide production may participate in uterine contraction via [Ca"^2+]i.

**Experimental Procedures**

Materials—Hypoxanthine (HX), xanthine oxidase, collagenase (type I), oxytocin, catalase, nifedipine, and fluorescein isothiocyanate-conjugated goat IgG anti-mouse immunoglobulin were obtained from Sigma. Superoxide dismutase, indo-1, and indo-1-AM were obtained from Wako Pure Chemical Industries (Japan). Anti-desmin monoclonal antibody was obtained from Bio-Science Product AG (Switzerland). Anti-myosin monoclonal antibody was obtained from Immunotech S.A. (France).

Cell Preparation—Myometrial tissues were obtained from women who underwent caesarean section after receiving consent from the women for their use. Cells were prepared by the method of Palmberg and Thyberg (15) as follows. The tissues were cut into 2-4 mm3 fragments and digested with 0.1% collagenase for 1 h at 37 "C in medium 199 supplemented with penicillin (200 units/ml), streptomycin (200 µg/ml), 0.1% bovine serum albumin, and 20 mM HEPES. They were then digested for another 16-18 h with fresh enzyme solution of the same composition as described previously. Cell aggregates were isolated by gentle pipetting in calcium-free Hank's salt solution. Nondispersed fragments were separated by filtration through gauze cloth (four layers).

The isolated cells were washed and counted and plated on 0.14 mm glass coverslips which were sealed under the 1.0-cm hole in the bottom of 35-mm culture dishes specially designed for studies on [Ca"^2+]i. The cells were maintained at 37 "C in an atmosphere of 95% air, 5% CO2 in medium 199 with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) and used for experiments after 24-48 h. On the other hand, in preliminary experiments, freshly isolated cells were used directly in suspension.

The cells selected for study of [Ca"^2+]i were completely attached to the coverslip and placed in areas with little cell debris in healthy cultures. The insufficiently attached cells were not adequate for measurement of [Ca"^2+]i, because of changing shapes caused by contraction. Data on single, well separated cells that showed a normal response to oxytocin were analyzed.

**Measurement of [Ca"^2+]i**—Cells were loaded with a calcium-sensitive fluorescent dye, indo-1-AM, in medium 199 at 37 "C for 60 min. The [Ca"^2+]i concentration was measured using a digital imaging microscopic system ACAS-70 workstation (Meridian Instruments Inc., West Germany). An argon ion laser beam with an output power controlled through the ACAS computer system was led to the specimen on an inverted microscope. Emission from the illuminated spot on the sample was directed to a sensitive photomultiplier tube, and the photocurrent was measured with a high speed data acquisition interface. The ratio of the intensities of fluorescent emission at 405 nm and 485 nm with excitation at 355 nm was measured. The free calcium concentrations in various portions of single cells were calculated from these ratios using a standard calibration curve for calcium concentration. The free calcium...
cium concentrations used to prepare the standard curve were determined using Ca"-EGTA solution (16). Two-dimensional fluorescence images were made by moving the specimen over the focal point of the laser, making fluorescence measurements, and correlating them with the coordinates of movement. For administration of drugs and their removal by washing, we used the perfusion system. Medium was perfused around the cells at a rate of 0.6 ml/min at 37 °C. The effects of each treatment on the [Ca"], in 10 cells were measured. Solution, of which Ca" concentration was controlled in the range of 50-100 nM by addition of EGTA, was prepared as a Ca"-free solution.

Indirect Immunofluorescence—On days 2, 4, 6, and 8 after isolation, cultured cells were fixed with methanol at -20 °C for 20 min. The cells were washed with 0.1 M phosphate-buffered saline and stained with monoclonal antibodies against desmin (muscle-specific intermediate filament) or myosin (contractile protein) for 40 min. After washing three times, fluorescein isothiocyanate-labeled goat IgG antimouse immunoglobulins were applied for 40 min. Then, the cells were washed and observed with a fluorescence microscope.

Analysis of Data—Data are shown as mean values plus or minus the standard error of the mean (mean ± S.E.) of multiple determinations in at least three replicate experiments. The significance of the differences was assessed by analysis of variance, and a p value of less than 0.01 was considered significant.

RESULTS

Superoxide was produced by adding HX and xanthine oxidase (HX-xanthine oxidase). At first, we investigated the effect of superoxide anion on [Ca"], in suspension of freshly isolated cells. HX-xanthine oxidase significantly induced increases of [Ca"], (Fig. 1). Subsequently, we examined by using single myometrial cells to clarify that the effect was induced especially in myometrium. The two-dimensional distributions of [Ca"], in a myometrial cell are shown in Fig. 2.

![Fig. 1. Change of [Ca"], of freshly isolated cells in suspension which was induced by the addition of xanthine oxidase (XO:10 million units/ml) in the presence of hypoxanthine.](image)

![Fig. 2. Two-dimensional analysis of [Ca"], distribution before and at various times after addition of xanthine oxidase (10 million units/ml) in the presence of hypoxanthine (1 mM). Graphics show the distribution of [Ca"], before (A) and 15 s (B), 30 s (C), and 120 s (D) after the stimulation.](image)

![Fig. 3. Dose dependence of changes of [Ca"], induced by hypoxanthine (HX) (1 mM) plus xanthine oxidase (XO) (1.0-1000 million units/ml) in the presence and absence of extracellular calcium. Neither HX nor xanthine oxidase alone increased [Ca"], Data are means ± S.E. (n = 10).](image)
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FIG. 4. Basal [Ca\(^{2+}\)]\(_i\) of resting cells and peak [Ca\(^{2+}\)]\(_i\) of cells stimulated with oxytocin (10 microunits/ml) 20 min before and 20 min after stimulation with HX-xanthine oxidase.

FIG. 5. Changes of [Ca\(^{2+}\)]\(_i\) induced by hypoxanthine (HX: 1 mM)-xanthine oxidase (XO: 10 milliunits/ml) in human cultured myometrial cells in the presence and absence of superoxide dismutase (SOD, 10 units/ml), superoxide dismutase (10 units/ml)-catalase (CAT, 100 ug/ml), and nifedipine (NIF, 1 \(\mu\)M). Data are means ± S.E. (n = 10). *p < 0.01 versus HX-xanthine oxidase-induced [Ca\(^{2+}\)]\(_i\).

Intracellular stores but mainly by stimulating influx via voltage-sensitive calcium channels. The responses of [Ca\(^{2+}\)]\(_i\) to pulsatile stimulations with superoxide anion and oxytocin are compared in Fig. 6. The response of [Ca\(^{2+}\)]\(_i\) to superoxide anion had a shorter unresponsive period than that to oxytocin. Pulsatile stimulation with superoxide anion caused desensitization of [Ca\(^{2+}\)]\(_i\) increases, but less than the desensitization on pulsatile stimulation with oxytocin.

Using indirect immunofluorescent technique, cultured cells were stained with monoclonal antibodies against desmin and fluorescein isothiocyanate-labeled IgG. At least 90% of cultured cells on day 2 showed positive labeling of desmin. Nearly all of oxytocin-responded cells were stained with similar high intensity on days 2, 4, and 6. The intensity started to decrease on day 8. Oxytocin-nonresponsible cells started to be weakly stained on days 4 or 6. On the other hand, nearly all of the cultured cells on days 2, 4, and 6 were stained with similar high intensity with monoclonal antibodies against myosin. On day 8, 70% of the cells was weakly stained, and other cells were strongly stained (Fig. 7). These results show that myometrial cells cultured for 2 days, especially oxytocin-responsive cells, could not modulate from contractile to synthetic phenotype.

DISCUSSION

In the present study, we measured the two-dimensional distribution of [Ca\(^{2+}\)]\(_i\) in cultured human myometrial cells and found that superoxide anion induced increases in [Ca\(^{2+}\)]\(_i\). HX-xanthine oxidase is known to generate superoxide anion, which is converted first to hydrogen peroxide and oxygen by the scavenger enzyme superoxide dismutase, and then to water and oxygen by catalase. The increase of [Ca\(^{2+}\)]\(_i\) induced by HX-xanthine oxidase was inhibited significantly and to similar extents by superoxide dismutase and superoxide dismutase + catalase indicating that superoxide anion itself increased [Ca\(^{2+}\)]\(_i\). Recently, superoxide anion has been proposed to cause endothelium-dependent contraction of vascular smooth muscle (18–20). Hirosumi et al. (21) reported measurement of change in [Ca\(^{2+}\)]\(_i\) induced by superoxide anion in endothelial cells in suspension. To our knowledge,
the present paper is the first report of the change in the distribution of [Ca\(^{2+}\)], induced by superoxide anion in single myometrial cells demonstrated by digital image microscopy. Hirosumi et al. (21) checked the release of 2-deoxy-D-[\(^{3}\)H]glucose as an estimation of cell injury. We determined the response of [Ca\(^{2+}\)] to a physiological contractant, oxytocin, before and after addition of IxIX-phenanthroline. Therefore, the increase of [Ca\(^{2+}\)], induced by superoxide anion is apparently not due to nonspecific cellular damage.

Superoxide anion is reported to inactivate the endoplasmatic reticular Ca\(^{2+}\) pump in coronary artery (22) and to activate Na\(^{+}\)-Ca\(^{2+}\) exchange in heart cells (23). We found that the increase of [Ca\(^{2+}\)], in myometrial cells was mainly caused by activation of L-type calcium channels. But the roles of superoxide anion on T-type calcium channels, Ca\(^{2+}\)-Na\(^{+}\) exchange, and the increase of [Ca\(^{2+}\)], in myometrial cells were not clear. Anwer and Sanborn (24) reported that oxytocin and carbabol increased [Ca\(^{2+}\)], in rat myometrial cells in suspension, and that the increase was due to both mobilization of intracellular stores and influx of extracellular calcium. We showed that the increase of [Ca\(^{2+}\)], in human myometrial cells induced by oxytocin was dependent in part on intracellular stores but mainly on extracellular Ca\(^{2+}\). In this point, superoxide anion and oxytocin may have similar effects. We found that resting stores and influx of extracellular calcium. We showed that the increase of [Ca\(^{2+}\)], in myometrial cells was mainly caused by calcium stores in sarcoplasmic reticulum in this region. The increase in calcium near the cell membrane was heterogeneous, suggesting that this distribution of calcium channels in the membrane is also heterogeneous.

Desensitization of the response of [Ca\(^{2+}\)], to superoxide anion is less than that of the response to oxytocin. Since the existence of a receptor for superoxide anion has not been demonstrated, the mechanism of the increase of [Ca\(^{2+}\)], induced by superoxide anion is not clear. Oxytocin may induce the increase of [Ca\(^{2+}\)], in the mechanism different from that of the oxytocin-activating calcium channel via receptor.

We studied the measurement of [Ca\(^{2+}\)], with myometrial cells cultured for 24–48 h. On the other hand, smooth muscle cells, including myometrial cells, in primary culture have been demonstrated to modulate from contractile to synthetic phenotype, paralleling a decrease of contractile protein (myosin, actin, and caldesmon) (25). Palmberg and Thyberg (15) reported that the process of modulation in cultured myometrial cells involved loss of desmin, and the proliferative potential of myometrial cells was markedly lower than that of arterial smooth muscle cells. Our examination of immunofluorescence showed that contractile phenotype remained in primary cultured myometrial cells, especially oxytocin-responsive cells, for 24–48 min. The increase of [Ca\(^{2+}\)], might be a trigger of uterine contraction. Superoxide is supposed to induce uterine contraction. Superoxide can be generated in vivo in the following ways: 1) stimulation of phagocytes in infectious focuses, 2) ischemia-reoxygenation, and 3) activation of arachidonic metabolism. There is much suggestive evidence that intrauterine infection is associated with the onset of premature labor (1). Prostaglandins have been considered to play an important role in uterine contraction due to infection. In fact, bacterial products stimulate prostaglandin production in amniotic membrane. Interleukin-1 and tumor necrosis factor produced by mononuclear cells and decidua also stimulate prostaglandin production in response to bacterial products (3, 4).

Cherouny et al. (26) reported that hydrogen peroxide induced prostaglandin production and contractions of pregnant rat uterus. But, the relationship between prostaglandin production and superoxide generation is still unknown. Our data showed that superoxide anion itself produced by phagocytes activated in infectious focuses could induce increases in [Ca\(^{2+}\)], and uterine contraction.

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