Impairment of endothelial nitric oxide production by acute glucose overload

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Kimura, Chiwaka, Masahiro Oike, Tetsuya Koyama, and Yushi Ito. Impairment of endothelial nitric oxide production by acute glucose overload. Am J Physiol Endocrinol Metab 280: E171–E178, 2001.—We examined the effects of acute glucose overload (pretreatment for 3 h with 23 mM D-glucose) on the cellular productivity of nitric oxide (NO) in bovine aortic endothelial cells (BAEC). We had previously reported (Kimura C, Oike M, and Ito Y. Circ Res, 82: 677–685, 1998) that glucose overload impairs Ca\(^{2+}\) mobilization due to an accumulation of superoxide anions (O\(_2^-\)) in BAEC. In control cells, ATP induced an increase in NO production, assessed by dianionfluorescein 2 (DAF-2), an NO-sensitive fluorescent dye, mainly due to Ca\(^{2+}\) entry. In contrast, ATP-induced increase in DAF-2 fluorescence was impaired by glucose overload, which was restored by superoxide dismutase, but not by catalase or deferoxamine. Furthermore, pyrogallol, an O\(_2^-\) donor, also attenuated ATP-induced increase in DAF-2 fluorescence. In contrast, a nonspecific intracellular Ca\(^{2+}\) concentration increase induced by the Ca\(^{2+}\) ionophore A-23187, which depletes the intracellular store sites, elevated DAF-2 fluorescence in both control and high D-glucose-treated cells in Ca\(^{2+}\)-free solution. These results indicate that glucose overload impairs NO production by the O\(_2^-\)-mediated attenuation of Ca\(^{2+}\) entry.

CARDIOVASCULAR DISEASE, including coronary heart disease, stroke, and peripheral vascular disease, is the most important cause of mortality and morbidity among patients with diabetes mellitus (1, 15, 31), in which impairment of endothelial functions may be involved (19). As vascular endothelium plays its physiological roles by producing various mediators, including nitric oxide (NO) (23), the production of these endothelium-derived substances might be impaired in hyperglycemic conditions. This idea is supported in a previous report, showing that the aortic ring from diabetic rabbit failed to induce endothelium-derived relaxation in response to acetycholine (34).

Accumulation of superoxide anion (O\(_2^-\)) is one of the reported candidates for the pathogenesis of vascular and/or endothelial damage in a diabetic environment. Excess glucose causes autoxidation of glucose and nonenzymatic protein glycation, both of which generate O\(_2^-\) (40). Metabolism of excess D-glucose via its collateral pathway, the polyl pathway, increases the NADH/NAD\(^+\) ratio, which results in the activation of protein kinase C and phospholipase A\(_2\). Prostaglandins are then synthesized by cyclooxygenase, which is capable of generating O\(_2^-\) as an intermediate (34, 39). Fructose, a product of the polyl pathway, also generates O\(_2^-\), because it induces protein glycation more efficiently than glucose (33). Furthermore, it has been reported that the activity of endogenous superoxide dismutase (SOD) is suppressed by hyperglycemia due to glycation (2). We have previously shown (12) that acute glucose overload abolishes ATP-induced Ca\(^{2+}\) oscillations by inhibiting Ca\(^{2+}\)-release-activated Ca\(^{2+}\) entry (CRAC) and Ca\(^{2+}\) extrusion and by accelerating Ca\(^{2+}\) leak from the intracellular Ca\(^{2+}\) store sites in bovine aortic endothelial cells (BAEC). Normally, vascular endothelium produces NO in response to the elevation of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), which complexes with calmodulin (CaM), and the Ca\(^{2+}\)-CaM complex stimulates NO synthase (NOS) to produce NO (24). Therefore, our previous findings raise a question that needs to be clarified, i.e., whether these impairments of Ca\(^{2+}\) homeostasis are responsible for the attenuation of endothelium-derived vasodilatation in the hyperglycemic condition.

For the measurement of cellular NO production, direct measurement of released NO by porphyrinic microsensor (20) or measurement of its metabolite, nitrite, by the Griess method (38) has been used. Dianionfluorescein 2 (DAF-2) has been developed recently as an NO-sensitive fluorescent dye, whose fluorescence was shown to correlate with the NO concentration from submicromolar levels (14). Furthermore, intracellular production of NO has been measured successfully from living cells by use of a membrane-permeable form of DAF-2, diacetylated DAF-2 (DAF-2/DA), in rat aortic smooth muscle cells (14) and BAEC (11). The major advantage of using DAF-2 is that it enables the measurement of intracellular NO production from living cells after any arbitrary pretreatment of the cell, including glucose overload.

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We examined the characteristics of cellular NO production in hyperglycemic conditions in BAEC by using DAF-2. In the present study, we used ATP to induce NO production in BAEC. We recently reported (27) that ATP is released from BAEC in response to mechanical stress. Because vascular endothelium shows its various physiological functions autonomically in response to mechanical stress (5), we supposed that ATP-induced cellular responses play an important role in endothelial physiology. The results obtained indicate that glucose overload attenuates endothelial NO production, and that this can be attributed mainly to the O$_2^-$-mediated impairment of Ca$^{2+}$ mobilization that we previously clarified.

**METHODS**

*Culture of BAEC.* Bovine thoracic aorta of 1-yr-old calf was obtained from the local slaughterhouse. Endothelial cells were scraped off from the intima with the edge of a razor. Collected endothelial cells were cultured in DMEM (Life Technologies, Rockville, MD) containing 10% fetal calf serum under 5% CO$_2$-95% air at 37°C. Cells of a second subculture were used in the present experiment. Cells were seeded, either on coverslips for measuring [Ca$^{2+}$]$_i$ and NO production or on a 96-well culture plate for measuring O$_2^-$ generation, and were cultured for ≥4 days after seeding. Identification of endothelial cells was confirmed by the specific uptake of acetylated low-density lipoprotein.

*Measurement of [Ca$^{2+}$]$_i$.* [Ca$^{2+}$]$_i$ was measured from non-confluent single cells by use of an Attofluor digital fluorescence microscopy system (Atto Instruments, Rockville, MD). Cells were loaded with fura 2 by incubation with 1 μM fura 2-AM (Dojindo, Kumamoto, Japan) for 20 min at 37°C. Fura 2 was excited at two alternative wavelengths (340 and 380 nm), and emitted fura 2 fluorescence images of 510-nm wavelength were recorded into a rewritable optical disc recorder (LQ-41000A, Panasonic, Osaka, Japan) at a rate of ~1 Hz. For each cell, fluorescence intensities excited at 340 and 380 nm (F$_{340}$ and F$_{380}$, respectively) were calculated from each image, and these data were converted to fluorescence ratio (F$_{340}$/F$_{380}$) and apparent [Ca$^{2+}$]$_i$ as previously reported (27).

*Measurement of O$_2^-$ by MCLA.* We measured the generation of O$_2^-$ in BAEC by using an O$_2^-$-sensitive luciferin derivative, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one (MCLA; Tokyo Kasei, Tokyo, Japan) (26). Cells were cultured for 4 days on a 96-well culture plate, and the culture medium was replaced with Krebs or high-glucose Krebs solutions and incubated for 3 h. Then the solution was replaced with 50 μl of 1 μM MCLA-containing solutions, and illuminated photons were counted for 10 min with a luminescence detection system (Argus-50/2D lumimeter, Hamamatsu Photonics, Hamamatsu, Japan). It has been reported that MCLA chemiluminescence is highly specific for O$_2^-$ and singlet molecular oxygen (1'O$_2$) (26). We used superoxide dismutase (SOD) to distinguish between chemiluminescence of O$_2^-$ and 1'O$_2$, because SOD does not scavenge 1'O$_2$ (8). Because it is difficult to calibrate MCLA chemiluminescence into absolute values of O$_2^-$ (25), we expressed the amount of O$_2^-$ as an equivalent concentration of xanthine oxidase, which reacts with xanthine and generates O$_2^-$ in a concentration-dependent manner (see inset of Fig. 2).

*Measurement of intracellular production of NO.* NO was measured by use of DAF-2, an NO-sensitive fluorescent dye (14). Cells were loaded with 10 μM DAF-2/DA (Daichi Pure Chemicals, Tokyo, Japan) for 30 min at 37°C. The DAF-2 fluorescence was measured by use of the same equipment as for [Ca$^{2+}$]$_i$ measurements but with a different filter set, i.e., excitation at 490 nm and emission at 515 nm. Because we found in the preliminary experiment that the frequent application of the excitation wavelength quenches DAF-2 fluorescence, we applied the excitation wavelength with an interval of 30 s. Therefore, temporal resolution of DAF-2 fluorescence was inevitably lower than that of [Ca$^{2+}$]$_i$, in the present study. It has been reported that DAF-2 fluorescence increases almost linearly with the NO concentration (14); therefore, we expressed the intracellular NO production as the net increment of DAF-2 fluorescence in 15 min relative to its basal value. Because NO synthase (NOS) produces O$_2^-$ instead of NO in the absence of a sufficient concentration of L-arginine (30), we added a maximal concentration of L-arginine (10 mM) to all solutions used for NO measurement, except for the experiment with N$^6$-nitro-L-arginine methyl ester (L-NAME)-treated cells. The solutions were alkalized by the addition of a high concentration of L-arginine, and we therefore readjusted the pH to 7.3 with HCl.

*Drugs and solutions.* The standard extracellular solution was a modified Krebs solution (1.5 mM Ca$^{2+}$ solution) containing (in mM): 132 NaCl, 5.9 KCl, 1.2 MgCl$_2$, 1.5 CaCl$_2$, 11.5 glucose, 11.5 HEPES, with pH adjusted to 7.3 with NaOH. High-glucose solution was made by replacing 6 mM NaCl with 11.5 mM D-glucose, and Ca$^{2+}$-free solution was made by replacing 1.5 mM CaCl$_2$ with 1 mM EGTA. The bath was perfused continuously with these solutions at a rate of 1.5 ml/min.

Thapsigargin and cyclopiazonic acid (CPA), inhibitors of endoplasmic Ca$^{2+}$-ATPase, were used to deplete intracellular Ca$^{2+}$ store sites. Neomycin was used to inhibit phospholipase C. We used SOD, a scavenger of O$_2^-$, catalase, a scavenger of hydrogen peroxide, and deferoxamine, an inhibitor of hydroxyl radical production. A-23187, a Ca$^{2+}$ ionophore, was used to deplete intracellular Ca$^{2+}$ store sites in a nonspecific manner. Pyrogallol was used as a spontaneous generator of O$_2^-$ due to autoxidation (21). L-NAME was used to inhibit NOS. All of these drugs were obtained from Sigma (St. Louis, MO). All experiments were performed at room temperature (25°C).

*Exposure of cells to high-glucose environment.* In this study, BAEC were pretreated for 3 h with either 11.5 mM D-glucose solution (normal glucose), 23 mM D-glucose solution (high D-glucose) or 11.5 mM D-glucose + 11.5 mM L-glucose solution (high L-glucose). Because a lower D-glucose concentration (5.8 mM) did not show any difference in Ca$^{2+}$ mobilizing properties and DAF-2 fluorescence from those treated with 11.5 mM glucose, we used 11.5 mM glucose, employed in our laboratory as normal Krebs, as “normal glucose” solution. We did not examine the chronic effects of glucose overload, because culture medium, which contains sufficient concentration of antioxidative amino acids and vitamins, would have had to be used for pretreating the cells for days, and this would have artificially reduced the impairing effects of high-glucose-induced O$_2^-$ accumulation.

*Data analysis.* Pooled data are given as means ± SE, and statistical significance was determined with Student’s unpaired t-test and one-way ANOVA for comparing two groups and more than three groups, respectively. Probabilities of <5% (P < 0.05) were regarded as significant.

**RESULTS**

*Effects of glucose overload on Ca$^{2+}$ mobilization in BAEC.* First, we confirmed our previous findings, i.e., that D-glucose overload impairs Ca$^{2+}$ mobilization due
to the accumulation of O$_2^-$ (12). As shown in Fig. 1A, ATP (1 μM) induced Ca$^{2+}$ oscillations in Ca$^{2+}$-containing Krebs solution in control cells treated with normal glucose. Ca$^{2+}$ oscillations were not spontaneous, since perfusion of normal Krebs solution alone without ATP did not induce any Ca$^{2+}$ responses. In high D-glucose-treated cells (Fig. 1B), but not in high L-glucose-treated cells (not shown), ATP (1 μM)-induced Ca$^{2+}$ oscillations were abolished. Impairment of ATP-induced Ca$^{2+}$ oscillations in high D-glucose-treated cells was restored by SOD (100 U/ml). However, catalase (1,200 U/ml) and deferoxamine (1 mM) did not reverse the impairment of Ca$^{2+}$ oscillation in high D-glucose-treated cells (not shown).

In our previous report (12), we also showed that the abolition of Ca$^{2+}$ oscillations was partially due to the impairment of CRAC. Thapsigargin (1 μM) induced transient [Ca$^{2+}$]$_i$ elevation in Ca$^{2+}$-free solution due to store depletion (Fig. 1C). Subsequent application of Ca$^{2+}$-containing solution induced further [Ca$^{2+}$]$_i$ increase due to CRAC in control cells (Fig. 1D). On the other hand, the CRAC phenomenon was not observed in high D-glucose-treated cells (Fig. 1D). This was also restored by SOD but not by catalase or deferoxamine (Fig. 1E). The same results were also obtained by use of CPA (not shown).

Generation of O$_2^-$ in high-glucose-treated cells. We then measured the amount of O$_2^-$ released from BAEC
into extracellular space by the use of MCLA (26). As shown in Fig. 2, the amount of $O_2^-$ generated from BAEC after the incubation with normal glucose for 3 h was equivalent to 0.018 ± 0.005 mU/ml of xanthine oxidase ($n = 10$). In contrast, the value was significantly higher in high d-glucose (0.139 ± 0.003 mU/ml; $n = 10$) but similar in high l-glucose solution (0.020 ± 0.008 mU/ml; $n = 10$). This was completely scavenged by SOD (below the detection limit) but not by catalase or deferoxamine (Fig. 2), thereby suggesting that glucose overload-induced MCLA chemiluminescence was due to the generation of $O_2^-$ but not $1O_2$. On the other hand, $O_2^-$ was not detected in normal Krebs or high D-glucose solutions without endothelium. These suggest that $O_2^-$ is actually generated and released from BAEC in high D-glucose condition and that SOD completely scavenges the released $O_2^-$. High D-glucose attenuates NO production in BAEC. ATP (1 μM) induced a gradual increase in DAF-2 fluorescence in normal glucose-treated cells (Fig. 3A). It should be noted that the reaction between DAF-2 and NO is irreversible (14); therefore, the accumulated level of DAF-2 fluorescence corresponds to the total amount of cellular NO production of the period. The ATP-induced increase in DAF-2 fluorescence was suppressed in cells pretreated with 0.1 mM L-NAME for 30 min, suggesting that DAF-2 fluorescence is linked to the cellular NO production (Fig. 3A). ATP increased DAF-2 fluorescence in a concentration-dependent manner in both Ca$^{2+}$-containing and Ca$^{2+}$-free solutions (Fig. 3C). Pretreatment with 1 mM neomycin for 30 min suppressed the 10 μM ATP-induced increase in DAF-2 fluorescence in Ca$^{2+}$-free solution (Fig. 3C), thereby indicating that the increase in DAF-2 fluorescence in Ca$^{2+}$-free solution was due to Ca$^{2+}$ release from intracellular store sites in BAEC. However, the increment of DAF-2 fluorescence was significantly smaller in Ca$^{2+}$-free solution than in Krebs solution, and ATP <1 μM did not induce a DAF-2 increase in Ca$^{2+}$-free solution (Fig. 3C).

On the other hand, the ATP (1 and 10 μM)-induced increase in DAF-2 fluorescence was significantly smaller in high D-glucose-treated cells (Fig. 3, B and C). As in the case of Ca$^{2+}$ mobilization, high L-glucose-treated cells showed a normal increase in DAF-2 fluorescence (Fig. 3B). The basal level of NO production, estimated by perfusing L-arginine alone without Ca$^{2+}$-mobilizing agents for 15 min, was not different between control and high-glucose-treated cells (control, 0.017 ± 0.006 ($n = 20$); high-glucose-treated, 0.022 ± 0.008 ($n = 18$); $P > 0.05$).

As shown in Fig. 1, D-glucose overload interferes with endothelial Ca$^{2+}$ homeostasis. We therefore further examined whether the attenuation of ATP-induced NO production by D-glucose overload is due to impairment of Ca$^{2+}$ homeostasis or to other factors such as the inhibition of NOS. For this purpose, we investigated the effects of D-glucose overload on NO production induced by the Ca$^{2+}$ ionophore A-23187, which nonselectively induces Ca$^{2+}$ entry from the extracellular space and Ca$^{2+}$ release from the intracellular store sites (18). To avoid the involvement of CRAC, we applied A-23187 in Ca$^{2+}$-free solution. Figure 4A shows that 1 μM A-23187 induced [Ca$^{2+}$]i elevation in Ca$^{2+}$-free solution and that the subsequent application of thapsigargin did not induce a further increase in [Ca$^{2+}$]i, thereby suggesting that the intracellular Ca$^{2+}$ store sites were depleted. The time integral of the
[Ca<sup>2+</sup>]<sub>i</sub> response over 5 min was not different between control and high d-glucose conditions (Fig. 4B; P > 0.05), indicating that glucose overload does not affect the A-23187-induced store depletion. A-23187 also increased DAF-2 fluorescence in the absence of extracellular Ca<sup>2+</sup> in both normal glucose- and high d-glucose-treated cells (Fig. 4C; P > 0.05). This was not because A-23187 scavenged O<sub>2</sub>, because MCLA chemiluminescence was not restored by A-23187 (Fig. 4D). These observations suggest that NO production still occurs in high d-glucose-treated cells, provided there is a proper [Ca<sup>2+</sup>]<sub>i</sub> elevation. Thus it can be speculated that attenuation of NO production in the high d-glucose condition may be due to the impairments of Ca<sup>2+</sup> mobilization. O<sub>2</sub> is responsible for the attenuation of NO production in glucose-overloaded BAEC. We have shown that impairment of Ca<sup>2+</sup> homeostasis by d-glucose overload is due to O<sub>2</sub> (Fig. 1 and Ref. 12), which is markedly accumulated in the extracellular space in the high d-glucose condition (Fig. 2). Thus we pretreated the cells with high d-glucose solution in the presence of SOD, catalase, or deferoxamine, and we measured ATP-induced NO production. SOD-treated cells showed an increase in DAF-2 fluorescence in response to 1 µM ATP similar to that in control cells (Fig. 5). In contrast, catalase and deferoxamine did not restore the glucose overload-induced attenuation of NO production (Fig. 5).

We then examined the effects of exogenously applied O<sub>2</sub> on ATP-induced NO production. For this purpose, cells were treated for 30 min at 37°C with 200 µM pyrogallol. Control cells treated with Krebs solution for the same period showed Ca<sup>2+</sup> oscillations in response to 1 µM ATP (Fig. 6A). However, in pyrogallol-treated cells, ATP showed a [Ca<sup>2+</sup>]<sub>i</sub> increase with a single peak but no Ca<sup>2+</sup> oscillations (Fig. 6B), as in the case of high d-glucose-treated cells (Fig. 1B). Thapsigargin and CPA-induced [Ca<sup>2+</sup>]<sub>i</sub> increase (Δ[Ca<sup>2+</sup>]<sub>i</sub>-CRAC) were also suppressed in pyrogallol-treated cells (not shown). Furthermore, 1 µM ATP did not show any apparent increase in DAF-2 fluorescence (Fig. 6C).

These results suggest that the generation of O<sub>2</sub> is responsible for the impairment of NO production in the high d-glucose condition.

**DISCUSSION**

Many investigators have reported that the high-glucose condition attenuates endothelium-derived vasorelaxation (7, 22, 29, 35). Therefore, it has been speculated that glucose overload would cause the impairment of NO release and/or the increased destruction of released NO. Langenstroer and Pieper (16) reported that the resting level of NO release was rather increased in diabetic rat aorta but that the endothelial functions were nevertheless attenuated.

Fig. 3. Effects of glucose overload on ATP-induced nitric oxide (NO) production in BAEC. A: control cells showed an increase in diaminofluorescein 2 (DAF-2) fluorescence in response to 1 µM ATP. ○: Actual values of DAF-2 fluorescence, and continuous line was drawn by averaging the adjacent 6 points. ●: Values from 0.1 mM N<sup>-</sup>-nitro-L-arginine (L-NAME)-treated cells. B: statistical analysis of 1 µM ATP-induced increase in DAF-2 fluorescence. Values were measured 15 min after the start of ATP application. **P < 0.01 vs. Krebs. C: concentration-response relationships of ATP-induced increase in DAF-2 fluorescence. ○ and ●: 1.5 mM Ca<sup>2+</sup>-containing solution with normal glucose and high d-glucose, respectively; □ and ■: Ca<sup>2+</sup>-free solution without and with treatment with neomycin, respectively. *P < 0.05, **P < 0.01.
since the destruction of NO was increased by O$_2$. Their report is supported by the fact that NO easily reacts with O$_2$ to generate peroxynitrite (9). The present results indicate that the production of NO is also impaired by acute glucose overload in BAEC due to the O$_2$-induced attenuation of Ca$^{2+}$ mobilization. The impairing effects of glucose overload are not due to the nonspecific action of glucose but to the metabolism of excess glucose, because nonmetabolizable L-glucose did not show an inhibitory effect on DAF-2 fluorescence (Fig. 3B). We measured the intracellular NO production induced by ATP and A-23187 with DAF-2; therefore, it should be noted that the present results did not take into account the possible destruction of released NO by O$_2$. It should also be noted that the present study was performed with the use of cultured endothelial cells, whose properties may not be identical to those in vivo, and DAF-2 fluorescence may have been influenced by dye quenching. Furthermore, the present study did not investigate the possible involvement of other cellular changes, such as the activation of protein kinase C (13) and the polyol pathway (3) in glucose overload-induced endothelial dysfunction.

Cosentino et al. (4) reported that long-term incubation of human endothelium with 22.2 mM glucose for 5 days increased the expression level of endothelial NOS mRNA and ionomycin-induced nitrite production. However, we consider that this does not contradict the present results, because the agonist-induced Ca$^{2+}$-dependent NO production requires CaM and NOS (24), we suppose that the attenuation of NO production by acute glucose overload can be attributed to the impairment of Ca$^{2+}$ homeostasis but not to the alteration of these molecules. We have shown that glucose overload abolishes Ca$^{2+}$ oscillations (Fig. 1 and Ref. 12). Generation of Ca$^{2+}$ oscillations requires integrity of various Ca$^{2+}$ pathways, i.e., Ca$^{2+}$ release from the intracellular store sites, extrusion of released Ca$^{2+}$ out of the cell, Ca$^{2+}$ entry from the extracellular space, and reloading of the intracellular Ca$^{2+}$ store sites (32). Therefore, although 1 μM ATP induced Ca$^{2+}$ transient in high glucose-treated cells also (Fig. 1B), the fact that Ca$^{2+}$ oscillations were abolished would indicate that some of the Ca$^{2+}$ mobilizing pathways were impaired. Because we observed that CRAC was attenuated by glucose overload (Fig. 1, D and E), we suppose that at least the
impairment of CRAC was involved in the disappearance of Ca\(^{2+}\) oscillations in high glucose-treated cells. Lantoine et al. (17) reported that, in human umbilical cord vein endothelial cells, NO production is triggered by Ca\(^{2+}\) entry from the extracellular space but not by Ca\(^{2+}\) release from store sites. The authors speculated that this was due to the submembrane localization of endothelial NOS. We also observed that ATP-induced NO production was much larger in the Ca\(^{2+}\)-containing solution than in the Ca\(^{2+}\)-free solution (Fig. 3C), suggesting the preferential production of NO by entered Ca\(^{2+}\), especially in the case of low levels of stimulation. However, considerable elevation of DAF-2 fluorescence was also induced by a high concentration of ATP or A-23187 in Ca\(^{2+}\)-free solution, thereby indicating that depletion of the intracellular Ca\(^{2+}\) store sites leads to NO production in BAEC. As shown in Fig. 4C, store depletion-induced NO production was not inhibited by glucose overload. Therefore, we hypothesized that the impairment of CRAC plays a central role in the attenuation of ATP-induced NO production by acute D-glucose overload. This is not selective for ATP-induced NO production, since glucose overload also attenuated 0.3 \(\mu\)M ACh-induced NO production (C. Kimura and M. Oike, unpublished observation).

A high-glucose environment leads to the accumulation of \(O_2^\cdot\), as summarized in the introductory section. In the present experiment, SOD, but not catalase or deferoxamine, restored high-glucose-induced impairment of Ca\(^{2+}\) homeostasis (Fig. 1) and NO production (Fig. 5). Furthermore, we confirmed the impairing effects of \(O_2^\cdot\) on Ca\(^{2+}\) homeostasis and NO production by pretreating the cell with pyrogallol (Fig. 6). Therefore, we conclude that \(O_2^\cdot\), which affects Ca\(^{2+}\) homeostasis, is responsible for the impairment of NO production in the hyperglycemic condition. Using MCLA chemiluminescence, we confirmed that the high d-glucose condition induces the increased generation and/or decreased scavenging of \(O_2^\cdot\), which was abolished by SOD (Fig. 2). MCLA is a water-soluble substance that does not permeate the cell membrane (25), and MCLA chemiluminescence was not detected in cell-free culture wells (Fig. 2). Therefore, although \(O_2^\cdot\) is supposed to be generated inside the cell as a result of glucose metabolism, it seems that \(O_2^\cdot\) permeates the membrane and is released into the extracellular space and then affects Ca\(^{2+}\) pathways of the cell and/or neighboring cells. We suppose that this is why SOD, a large molecule (31 kDa) that could not penetrate the cell membrane, scavenged the intracellularly generated \(O_2^\cdot\) and restored Ca\(^{2+}\) mobilization and NO production. Graier et al. (10) reported that the high-glucose-induced generation of \(O_2^\cdot\) enhanced endothelium-derived relaxing factor formation in porcine aortic endothelium. They incubated the cells for 24 h with a high-glucose culture medium and observed that bradykinin-induced Ca\(^{2+}\) transient and cGMP concentration were augmented in high-glucose-treated cells due to \(O_2^\cdot\) production. However, the present study and many reports from other laboratories (7, 22, 28, 35) indicate that glucose overload attenuates endothelium-derived relaxation or NO production. Although it may be possible that this discrepancy was due to the differences in the period of glucose overload, species, and agonist, we could not identify the precise reason for the discrepancy.

It is well known that the impairment of endothelial constitutive NO production would result not only in the loss of local control of vascular tonus but also in the development of atherosclerosis (36), heart failure (6), and other complications.
or cerebrovascular events (37). Therefore, the mechanism shown in the present study is probably involved in the pathogenesis of diabetic vascular complications. In conclusion, we have shown that acute glucose overload attenuates endothelial NO production by the impairment of Ca\(^{2+}\) homeostasis, especially CRAC. Our results also suggest that scavenging O\(_2^-\) might be a good therapeutic approach to diabetic vascular complications.

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