Hyperglycemic switch from mitochondrial nitric oxide to superoxide production in endothelial cells

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Brodsky, Sergey V., Shujuan Gao, Hong Li, and Michael S. Goligorsky. Hyperglycemic switch from mitochondrial nitric oxide to superoxide production in endothelial cells. Am J Physiol Heart Circ Physiol 283: H2130–H2139, 2002.—The accumulated ultrastructural and biochemical evidence is highly suggestive of the existence of mitochondrial nitric oxide (NO) synthase (mtNOS), where local production of NO regulates the electron transport along the respiratory chain. Here, the functional competence of mtNOS in situ in a living cell was examined using an intravital fluorescent NO indicator, 4,5-diaminofluorescein, employing a new procedure for loading it into the mitochondria to demonstrate local NO generation in undisrupted endothelial cells and in isolated mitochondria as well as in human embryonic kidney cells stably expressing endothelial NOS. With the use of this approach, we showed that endothelial cells incubated in the presence of high concentration of D-glucose (but not L-glucose) were characterized by the reduced NO synthetic function of mitochondria despite the unaltered abundance of the enzyme. In parallel, mitochondrial generation of superoxide was augmented in endothelial cells incubated in the presence of a high concentration of D-glucose. Both the NO generation and superoxide production in hyperglycemic environment could be restored to control levels by treating cells with a cell-permeable superoxide dismutase mimetic. In addition, enhanced mitochondrial superoxide production could be suppressed with an inhibitor of NOS in stimulated endothelial cells. In conclusion, the data 1) provide direct evidence of mitochondrial NO production in endothelial cells, 2) demonstrate its suppression and enhanced superoxide generation in hyperglycemic environment, and 3) provide evidence that “uncoupled” mtNOS represents an important source of superoxide anions in endothelial cells incubated in high glucose-containing medium.

mitochondria; nitric oxide synthase; superoxide anions

SINCE THE DISCOVERY of the endothelium-derived relaxing factor and its identification with nitric oxide (NO) (11, 18, 27), the spectrum of signaling functions attributed to this gaseous messenger is continuously growing (35). It has been recognized that NO synthase (NOS) is associated, in addition to the well-established plasmalemmal localization, with mitochondria of different cell types. Kobzik et al. (19) observed that normal skeletal muscle fibers express two types of NOS, endothelial (eNOS) and neuronal NOS, in a spatially distinct pattern. The endothelial isoform was colocalized with the mitochondrial marker succinate dehydrogenase and participated in calcium-dependent inhibition of oxygen consumption. With the use of immunoelectron microscopy of isolated mitochondrial preparations obtained from the rat heart, liver, and kidney and stained with antibodies directed against eNOS, Bates et al. (2, 3) found that 63–86% of mitochondria displayed the silver-gold signal. Electron paramagnetic resonance studies of isolated rat liver mitochondria provided functional support to the idea that mitochondrial NOS (mtNOS) was capable of generating NO (13, 14). Together with existing data on NO modulation of the electron transport along the respiratory chain (5, 7), the potential role of mtNOS in the regulation of oxidative phosphorylation has been deduced (3). In an attempt to measure NO production in mitochondria of nondisrupted cells, we sought to establish technical approaches utilizing an NO-sensitive fluorophore, 4,5-diaminofluorescein diacetate (DAF; see METHODS) and applied them to the vascular endothelium. Specifically, one of the questions to be addressed was related to NO generation by mtNOS in endothelial cells subjected to the hyperglycemic microenvironment, because it has been suggested that suppressed NO production and enhanced generation of superoxide anions could be responsible for diverse metabolic derangements occurring in the vascular endothelium (25).

METHODS

Materials. DAF and manganese (III) tetrakis (4-phenylazoacid) porphyrin were purchased from Alexis (San Diego, CA). A-23187, bradykinin, and Nω-nitro-l-arginine (L-NNA) were purchased from Sigma (St. Louis, MO). 2′,7′-Dichlorodihydrofluorescein diacetate (H2DCFDA) and MitoTracker red were manufactured by Molecular Probes (Eugene, OR).

Cell cultures. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics and maintained un-
nder conditions of 37°C and 5% CO₂ in EBM-2 medium (Clonetix) supplemented with 2% serum and containing 5 mM d-glucose. HUVEC were used between passages 2 and 5. Rat renal microvascular endothelial cells (RMVEC) were previously established and characterized by our laboratory; these simian virus-40-immortalized cells established from explant cultures of microdissected rat renal resistance arteries incorporate acetylated low-density lipoprotein, express immunodetectable von Willebrand antigen, and are capable of capillary tube formation (36). Wild-type and stably expressing human eNOS human embryonic kidney (HEK) cells, established by Liu et al. (24), were kindly provided by Dr. S. S. Gross (Cornell Medical College).

**Cell and mitochondrial loading with DAF and DDF.** HUVEC or RMVEC were cultured in glass-bottom dishes (Becton-Dickinson; Belford, MA). Cell loading was accomplished by incubating HUVEC with 5 μM DAF for 30 min (4, 20) or 10 μM DDF for 30 min at 37°C in cell culture medium (40, 41). Thus loaded cells were used either for measurements of intracellular NO or O₂⁻ or for pulse-chase mitochondrial loading. Briefly, after being washed with sterile PBS to remove excess reagents, cells were resuspended in a Beckman J-20 rotor, fresh culture medium with hourly inspection of the distribution of fluorophores. Usually, 6–8 h after initiation of DAF washout and 1–2 h after DDF washout, diffuse cytoplasmic fluorescence disappeared and only mitochondria-confined fluorescence was retained, as confirmed by its colocalization with MitoTracker fluorescence and by cell fractionation studies (see RESULTS). MitoTracker red (25 nM) was loaded for 10 min by its colocalization with mitochondrial marker enzymes (see RESULTS), was washed twice and resuspended in the same buffer. The postmitochondrial supernatant fraction was designated as the cytosolic fraction. Western blot analysis of lysates prepared from isolated mitochondria or whole cells was performed as previously described (22). Briefly, SDS-PAGE was performed using a 4–12% Tris-glycine gel (Invitrogen) under reducing conditions. Proteins were transferred to nitrocellulose membranes and incubated with primary antibodies (anti-human cytochrome c oxidase subunit II monoclonal antibody from Molecular Probes, 1:1,000 dilution; anti-eNOS antibody from Transduction Laboratories, 1:500 dilution; and anti-Na⁺/K⁺-ATPase α₁-subunit monoclonal antibody from Research Diagnostics, 1:250 dilution). The blotted bands were incubated with horseradish peroxidase-conjugated IgG. Bands were detected with an enhanced chemiluminescence detection system (Pierce).

For isolation of rat liver mitochondria, 1 g of rat liver was obtained from Zucker diabetic fatty (ZDF) and lean nondiabetic (ZL) rats and homogenized in the buffer containing 10 mM Tris-HCl, 250 mM sucrose, and 0.1 mM EDTA by applying 20–30 strokes in Li et al. (23). The nuclei and debris were removed by centrifugation at 610g for 10 min. The supernatant was centrifuged at 8,600g for 10 min. The mitochondrial pellet was washed with the above buffer three times and used immediately for fluorescence studies.

**Fluorescence plate reader assay.** Assays were performed in 96-well microtiter plates (Falcon) using a CytoFluor II fluorometer (PerSeptive Biosystem; Framingham, MA). Both NO and O₂⁻ release was measured with a fluorescein filter set (excitation 485 ± 20 nm; emission 530 ± 25 nm) in the plate reader. Cells or isolated mitochondria were loaded with fluorophores as described above for DAF and DDF; L-NNA or antimycin was added at the concentrations specified in RESULTS. A-23187 or calcium-calmodulin were added after 20 min of baseline recording. Data were analyzed using the Excel and Microcal Origin software packages.

**Statistical analysis.** Statistical analysis was performed using paired or unpaired t-test and/or ANOVA, followed by Tukey’s post-test with P < 0.05 considered statistically significant. All values are presented as means ± SE. All experiments were repeated at least three times.

**RESULTS**

**Detection of intracellular NO production using DAF fluorescence.** Macrophages and microvascular endothelial cells, HUVEC and RMVEC, respectively, were loaded with DAF, and real-time changes in fluorescence intensity were monitored using short cycles of illumination, as detailed in METHODS. Under these conditions, recordings showed a stable baseline indicative of negligible photoactivation of DAF. Application of a calcium ionophore, A-23187 (5 μg/ml), resulted in increased fluorescence of both cell types (Fig. 1, A and B), which was reversed by the addition of a NOS inhibitor, 1 mM L-NNA. The effect of A-23187 was significantly blunted in cells pretreated with 1 mM L-NNA (Fig. 1, A and B). Bradykinin-stimulated HUVEC (10 μM) responded with increased fluorescence of DAF, which was similarly prevented by L-NNA pretreatment (Fig. 1C).

**Detection of NO in mitochondria.** In a series of pulse-chase studies of DAF washout from live endothelial cells, it was observed that after 8 h of incubating DAF-loaded cells in the fluorophore-free culture medium, cell fluorescence, hitherto diffuse cytoplasmic, was redistributed to a network of elongated intracellu-
lar structures. Costaining with a fluorescent mitochondrial marker, MitoTracker, showed a conspicuous colocalization of fluorophores reaching 84.8% (from 23.2%) by 8 h (Fig. 2A). These data suggested that DAF was retained in the mitochondria in the process of its washout from the cytoplasm.

To confirm this conclusion, we fractionated HUVEC loaded with DAF and examined the distribution of DAF fluorescence in different fractions (Fig. 2B). After 45 min of cell loading, DAF fluorescence was highest in the mitochondrial fraction, whereas the plasma membrane fraction and the cytosol retained >50% of DAF fluorescence. In contrast, after similar DAF loading and 8 h of washout, fluorescence was predominantly associated with the mitochondrial fraction, with <20% fluorescence retained in association with the plasma membrane and the cytosolic fractions. Importantly, DAF fluorescence associated with the mitochondrial fraction showed only an insignificant decline after 8 h of washout, further confirming the validity of the procedure.

With the use of this loading protocol, NO responses of HUVEC were reexamined. A-23187 elicited a L-NNA-inhibitable increase in DAF fluorescence (Fig. 2C). Similar results were obtained in mitochondria isolated from HUVEC and studied using a fluorescence microplate reader (data not shown).

In an attempt to discern the source (mitochondrial vs. cytosolic) of A-23187-evoked NO production as detected by mitochondrial loaded DAF, the purity of isolated mitochondria was examined using Western blotting. A mitochondrial marker protein, cytochrome c oxidase, was enriched in mitochondrial fractions compared with the whole cell lysates (Fig. 4). Immunodetectable eNOS was present in mitochondrial fractions of HUVEC and a surrogate cell line expressing eNOS, HEK/eNOS cells, but only trace amounts of eNOS were detectable after prolonged exposure of X-ray film in the wild-type HEK cells (only after overexposing the film). Hence, eNOS is immunodetectable in mitochondrial fractions of HUVEC and HEK/eNOS cells.

We next compared DAF fluorescence in wild-type HEK and HEK cells stably transfected with human eNOS. In wild-type HEK cells, mitochondrial loading with DAF resulted in a faint fluorescence and marginal changes in fluorescence intensity in response to 5 µg/ml A-23187 (Fig. 3A). This was in sharp contrast with HEK/eNOS cells, which exhibited mitochondrial DAF fluorescence and responded to 5 µg/ml A-23187 with an increase in DAF fluorescence intensity comparable to that seen in HUVEC. Changes in fluorescence intensity were inhibited by Nω-nitro-l-arginine methyl ester.

Fig. 1. 4,5-Diaminofluorescein (DAF) fluorescence in macrovascular [human umbilical vein endothelial cells (HUVEC)] and microvascular (rat renal microvascular endothelial cells (RMVEC)) endothelial cells. A: in HUVEC, 5 µg/ml of the calcium ionophore A-23187 (n = 10) elicited a Nω-nitro-l-arginine (l-NNA)-inhibitable (n = 10, 1 mM L-NNA) increase in DAF fluorescence intensity. [Inset, representative dose-response curve of DAF fluorescence upon the addition of different concentrations of sodium nitroprusside (SNP).] *P < 0.05 compared with L-NNA-treated cells. B: in RMVEC, A-23187 produced a quantitatively and qualitatively similar response (n = 9), which was blocked by pretreatment with L-NNA (1 mM, n = 7) and reversed by posttreatment with L-NNA (n = 7). *P < 0.05 compared with L-NNA-pretreated cells; #P < 0.05 compared with L-NNA-nontreated cells. C: bradykinin (BK; 10 µM) elicited in HUVEC a comparable to A-23187 increase in the fluorescence intensity (n = 10), which was blocked by pretreatment with l-NNA (1 mM, n = 9). *P < 0.05 compared with L-NNA-treated cells.
ester (l-NAME) but not by \(N^\omega\)-nitro-\(d\)-arginine methyl ester (d-NAME) (Fig. 3B).

**Mitochondrial generation of NO in a hyperglycemic environment.** It has previously been demonstrated that NOS function is modified by reactive oxygen species (ROS) (8). Furthermore, there is growing evidence that the elevated glucose concentration results in oxidative stress (11), specifically, mitochondrial oxidative stress (21). Therefore, it was important to examine whether mitochondrial oxidative stress induced by hyperglycemia could affect NO generation by mtNOS. The above protocol provided the only existing technical means to address this question.

HUVEC were incubated in culture medium supplemented with 30 mM \(d\)-glucose (or \(l\)-glucose as a control for osmolality) for different periods of time, and mitochondrial NO generation was examined. The A-23187-stimulated increase in DAF fluorescence was blunted in the presence of 30 mM \(d\)-glucose, exhibiting a decrease in both the peak NO generation and the duration of the response (Fig. 5A). To evaluate the contribution of superoxide anions to the decreased mitochondrial NO production, in a separate series of experiments, \(d\)-glucose-treated HUVEC were cotreated with a cell-permeable superoxide dismutase mimetic, Mn-TBAP (50 \(\mu\)M), and mitochondrial NO production was examined. As shown in Fig. 5B, Mn-TBAP completely restored the mitochondrial ability to generate NO in response to A-23187. Western blot analysis of mitochondrial fractions obtained from HUVEC cultured in euglycemic or hyperglycemic environment with detection of mtNOS using anti-eNOS antibodies revealed that the decrease in NO production could not be attributed to the reduced expression of the enzyme in HUVEC incubated in the presence of 30 mM \(d\)-glucose (Fig. 5C).

Similar results were obtained in HEK/eNOS cells. Mitochondrial loading with DAF of wild-type and eNOS-expressing HEK cells produced a drastically distinct staining: faint in HEK cells and bright in HEK/eNOS cells. Application of A-23187 to both cell types preincubated with 5 or 30 mM \(d\)-glucose for 24 h evoked a significant l-NAME-inhibitable increase in fluorescence intensity of DAF in HEK/eNOS cells but not in HEK cells (Fig. 6). However, cells pretreated with 30 mM \(d\)-glucose for 24 h showed a 50% blunting of responses to A-23187, similar to what was observed in HUVEC.

To verify whether mitochondria obtained from diabetic animals also have a defect of mitochondrial NO production, 18-wk-old hyperglycemic ZDF and control ZL rats were used to prepare liver mitochondria. Isolated mitochondria were loaded with DAF, and the

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**Fig. 2.** Pulse-chase loading of HUVEC with DAF and measurement of mitochondrial nitric oxide (NO) production. A: cells were loaded with DAF, as detailed in METHODS, for 30 min, washed, and incubated for different periods of time in fresh medium devoid of the fluorophore. At different times during washout, a mitochondrial marker, MitoTracker red, was added to the incubation medium, and dual-wavelength confocal microscopy was performed. Initially, DAF fluorescence was uniformly distributed throughout the cytoplasm. Six hours after initiation of washout, DAF fluorescence showed a flocculated appearance but only partially colocalized with MitoTracker (data not shown). After 8 h of washout, DAF fluorescence was almost entirely confined to the mitochondrial compartment, as judged from the colocalization of both fluorophores (merged images). B: HUVEC \((n = 8)\) were loaded with DAF for 45 min or pulse chase loaded with DAF (8 h), followed by cell fractionation and measurement of DAF fluorescence [in arbitrary units (a.u.)/mg protein] in individual fractions (Mt, mitochondria; PM, plasma membrane; CP, cytoplasmic fraction). Whereas initially DAF was present in all fractions, after 8 h of washout the fluorophore was highly enriched in the mitochondrial fraction. *\(P < 0.05\) compared with mitochondrial fluorescence intensity. C: HUVEC were pulse chase loaded with DAF, and mitochondrial DAF fluorescence was monitored. A-23187 elicited an increase in DAF fluorescence \((n = 11)\). Pretreatment with l-NAME blunted this response to A-23187 \((n = 10)\). (Breaks in the graph are shown for the 10-min pretreatment with l-NAME.) *\(P < 0.05\) compared with l-NAME-treated cells.
fluorescence intensity was studied in a 96-well plate reader under basal conditions and after application of A-23187. The data presented in Fig. 5 demonstrate that basal NO production by mitochondria isolated from ZDF rats was significantly reduced compared with ZL rats (basal NO production by ZL mitochondria was used for normalization of data). A-23187 elicited NO production in mitochondria isolated from both groups, but it remained drastically blunted in ZDF rats.

Mitochondrial generation of superoxide anions in a hyperglycemic environment. To further address the possibility of mitochondrial oxidative stress represent-
ing a cause of reduced NO production, we next applied the similar mitochondrial loading strategy to DDF. This nonfluorescent, cell-permeable compound acquires fluorescence properties upon oxidation and has been broadly used to detect ROS (40, 41). Specifically, cell loading was followed by a washout period, when fluorophore virtually disappeared from the cytoplasm but was retained in a tubular network. Dual labeling of HUVEC with MitoTracker and DDF, as detailed in METHODS, showed 79.9% (from 24.7%) colocalization of both fluorophores (Fig. 7A). Application of A-23187 to DDF-loaded HUVEC resulted in a modest increase in mitochondrial fluorescence intensity (Fig. 7B). When cells were treated with 30 mM D-glucose for 24 or 48 h, application of the calcium ionophore elicited a robust response, which was fivefold higher than that observed in HUVEC incubated in the euglycemic medium. There was no significant difference between responses observed after 24 or 48 h of treatment with D-glucose. L-Glucose added at the concentration of 30 mM did not reproduce the effects of the equivalent concentration of D-glucose (Fig. 7B). The exaggerated generation of superoxide anions in D-glucose-treated cells stimulated with A-23187 was significantly attenuated by pretreatment of HUVEC with a superoxide dismutase mimic, Mn-TBAP (Fig. 7C). Furthermore, isolated liver mitochondria from 22-wk-old ZDF and ZL rats showed that basal and stimulated levels of superoxide produced in ZDF mitochondria were significantly higher than in ZL mitochondria (Fig. 7D).

Hyperglycemic environment switches mtNOS from a NO-generating to a superoxide-producing enzyme. To assess the source(s) of A-23187-triggered production of superoxide anions in D-glucose-treated HUVEC, cells were pretreated with antimycin A (to block the pathway to complex III, cytochrome c, and complex IV) or L-NNA (to block any possible superoxide production by NOS). As shown in Fig. 8, the increased basal DDF fluorescence in HUVEC incubated with 30 mM D-glucose (but not the equivalent concentration of L-glucose), even before the application of A-23187, was attenuated by L-NNA (1 mM) and virtually returned to the control level with pretreatment with antimycin (10 μM). In contrast, a further increase in mitochondrial superoxide generation induced by A-23187 in HUVEC incu-

![Figure 3](http://ajpheart.physiology.org/)  
**Fig. 3.** Human embryonic kidney (HEK) cells, stably expressing human endothelial NO synthase (eNOS), exhibit Nω-nitro-L-arginine methyl ester (L-NAME)-inhibitable mitochondrial NO production. **A:** effects of 5 μg/ml A-23187 on mitochondrial DAF fluorescence in HEK (n = 11) and HEK/eNOS (n = 11) cells. *P < 0.05 compared with HEK cells. **B:** effects of 23.187 on mitochondrial NO production in HEK/eNOS (n = 11) cells are inhibited by pretreatment with L-NAME (n = 11) but not with Nω-nitro-D-arginine methyl ester (D-NAME; n = 10). *P < 0.05 compared with L-NAME-treated cells.

HUVEC | HEK  
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Mt | Cell | Mt | Cell  
GI5 | WT | Gl5 | Gl30 | Gl5 | Gl30 | 5 and 30 mM D-glucose, respectively; WT, wild type; eNOS, HEK-expressing eNOS.

![Figure 4](http://ajpheart.physiology.org/)  
**Fig. 4.** Expression of eNOS and cytochrome c oxidase (COX) in mitochondrial and whole cell lysates prepared from HUVEC and HEK cells. Mt, lysates of the mitochondrial fraction; Cell, whole cell lysates; Gl5 and Gl30, 5 and 30 mM D-glucose, respectively; WT, wild type; eNOS, HEK-expressing eNOS.
bated with 30 mM D-glucose (but not L-glucose) was abolished by L-NNA and attenuated by antimycin, suggesting that the respiratory chain is the major source of superoxide anions under basal conditions and that mtNOS is the major source of superoxide anions under the stimulated conditions modeled by calcium overload.

**DISCUSSION**

This study provides a complementary set of experimental data on mitochondrial generation of NO or O$_2^-$/H$_2$O$_2$ by endothelial cells exposed to normal (5 mM) or high (30 mM) glucose concentrations and by HEK/eNOS cells as well as by liver mitochondria isolated from ZDF rats. A remarkable reciprocity in the ability to generate these products appears to exist in the mitochondria: conditions leading to the increased production of O$_2^-$/H$_2$O$_2$ are associated with the suppression of NO. Could these processes be functionally coupled?

Mitochondrial generation of NO by an intrinsic NOS has been documented in several studies (2, 3, 13, 14, 19). Our data demonstrated immunodetectable eNOS in the mitochondrial fractions of HUVEC and HEK/eNOS (but not wild-type HEK) cells. In addition, we recently provided evidence for eNOS binding to the

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**Fig. 5.** Hyperglycemic environment inhibits mitochondrial NO production. A: HUVEC were incubated for 24–48 h in the presence of 30 mM D-glucose (D-Gl; $n = 11$), L-glucose (L-Gl) (25 mM + 5 mM D-glucose, $n = 10$) or 5 mM D-glucose ($n = 11$) and pulse chase loaded with DAF, and mitochondrial fluorescence was monitored. High D-glucose (but not L-glucose) resulted in a significant blunting of the A-23187-elicited increase in mitochondrial DAF fluorescence intensity. *$P < 0.05$ compared 5 mM D-glucose. B: pretreatment of HUVEC cultured for 24 h in 30 mM D-glucose ($n = 12$) with a cell-permeable superoxide dismutase mimetic, Mn-TBAP (50 μM, $n = 13$), restored mitochondrial NO production in response to A-23187. C: Western blot analysis of eNOS expression in whole cell and mitochondrial lysates. Cytochrome c oxidase expression was used as a marker of mitochondria. Note that incubation in 30 mM D-glucose did not affect the abundance of eNOS in either mitochondria or the whole cell. D: NO production by liver mitochondria isolated from Zucker diabetic fatty (ZDF) and control lean (ZL) rats. Differential NO responses in isolated liver mitochondria from diabetic and control rats are shown. Isolated mitochondrial preparations were obtained from the liver of ZDF and ZL rats. Mitochondria were loaded with DAF and stimulated with A-23187. Fluorescence was detected with a plate reader at different times after stimulation. Note that NO production by mitochondria obtained from ZDF rats was significantly blunted. *$P < 0.05$, ZDF vs. ZL rats, $n = 9$ in each group.

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**Fig. 6.** Influence of the ambient D-glucose concentration on DAF fluorescence intensity in HEK cells. HEK and HEK/eNOS cells incubated for 24 h in 5 or 30 mM D-glucose were loaded with DAF using the mitochondrial loading protocol. Basal (Bl) and A-23187-elicited (5 μg/ml) fluorescence (A23) were detected using a fluorescence plate reader. Experiments were performed in the presence or absence of either D-NAME or L-NAME. Note that HEK/eNOS cells exhibited higher fluorescence intensity at baseline (**$P < 0.05$ vs. HEK, $n = 7$) and responded to A-23187 with a L-NAME-inhibitable increase in fluorescence intensity (•$P < 0.05$ vs. HEK, $n = 7$). The responses observed in HEK/eNOS cells incubated in 30 mM D-glucose were blunted compared with cells in 5 mM D-glucose (**•$P < 0.05$ vs. 30 mM D-glucose, $n = 7$).
outer mitochondrial membrane of HUVEC and HEK/eNOS cells (S. Gao, unpublished observations), further implying that NO detected in mitochondria arises from the local rather than the plasmalemmal source.

Known pathways of mitochondrial O$_2$ generation include NADH dehydrogenase at complex I and ubiquinone-complex III (21). It has been demonstrated that hyperglycemia produces oxidant stress in endothelial cells and antioxidants prevent intracellular formation of advanced glycation endproducts (12, 32). Previous studies by Nishikawa et al. (25) have provided evidence that the tricarboxylic acid cycle rather than the malate-aspartate shuttle was mainly responsible for the oxidant stress in hyperglycemic endothelial cells. These authors have also demonstrated that a cell-permeable form of Mn-SOD not only reduced oxidant stress in hyperglycemia but also suppressed formation of sorbitol, advanced glycation endproducts, and activity of protein kinase C. Our data obtained with a superoxide dismutase mimetic, Mn-TBAP, are consistent with this observation. In addition, we demonstrated that mitochondrial O$_2$ generation was suppressed by the inhibition of NOS, thus raising the question of the contribution of mtNOS to the oxidant stress.

Several technical aspects of our studies and their interpretation deserve further elucidation. First, DAF
increase in DDF fluorescence intensity. In 30 mM D-glucose-treated cells (but not those from L-glucose), A-23187 resulted in a significantly increased over control; fluorescence intensity was significantly increased over control; basal fluorescence of control preparations, which was inhibited by AM. In mitochondria of the high D-glucose group (but not those from L-glucose), A-23187 resulted in a significant increase in DDF fluorescence. Pretreatment with L-NNA virtually abolished any calcium ionophore-induced increase in DDF fluorescence, whereas AM attenuated the response to A-23187.

is not an ideal NO reporter. As noted above, this indicator is light and calcium activatable (4). The former obstacle can be attenuated by minimizing the illumination (lower energy and shorter exposure), the strategy that was employed in our study. The latter property compounds the interpretation of results by creating uncertainty as to the causes of increased NO readout: artifact related to the NO-independent activation of DAF or calcium-induced stimulation of eNOS. The observation that the DAF fluorescence yield was decreased after treatment with L-NAME, but not D-NAME, despite the fact that calcium underwent similar pharmacological manipulations, argues in favor of calcium activation of eNOS. Utility of DDF as a ROS indicator has recently been questioned on the basis of findings that heme and hemoproteins or glycated albumin may interfere with the fluorescence quantum yield and that this indicator may stimulate per se the formation of ROS (26, 28, 33). However, the impact of these serious drawbacks on the fidelity of results was minimized in our experiments by performing parallel studies in resting, A-23187-stimulated, and L-NAME-treated mitochondria. The simultaneous analysis of all these variables allowed us to draw conclusions on the direction of ROS changes.

There is ample evidence that ROS can function as a NO-generating or as an \( \text{O}_2^- \)-generating enzyme, the latter being a result of uncoupling of oxygen reduction and arginine oxidation by NO (16, 28, 37, 38). Du et al. (10) indicated that high glucose leads to an increase in generation of reactive oxygen intermediates, which can be prevented not only by antioxidants but also by L-NNA. In contrast, Graier et al. (15) reported that increased superoxide formation by high D-glucose as well as a nonmetabolizable D-glucose analog, 3-O-methylglucopyranose, was insensitive to L-NNA. In our experiments, production of ROS was also uninhibitable by L-NNA under unstimulated conditions but became inhibitable only after stimulation. The fact that L-NNA inhibited not only mitochondrial NO production but also the generation of ROS argues in favor of the idea that, in hyperglycemia, the enzyme is uncoupled rather than NO is merely scavenged by superoxide. Experiments designed to stimulate mtNOS by increasing calcium influx demonstrated that this resulted in NO generation by endothelial cells maintained in the normoglycemic environment, whereas the similar stimulation of cells kept in the hyperglycemic environment resulted in the diminished NO production and markedly enhanced \( \text{O}_2^- \) generation. In addition, pretreatment with L-NNA, which had only a partial effect in suppressing \( \text{O}_2^- \) generation in normoglycemia, resulted in a dramatic inhibition of \( \text{O}_2^- \) generation in hyperglycemic endothelial cells, especially after calcium stimulation. Investigations by Hotta et al. (17) demonstrated that intramitochondrial calcium concentration, as measured with fura 2 in myocardial mitochondrial preparations, was not significantly affected by NOS inhibition under steady-state conditions. Of note, these data obtained intravitally were corroborated by the results of experiments in isolated mitochondria, thus reducing the chance that NO and \( \text{O}_2^- \) generated at the plasma membrane diffused into fluorophore-loaded mitochondria in the whole cell.

While the excessive generation of ROS has been shown to activate protein kinase C, induce aldose reductase activity, stimulate formation of advanced glycation endproducts and activate NF-κB (25), the consequences of reduced mitochondria-associated production of NO readily complement this list. Specifically, it has been demonstrated that NO suppresses aldose reductase activity (6) and inhibits the formation of advanced glycation endproducts (1).

In addition to these actions, endogenous NO has been shown to regulate the activity of cytochrome \( c \) oxidase (13, 14). Nanomolar concentrations of NO have been shown to increase the apparent Michaelis-Menten constant of cytochrome oxidase for oxygen, in both isolated mitochondria and cultured endothelial cells, and transiently decrease oxygen consumption (5, 7). These cellular data correspond well with the whole animal findings demonstrating that inhibition of NO increases total oxygen consumption (30, 31). It can be deduced, therefore, that under hyperglycemic conditions, endothelial cells exhibit dysregulation of this mechanism adjusting oxygen consumption to its availability. Indeed, this prediction is supported by the recent demonstration that bradykinin- or carbachol-
induced inhibition of oxygen consumption is impaired in diabetic heart muscle in the dog (42) and in the retina of diabetic patients without retinopathy during hyperglycemia (34). In addition, the combination of oxidative and nitrosative stress, leading to peroxynitrite formation, results in the irreversible inhibition of mitochondrial respiration through the iron-sulfur centers of complexes I and II of the respiratory chain (39). Our findings of the functional switch of mtNOS from a NO-generating to an O2-generating enzyme may also explain the observed failure to regulate oxygen consumption in diabetes.

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