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Title Page:

DAF-FM Diacetate Detects Impairment of Agonist-stimulated Nitric Oxide Synthesis by Elevated Glucose in Human Vascular Endothelial Cells: Reversal by Vitamin C and L-Sepiapterin

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Running Title Page:

Running title: Reversal of impaired NO synthesis in endothelial cells

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Abbreviations: DAF-FM, 4-amino-5-methylamino-2',7'-difluorofluorescein; eNOS, endothelial nitric oxide synthase; BH4, tetrahydrobiopterin; L-NAME, L-nitro-arginine methyl ester; NO, nitric oxide;

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Abstract

Elevated plasma glucose, as commonly seen in types I and II diabetes mellitus, is known to result in endothelial dysfunction, a condition characterized by a loss of nitric oxide (NO)-dependent regulation of vascular tone. In the present study, we have utilized a recently developed NO-sensitive fluorescent dye, 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate to examine directly the consequences of elevated glucose on agonist-evoked NO synthesis in cultured human vascular endothelial cells. Exposure of cells for 5-7 days to high (20 mM) external glucose markedly reduced NO production in response to ATP, histamine or the Ca ionophore calcimycin A23187, compared to 5 and 10 mM glucose concentrations. However, high glucose did not affect agonist-evoked elevations in cytosolic free calcium, as monitored by Fluo-3. Addition of vitamin C (150 μM) and L-sepiapterin (20 μM) for ~24 h to 20 mM glucose-treated cells improved stimulus-evoked NO synthesis, but had no effect on cells exposed to either 5 or 10 mM glucose. Similarly, impaired NO production in high glucose-treated cells was largely reversed by exposure (~3 hr) to superoxide dismutase. Cellular levels of eNOS protein were unaltered by elevated glucose treatment, and no further change was observed following addition of vitamin C and L-sepiapterin. Taken together, the results of our study serve to explain directly at the cellular level how glucose-impaired NO production in human endothelial cells may be reversed by agents that are reported clinically to improve endothelium-dependent vasorelaxation in patients.

Introduction

The vascular endothelium exerts precise control over mean systemic blood pressure through the synthesis and release of vasoactive factors (e.g. nitric oxide, prostaglandins, endothelin, arachadonic acid metabolites, etc.) that act on the surrounding smooth muscle layer(s) (Mombouli and Vanhoutte, 1999). In diabetes mellitus, an early consequence of elevated plasma glucose is the development of endothelial dysfunction, which is characterized by an impairment of nitric oxide-dependent vasorelaxation that can lead to retinopathy, end stage renal failure, peripheral neuropathy, atheroschlerosis and heart disease (Lüscher et al., 2003). It is now well recognized that a major consequence of the glucose-mediated metabolic alterations in vascular endothelium is the disruption of normal NO signal transduction (Cosentino and Lüscher, 1998;Creager et al., 2003), which may represent the primary cellular event that initiates the pathogenesis of more severe cardiovascular complications. The increased generation of superoxide radicals, as a result of glucose-mediated metabolic disruption, has been identified as a causative event contributing to endothelial cell dysfunction and the loss of NO synthesis/bioavailability (Brownlee, 2001). For example, impaired endothelium-dependent vasorelaxation in diabetic patients and rodent models of diabetes is improved upon administration of either an anti-oxidant (e.g. vitamin C) (Ting et al., 1996;Timimi et al., 1998;Beckman et al., 2001) or essential enzyme cofactors that support proper eNOS function (e.g. tetrahydrobiopterin) (Pieper, 1997;Heitzer et al., 2000;Shinozaki et al., 1999;Pannirselvam et al., 2003). Elucidation of the cellular events underlying these observations would greatly increase our understanding of endothelial dysfunction and suggest more effective strategies for clinical treatment of this condition.

To examine how elevated glucose affects the kinetics and magnitude of agonist-evoked NO production in real-time, we have utilized the NO-sensitive fluorescent dye 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate to monitor NO synthesis directly in cultured human endothelial cells exposed to 5, 10 or 20 mM external glucose for 5-7 days. Prolonged exposure to elevated glucose depressed agonist-evoked NO production in response to histamine, ATP or the calcium ionophore calcimycin A23187, however, short-term addition of vitamin C (ascorbic acid) and L-sepiapterin (i.e. 24 h) or superoxide dismutase (i.e. 3 h) largely reversed this impairment. Elevated glucose did not affect agonist-induced intracellular Ca^{2+} transients. The amounts of immunoreactive NO synthase protein (eNOS) detected in all three groups of glucose-treated cells were unchanged, and were not noticeably affected following exposure to vitamin C and/or L-sepiapterin, a biosynthetic precursor of tetrahydrobiopterin (BH4). Taken together, the findings of our study are consistent with an impairment of agonist-evoked NO production in human endothelial cells by glucose-induced oxidative stress, and that counteracting this stress leads to a direct increase in NO bioavailability.

Materials and Methods

Preparation of EA.hy926 cells

The cultured human umbilical cell line, EA.hy926 cell line (Edgell et al., 1983), was kindly provided by Dr. Cora-Jean Edgell, Dept. of Pathology, the University of North Carolina. Cells were grown at 37^{o}C in 5% CO_2 on 100 mm tissue culture-quality plastic dishes in Dulbecco's modified Eagle's medium (DMEM) containing high glucose, 10% (v/v) heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) and a hypoxanthine/thymidine (HT) media supplement (Sigma-Aldrich Chemical Co., St. Louis, MO). Following replating onto 35 mm dishes, cells at 50-80% confluence were treated for 4-6 days in DMEM containing 1% heat-inactivated FBS (to maintain cells in a quiescent state) and 5, 10 or 20 mM D-glucose (mannose was added as required to achieve consistent osmolality). Glucose-treated cells were incubated with either 150 μM vitamin C or 20 μM L-sepiapterin, or both, and then harvested ~24 h later for Western blot analysis. Alternatively, some glucose-treated cells were reseeded onto fibronectin-coated glass coverslips, maintained under the same concentrations of glucose, and then loaded the next day with the membrane permeable form of the NO-sensitive fluorescent dye DAF-FM diacetate (see below). For some experiments, superoxide dismutase (SOD, isolated from bovine erythrocytes, Sigma-Aldrich Chemical Co.) was added to the culture medium of glucose-treated cells for a period of 3 hr at 37 ^{o}C immediately prior to recordings of DAF-FM fluorescence. The final concentration of SOD in the medium was 200 U/ml.

Measurement of NO Production in EA.hy926 Cells

Intracellular NO was monitored with 4-amino-5-methylamino- 2',7'-difluorofluorescein

(DAF-FM) diacetate, a pH-insensitive fluorescent dye that emits increased fluorescence following reaction with an active intermediate of NO formed during the spontaneous oxidation of NO to NO_2^- (Kojima et al., 1998). EA.hy926 cells seeded on glass cover slips were incubated at 22^{o}C for 30 min in DMEM containing a low concentration (i.e. 0.5 μM) of DAF-FM diacetate (Molecular Probes/Invitrogen, Eugene, OR); this condition significantly reduced the background autofluorescence and improved the signal-to-noise ratio of NO detection in single cells (Leikert et al., 2001). After loading, cells were rinsed three times with DMEM and then placed on the stage of a Nikon inverted microscope equipped with a SFX-1 microfluorimeter (Solamere Technology Group, Salt Lake City, UT). Typically, one to three EA.hy926 cells were identified in a field and NO fluorescence was measured using excitation and emission wavelengths of 488 nm and 520 nm, respectively. During the experiments, cells were superfused in a 0.3 ml bath chamber at 35 °C under a constant flow (~1 ml/min) of HEPES-buffered saline (135 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4). Because the fluorescence intensity of the triazole or NO-bound form of DAF-FM diacetate originating from a single cell was usually quite modest, it was difficult to use a neutral density filter in the excitation light path. Therefore, in order to reduce photobleaching of the NO-modified DAF-FM diacetate dye that can occur during continuous cell illumination, cells were occasionally exposed to 488 nm light for periods of 5 sec, once every 20 sec,. All solutions used in the present study contained 1 mM L-arginine, except for experiments in which cells were exposed to L-NAME.

Measurement of Intracellular Calcium Transients in EA.hy926 Cells

Relative changes in cytosolic free calcium were measured using the fluorescent indicator Fluo-3 (Kao et al., 1989). Cells re-plated on glass coverslips were incubated for 30 min at room temperature in serum-free DMEM containing 2 μM Fluo-3/AM (Molecular Probes/Invitrogen). After loading, cells were washed three times with DMEM. Fluo-3 fluorescence signals in EA.hy926 cells were monitored as described above for measurements of NO production.

Western Blotting

Glucose-treated EA.hy926 cells, growing on tissue culture dishes, were rinsed once with sterile phosphate-buffered saline (PBS) and then detached by a ~1 min incubation with 0.05% trypsin/0.5 mM EDTA in PBS. Detached cells were collected by centrifugation at ~100 x g for 5 min, and the stored at –80°C as intact cell pellets. These pellets were suspended in ~0.5 ml of ice-cold lysis buffer (20 mM Tris HCl, pH 7.4, 140 mM NaCl, 5 mM KCl, 1% (v/v) Triton X-100, 1 mM EGTA, 2 mM EDTA, 1 mM DTT, 1 mM benzamidine, 0.2 mM PMSF and 5 μg/ml each of leupeptin, aprotinin, and pepstatin A), followed by incubation on ice for 30 min. Protein concentrations of the whole cell lysates were measured using a modified Lowry procedure and equal amounts of total protein were mixed with Laemmli sample buffer containing 0.5% (v/v) β-mercaptoethanol. Protein samples were incubated for ~5 min at 95 °C and then resolved by denaturing SDS-PAGE using a 9% separating gel. Following overnight transfer of resolved proteins to nitrocellulose membrane (18 V, 4°C), Western blotting was performed using an anti-human eNOS monoclonal antibody (1:1000 dilution) (BD Biosciences, Mississauga, Ont., Canada), as we have earlier described for other proteins (Swayze and Braun, 2001).

Quantification of eNOS immunoreactivity was carried out by image analysis densitometry

using Quantity One software (BioRad Laboratories, Hercules, CA). Under each glucose condition, differences between drug-treated and control groups were calculated following normalization to the corresponding Coomassie blue-stained gel containing the same lysate samples (see below) using the following formula:

% Difference = [(eNOS Intensity$_{drug}$ - eNOS Intensity$_{control}$) / eNOS Intensity$_{control}$] x 100

Where eNOS Intensity$_{drug}$ is the amount of eNOS immunoreactivity detected in glucose-treated cells exposed to vitamin C and/or L-sepiapterin and eNOS Intensity$_{control}$ is the level of eNOS immunoreactivity detected in cells treated by same concentration of glucose alone. Note that in the far right-hand lane of the blot displayed in Fig. 7A, a lysate sample derived from cultured HUVECs that were collected one passage prior to all the other cells used served as a positive control for eNOS expression.

To ensure that observed changes in eNOS immunoreactivity were not skewed by unequal loading of total protein between lanes, the same cells samples analyzed by Western blotting were also resolved on a parallel SDS polyacrylamide gel and then stained with Coomassie Blue R-250. The total protein staining intensity observed in cells treated by glucose alone (set to 100%) was compared to that of cells treated at the same glucose concentration and also exposed to vitamin C and/or L-sepiapterin; differences in protein staining >5% (higher or lower) were noted. These differences were then used to correct the amount of eNOS immunoreactivity detected in glucose-treated cells exposed to vitamin C and/or L-sepiapterin prior to calculating the percent differences in eNOS immunoreactivity, as described above.

Statistical Analyses

All experiments were repeated at least four times. Mean values from independent

experiments were statistically analyzed by Student's *t*-test, and multiple comparisons were performed using one-way ANOVA for repeated measurements, together with a Dunnett's post-hoc *t*-test. Differences were considered to be significant when $P < 0.05$.

Results

Agonist-induced NO synthesis in the endothelial cell line EA.hy926 cell line (Edgell et al., 1983) was monitored in either single cells or small groups of 2-5 cells using the membrane permeable fluorescent dye DAF-FM diacetate (Kojima et al., 1998). Brief exposure of dye-loaded cells to either histamine or the purinergic agonist ATP evoked increases in cellular fluorescence that were reproducible upon a second agonist exposure (Fig. 1A). In the absence of agonist stimulation, basal fluorescence from DAF-FM loaded cells did not noticeably change over a period of 5-10 min. Addition of the direct NO donor spermine nonoate (10 μM) produced a much larger fluorescence signal in cells compared to ATP or histamine, consistent with the spontaneous release of NO produced by this compound (Feelisch, 1998). Similar results were observed with the clinical nitrovasodilator sodium nitroprusside (10 μM) (data not shown). Treatment (~10 min) of dye-loaded cells with the competitive eNOS inhibitor L-nitro-arginine methyl ester (L-NAME) (Knowles and Moncada, 1994) following the initial agonist exposure abolished subsequent increases in fluorescence upon re-addition of the same agonist (e.g. histamine or ATP) (Fig. 1B). However, L-NAME-treated cells still produced a qualitatively similar increase in fluorescence upon exposure to spermine nonoate, indicating that L-NAME treatment did not compromise cell viability or the responsiveness of the intracellular DAF-FM diacetate fluorescent reporter. Under the experimental conditions used to detect agonist-stimulated NO synthesis in single endothelial cells (see Methods), the evoked DAF-FM fluorescence increases were found to be transient in nature. Similarly, Qiu et al. have reported that DAF-2 fluorescence signals may be transient in populations of stimulated bovine aortic endothelial cells detected using a real-time imaging strategy; this phenomenon likely

reflects photobleaching of the NO-bound form of the dye (Qiu et al., 2001). However, as photobleaching appears to affect primarily the triazole or NO-modified form of DAF (Kojima et al., 1998), which represents only a small fraction of the total intracellular pool (Leikert et al., 2001), the large excess of unconverted dye in the cytoplasm remains fully sensitive to *de novo* NO synthesis, thereby allowing repeated measurements of stimulated NO production from the same cell(s) (Fig. 1A, B).

There is now ample evidence implicating a causative role for elevated plasma glucose, as seen in types I and II diabetes mellitus, in the pathogenesis of endothelial dysfunction, which is strongly associated with impaired NO availability (Brownlee, 2001;Creager et al., 2003). Use of DAF-FM diacetate as a real-time reporter of NO synthesis provides the opportunity to examine directly how prolonged exposure to elevated glucose impacts agonist-evoked NO synthesis. Cells maintained in 5 mM glucose displayed progressive increases in the NO fluorescence signal in response to increasing concentrations of ATP (Fig. 2A). However, agonist-evoked fluorescence signals in cells treated with 20 mM glucose were significantly blunted compared to those observed with 5 mM glucose treatment (Fig. 2B). Quantification of the observed concentration-dependent increases in NO fluorescence evoked by ATP in 5 and 20 mM glucose-treated cells are plotted in Figure 2D, along with responses observed in cells treated with an intermediate glucose concentration (10 mM). Over the concentration range of 1 – 100 μM, ATP evoked similar increases in NO-associated fluorescence in 5 and 10 mM glucose-treated cells, however, fluorescence signals were significantly lower in cells treated with 20 mM glucose. In the continued presence of elevated glucose, the blunted NO fluorescence signals observed in 20 mM glucose-treated cells were largely reversed by a ~24

hour exposure to vitamin C (150 μM), a potent anti-oxidant (Frei et al., 1989), and L-sepiapterin (20 μM), an endogenous precursor that can be converted to the eNOS cofactor BH4 via a cellular 'salvage' pathway (Werner-Felmayer et al., 1993)(Fig. 2C). Similar findings have been reported in murine mesangial cells treated by 25 mM glucose, in which vitamin C and BH4 were shown to reverse the impairment of cytokine-induced NO production (Prabhakar, 2001). In contrast to these effects observed under high glucose conditions, exposure to vitamin C and L-sepiapterin did not enhance ATP-evoked increases in NO synthesis in endothelial cells treated with either 5 or 10 mM glucose (Fig. 2E) and appeared to dampen somewhat the maximal production of NO in response to agonist stimulation. Previous studies have reported that excess BH4/L-sepiapterin may autoxidize to generate superoxide radicals, thereby reducing NO availability and endothelium-dependent vasodilation (Kirsch et al., 2003;Mitchell et al., 2004).

To rule out that the observed blunted response to ATP was somehow selective for purinergic agonists, we further examined NO synthesis in response to histamine. As shown in Figures 3A and B, histamine-evoked increases in NO-associated fluorescence were also depressed in cells treated with 20 mM glucose, compared to those treated with 5 mM glucose. As in the case of ATP, addition of vitamin C and L-sepiapterin in the continued presence of 20 mM glucose largely reversed the blunted response to histamine, but produced no enhancement of agonist-evoked NO production in cells exposed to either 5 or 10 mM glucose (Figs. 3C-E).

Given the effectiveness of vitamin C and L-sepiapterin to improve agonist-evoked increases in NO production in high glucose-treated cells, we examined the effect of each agent individually on NO production in response to ATP. In glucose-treated cells exposed separately

to either vitamin C (Fig. 4A) or L-sepiapterin (Fig. 4B), agonist-induced increases in NO synthesis were only partially restored, in contrast to the results obtained with combined vitamin C/L-sepiapterin treatment (see Figs. 2E and 3E).

As disruption of agonist-evoked NO synthesis by elevated glucose could potentially be explained by altered calcium handling, we performed fluorescence-based measurements of ATP- and histamine-evoked cytosolic calcium transients in glucose-treated cells. Figure 5 shows fluorescence tracings of calcium transients evoked by either ATP (panels A and C) or histamine (panels B and D) in single endothelial cells treated with either 5 or 20 mM glucose, as monitored with the membrane permeable, calcium-sensitive dye Fluo-3 (Kao et al., 1989). Quantitative analyses of these signals indicated no significant differences in the magnitudes of agonist-induced calcium transients in cells treated by 5, 10 or 20 mM glucose (Fig. 5E, F).

Based on these observations, we next examined the direct calcium-dependent activation of eNOS using the Ca^{2+} ionophore calcimycin A23187, which induces elevation of cytosolic Ca^{2+} by directly releasing intracellular Ca^{2+} stores and promoting entry of external calcium. Similar to the results observed with ATP and histamine (see Figs. 2 and 3), stimulation of NO production by calcimycin A23187 (0.1 to 1 μM) was significantly lower in cells treated with high glucose *versus* those exposed to either 5 or 10 mM external glucose (Fig. 6A). Furthermore, addition of 150 μM vitamin C and 20 μM L-sepiapterin to glucose-treated cells improved calcimycin A23187-induced NO synthesis in EA.hy926 cells treated with 20 mM glucose (Fig. 6B), similar to the recovery of NO production in response to either ATP (Fig. 2E) or histamine (Fig. 3E). Finally, we observed that exposure to either vitamin C or L-sepiapterin alone only partially restored the calcimycin A23187-induced NO response in 20 mM

glucose-treated cells (data not shown), similar to the data presented in Figure 4. Mechanistically, these observations suggest that glucose-induced impairment of stimulated NO production occurs subsequent to the elevation of cytosolic free Ca^{2+}, and may possibly involve either a disruption of eNOS activity itself or a decrease in the availability of newly synthesized NO.

Earlier studies have reported that chronic elevated glucose may alter the cellular expression of eNOS protein (Cosentino et al., 1997;Chakravarthy et al., 1998;Ding et al., 2000), which may negatively impact upon NO production. Using Western blot analyses, endogenous eNOS was detected as a single ~140 kDa immunoreactive band in EA.hy926 human endothelial cells under all three glucose treatment conditions and in either absence or presence of vitamin C and/or L-sepiapterin (Fig. 7A). Using densitometric image analysis (see Methods), we found that the levels of eNOS immunoreactive protein in cells treated by either 10 or 20 mM glucose alone were 96.9 ± 8.0% and 98.1 ± 3.6% (mean ± SE, n = 6), respectively, of the eNOS immunoreactivity detected in 5 mM glucose-treated cells (set to 100%). Figure 7B plots the percent difference in eNOS immunoreactivity calculated between glucose-treated cells without exposure to either vitamin C or L-sepiapterin (control, set to 100%) and cells exposed to these agents at the same glucose concentration. Although modest differences in eNOS immunoreactivity were observed in cells exposed to vitamin C and/or L-sepiapterin, none of these changes were found to be statistically significant.

The observed improvement in agonist-induced NO production in high glucose-treated cells following addition of vitamin C (Figs. 2, 3, 4 and 6) suggested that actively lowering cellular oxidants, such as superoxide radicals, may itself be beneficial. Elevated glucose is often associated with increased oxidative stress in the vascular wall (Giugliano et al., 1996) and this

effect can be reduced by treatment with agents such as superoxide dismutase (SOD) that scavenge superoxide anions (Ammar et al., 2000;Nishikawa et al., 2000). To examine this possibility, glucose-treated EA.hy926 cells were incubated for 3 hr with superoxide dismutase (200 U/ml) in the continued presence of 5, 10 or 20 mM glucose and then immediately utilized for recordings of DAF-FM fluorescence. As shown in Figure 8, addition of SOD reversed the impairment of ATP-stimulated NO production typically observed in EA.hy926 cells treated with 20 mM glucose; however, SOD appeared to have little or no effect on agonist-induced NO production in cells treated by 5 or 10 mM glucose (compare to data in Fig. 2D). These observations are thus consistent with an increased presence of superoxide radicals in cells undergoing prolonged exposure to elevated glucose.

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Discussion

It is now recognized clinically that chronic elevation of plasma glucose, characteristic of types I and II diabetes mellitus, leads to impaired NO-dependent vasorelaxation (i.e. endothelium dysfunction), as a result of decreased synthesis and/or bioavailability of NO. Clinical studies examining endothelial dysfunction associated with hypertension or coronary artery disease have reported that the severity of dysfunction in patients can be reduced by administration of vitamin C (ascorbic acid) (Levine et al., 1996;Taddei et al., 1998). Similarly, in rodent and human diabetic models of endothelial dysfunction, administration of either L-sepiapterin (Pannirselvam et al., 2003) or BH4 (Pieper, 1997;Shinozaki et al., 1999;Heitzer et al., 2000) is reported to improve endothelium-dependent vasorelaxation. However, it remains unclear from the majority of these reports what aspect(s) of vascular function and/or properties are affected by such treatments. In the present study, we have utilized the NO-sensitive fluorescent dye DAF-FM diacetate (Kojima et al., 1998) to examine directly, in real-time, how agonist-induced NO synthesis is affected by elevated extracellular glucose in cultured human vascular endothelial cells and how vitamin C and L-sepiapterin influence this process. Importantly, agonist-evoked increases in DAF-FM fluorescence were inhibited by pre-treatment of dye-loaded cells with L-NAME, a competitive inhibitor of endothelial nitric oxide synthase (eNOS) (Knowles and Moncada, 1994), whereas L-NAME had no effect on the fluorescence increase produced by addition of the direct NO donor spermine nonoate (Feelisch, 1998). These data are thus consistent with the reported properties of DAF-FM diacetate in endothelial cells (Nakatsubo et al., 1998), and support the conclusion that the increases we have observed in DAF-FM diacetate fluorescence reflect agonist-stimulated elevations in cellular

NO.

In the presence of either normal (i.e. 5 mM, ~90 mg/dl), or modestly elevated extracellular glucose (i.e. 10 mM, ~180 mg/dl), both ATP and histamine produced similar concentration-dependent increases in DAF-FM fluorescence (Figs. 2A and 3A). However, NO synthesis was markedly blunted in response to the same concentrations of these agonists in cells exposed to a higher concentration (i.e. 20 mM, ~360 mg/dl) of extracellular glucose. Similarly, NO production stimulated by calcimycin A23187, which directly elevates the cytosolic free [Ca^{2+}] by promoting release from intracellular stores and calcium influx, was also decreased by 20 mM glucose treatment (Figure 6). These data thus demonstrate that above a threshold concentration, elevated external glucose interferes with stimulated NO production at the level of individual vascular endothelial cells.

Although prolonged exposure to elevated glucose may depress endothelial cell NO production by altering a number of cellular properties, an obvious candidate is altered release of intracellular Ca^{2+} stores, which would be expected to have a direct impact on eNOS activation and NO synthesis. Using Fluo-3 to monitor agonist-stimulated elevations in cytosolic free Ca^{2+} in glucose-treated cells, we did not detect any apparent differences in either the magnitude or kinetics of intracellular Ca^{2+} transients evoked by brief exposure to either ATP or histamine (Fig. 5). Such observations provide important insights regarding the integrity of cellular function in these different treatment groups. First, the similar concentration dependence for evoked Ca^{2+} transients by either ATP or histamine suggest that high glucose exposure does not alter receptor/G-protein activation or the signaling pathway leading to release of intracellular Ca^{2+} stores. Second, the cellular mechanisms responsible for maintaining intracellular Ca^{2+}

stores and promoting entry of external Ca^{2+} following store depletion do not appear to be compromised by prolonged treatment with elevated glucose. Collectively, these observations suggest that high glucose does not impair the signal transduction events leading to the generation of intracellular Ca^{2+} transients in human endothelial cells, but rather, point to a defect(s) downstream of this event that may be responsible for decreased NO availability.

Based on steady-state measurements of NO metabolites, such as nitrites, it has been suggested that agonist-stimulated NO synthesis may be lower in arterial vessels from diabetic animals (Shinozaki et al., 1999). An important finding of our study is that the observed impairment of stimulus-evoked NO production by 20 mM external glucose could be largely reversed by addition of vitamin C (150 μM), an effective anti-oxidant in plasma and tissues (Frei et al., 1989) and L-sepiapterin (20 μM), a biosynthetic precursor of the eNOS co-factor BH4 (Werner-Felmayer and Gross, 1996). Clinically, administration of vitamin C has been shown to improve endothelium-dependent vasorelaxation in diseased humans (Ting et al., 1996;Timimi et al., 1998;Levine et al., 1996;Taddei et al., 1998), and similar beneficial effects have been reported for BH4, or its biosynthetic precursor, L-sepiapterin (Heitzer et al., 2000;Maier et al., 2000;Tiefenbacher et al., 2000;Pieper, 1997;Pannirselvam et al., 2003). Under normal physiologic conditions, however, administration of these compounds does not further enhance endothelial function (Ting et al., 1996;Pieper, 1997;Timimi et al., 1998;Taddei et al., 1998;Heitzer et al., 2000;Tiefenbacher et al., 2000;Beckman et al., 2001). Our results demonstrating that vitamin C and L-sepiapterin do not increase agonist-evoked NO synthesis in endothelial cells treated with either 5 or 10 mM glucose (Figs. 2 and 3) are entirely consistent with these earlier reports. While it has not been readily apparent which cell type(s) is primarily

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affected by these therapeutic agents in whole animal or isolated vessel studies, our results provide clear evidence that vitamin C and L-sepiapterin act directly on vascular endothelial cells to improve agonist-stimulated NO synthesis and/or availability.

It is now well recognized that high glucose produces a number of metabolic changes in human cells, including the production of advanced glycation end products, the activation of protein kinase C and elevated production of superoxide anions ($O_2^{\cdot -}$) (for review, see Brownlee, 2001). In human endothelial cells, elevated glucose may increase superoxide anions (Cosentino et al., 1997;Creager et al., 2003), which can rapidly react with NO *in situ* to produce the oxidizing agent peroxynitrite ($ONOO^-$) (Squadrito and Pryor, 1995). Peroxynitrite can cause oxidation of BH4 (Werner-Felmayer and Gross, 1996) and under conditions of limited BH4 levels, eNOS itself may be capable of generating superoxide anions (Pou et al., 1992;Vasquez-Vivar et al., 2002). Such a process would be expected to increase the formation of peroxynitrite, thereby contributing to the progression of endothelial dysfunction. Our results (see Fig. 8) showing that prior addition of superoxide dismutase for ~3 h reversed the impairment of agonist-stimulated NO synthesis in high glucose-treated endothelial cells is thus consistent with an elevated level of oxidative stress in these cells (Nishikawa et al., 2000). Furthermore, vitamin C, a potent anti-oxidant, is reported to enhance eNOS enzyme activity and NO synthesis by stabilizing the cellular levels of BH4 (Huang et al., 2001;Heller et al., 2001). Mechanistically, vitamin C may thus prevent peroxynitrite formation and BH4 oxidation by neutralizing superoxide radicals; supplementation by L-sepiapterin may further ensure adequate BH4 levels to fully support eNOS activity. In some preparations, high glucose is reported to altered expression of eNOS itself (Cosentino et al., 1997;Shinozaki et al.,

1999;Ding et al., 2000;Chakravarthy et al., 1998), which may result in lower NO availability via reduced synthesis or increased chemical transformation of NO (Li et al., 2002). In our study, however, the cellular levels of eNOS protein in human endothelial cells were not significantly changed following 5-7 days of elevated glucose treatment (Figure 7). A similar lack of change in eNOS protein in the presence of elevated glucose has been previously reported (Wu and Meininger, 1995). Changes in eNOS protein levels may thus reflect differences in the degree or progression of cellular dysfunction induced by elevated glucose in endothelial cell preparations, with no change perhaps representing the mildest response.

In patients displaying endothelial dysfunction, acute administration of vitamin C is reported to improve endothelium-dependent vasodilation (Levine et al., 1996;Taddei et al., 1998), and L-sepiapterin/BH4 has been shown to produce similar beneficial effects in human (Heitzer et al., 2000;Tiefenbacher et al., 2000;Maier et al., 2000) and rodent models of diabetes mellitus (Shinozaki et al., 1999;Meininger et al., 2000;Pannirselvam et al., 2003). Although it has been widely assumed that the improved vascular reactivity observed in these studies reflects increased NO bioavailability, our results provide the first direct evidence that vitamin C and L-sepiapterin act by restoring the levels of NO in stimulated human vascular endothelial cells in the continued presence of elevated glucose, most likely via a reduction in oxidative stress. Such data thus increase our understanding of how these agents may improve endothelium-dependent vasorelaxation in hyperglycemic patients. In future studies, fluorescence-based techniques may be exploited to monitor changes in endothelial NO synthesis under basal conditions, which contributes to the long-term maintenance of vascular function in health and disease.

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Figure Legends

Figure 1 -Fluorescence detection of agonist-stimulated NO synthesis in EA.hy926 cells is blocked by treatment with the NOS inhibitor L-NAME. EA.hy926 cells maintained in 5 mM glucose and loaded with the NO-sensitive dye DAF-FM diacetate generate reproducible fluorescence signals in response to successive exposures to the receptor agonists histamine (His.) and ATP, as indicated by the horizontal bars (panel A). Addition of the direct NO donor spermine nonoate (10 μM) following washout of the receptor agonists is indicated by the arrow. In panel B, DAF-FM-loaded cells were briefly treated (10 min) with the competitive NOS inhibitor L-NAME in between the two rounds of agonist exposure; subsequent addition of spermine nonoate is shown by the arrow. Scale bars shown at the bottom of the figure apply to the fluorescence tracings in both panels A and B.

Figure 2 -The purinergic agonist ATP stimulates NO production in a dose-dependent manner. EA.hy926 cells treated with 5 mM glucose (panel A), 20 mM glucose (panel B) or 20 mM glucose + 0.15 mM vitamin C and 20 μM L-sepiapterin (panel C) were loaded with DAF-FM diacetate and then exposed to increasing concentrations (1-100 μM) of ATP, as indicated by the horizontal bars above the fluorescence tracings. The time bar shown in panel C applies to all three sets of recordings. Quantification of the concentration-dependent increases in NO-associated fluorescence evoked by ATP in cells treated with 5, 10 or 20 mM glucose is plotted in panel D. Agonist-evoked increases in NO synthesis recorded in glucose-treated cells also exposed to 0.15 mM vitamin C + 20 μM L-sepiapterin are plotted in panel E. The legend shown in panel E also describes the symbols in panel D. Results are shown as means ± S.E. of 4-9 recordings from individual preparations of cells treated at each glucose concentration.

Asterisks denote values that are significantly different than corresponding values in 5 mM glucose-treated cells, $P < 0.05$.

Figure 3 - Histamine stimulates NO production in a dose-dependent manner. EA.hy926 cells treated with 5 mM glucose (panel A), 20 mM glucose (panel B) or 20 mM glucose + 0.15 mM vitamin C and 20 μM L-sepiapterin (panel C) were loaded with DAF-FM diacetate and then exposed to increasing concentrations (1-100 μM) of histamine, as indicated by the horizontal bars above the fluorescence tracings. The time bar shown in panel C applies to all three sets of recordings. Quantification of the concentration-dependent increases in NO fluorescence evoked by histamine in cells treated with 5, 10 or 20 mM glucose is plotted in panel D. The agonist-dependent increases in NO synthesis recorded in glucose-treated cells also exposed to 0.15 mM vitamin C + 20 μM L-sepiapterin are plotted in panel E. The description of symbols in panel E also applies to panel D. Results are shown as means ± S.E. of 4-8 recordings from individual preparations of cells treated at each glucose concentration. Asterisks denote values that are significantly different than corresponding values in 5 mM glucose-treated cells, $P < 0.05$.

Figure 4 – Vitamin C or L-sepiapterin partially restore ATP-mediated increases in NO production in high glucose-treated cells. EA.hy926 cells treated with 5, 10 or 20 mM glucose were exposed for approximately 24 hr to either 0.15 mM vitamin C (panel A) or 20 μM L-sepiapterin (panel B). Data points represent the means ± S.E. of agonist-induced changes in DAF-FM fluorescence recorded from 4-5 different populations of cells treated at the indicated concentrations of glucose. In panels A and B, the data depicting ATP-evoked NO production in cells treated by 20 mM glucose alone (solid symbols) have been taken from Figure 2D and are

presented here for comparison purposes. Asterisks denote values for 20 mM glucose-treated cells that are significantly different than corresponding values observed in cells treated with 5 mM glucose, $P < 0.05$.

Figure 5 – Agonist-evoked intracellular Ca^{2+} transients in EA.hy926 cells are unaffected by elevated glucose treatment. Glucose-treated EA.hy926 cells were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-3 and then exposed briefly to increasing concentrations of either ATP (panels A and B) or histamine (panels C and D), as indicated by the horizontal bars. Representative fluorescence tracings from single endothelial cells treated with either 5 or 20 mM external glucose are shown in panels A-D. Quantification of the agonist-evoked increases in Fluo-3 fluorescence evoked in cells treated with 5, 10 or 20 mM glucose is plotted in panels E and F. Values are shown as means ± S.E. calculated from 'n' individual cells analyzed at each glucose concentration.

Figure 6 – Effect of high glucose treatment on NO production in response to the calcium ionophore calcimycin A23187 in the absence and presence of vitamin C and L-sepiapterin. Panel A shows a plot of evoked increases in DAF-FM fluorescence in glucose-treated EA.hy926 cells in response to 1 min exposures to calcimycin A23187 (0.1, 0.3 or 1 μM). In panel B, calcimycin A23187-induced increases in DAF-FM fluorescence were recorded from glucose-treated cells that had been incubated with 0.15 mM vitamin C and 20 μM L-sepiapterin (~24 hr exposure). Results are shown as the means ± S.E. of 4-5 individual recordings under each experimental condition. Asterisks denote values that are significantly different than corresponding values in 5 mM glucose-treated cells, $P < 0.05$.

Figure 7 - Immunodetection of eNOS protein in glucose-treated EA.hy926 cells exposed to

vitamin C and/or L-sepiapaterin (panel A). Cells maintained in either 5, 10 or 20 mM glucose for 5-6 days were exposed for an additional 24 hr to 0.15 mM vitamin C and 20 μM L-sepiapterin, either alone or in combination, as indicated by the minus (-) and plus (+) signs above the western blot. Equal amounts of whole cell lysate protein were probed using an anti-eNOS monoclonal antibody and endogenous eNOS was detected as a single immunoreactive band of ~140 kDa. The sample marked 'eNOS Positive Control' in the far right hand lane represents a culture of untreated EA.hy926 cells collected one passage prior to those cells used in the various treatment groups. The electrophoretic positions of molecular weight markers are shown on the left hand side of the blot. In panel B, immunoreactive eNOS bands in the various treatment groups were quantified by image analysis densitometry and corrected for minor differences in total protein loading, as described in the Methods section. Values represent the means ± S.E. of 6 experiments, in which all 12 treatment conditions were carried out and analyzed simultaneously (refer to panel A). None of the modest differences in eNOS immunoreactivity observed in the presence of vitamin C and/or L-sepiapterin at a given glucose concentration were found to be statistically significant with respect to cells treated by glucose alone, $P > 0.05$.

Figure 8 – Superoxide dismutase (SOD) restores ATP-induced NO synthesis in high glucose-treated endothelial cells. SOD (from bovine erythrocytes) was added to the culture medium of EA.hy926 cells treated with 5, 10 or 20 mM glucose at a final concentration of 200 U/ml. Cells were pre-treated with SOD for approximately 3 h immediately prior to recordings of DAF-FM fluorescence. Data points represent the means ± S.E. of agonist-induced changes in DAF-FM fluorescence recorded from 5 different populations of cells treated at the indicated

concentrations of glucose.

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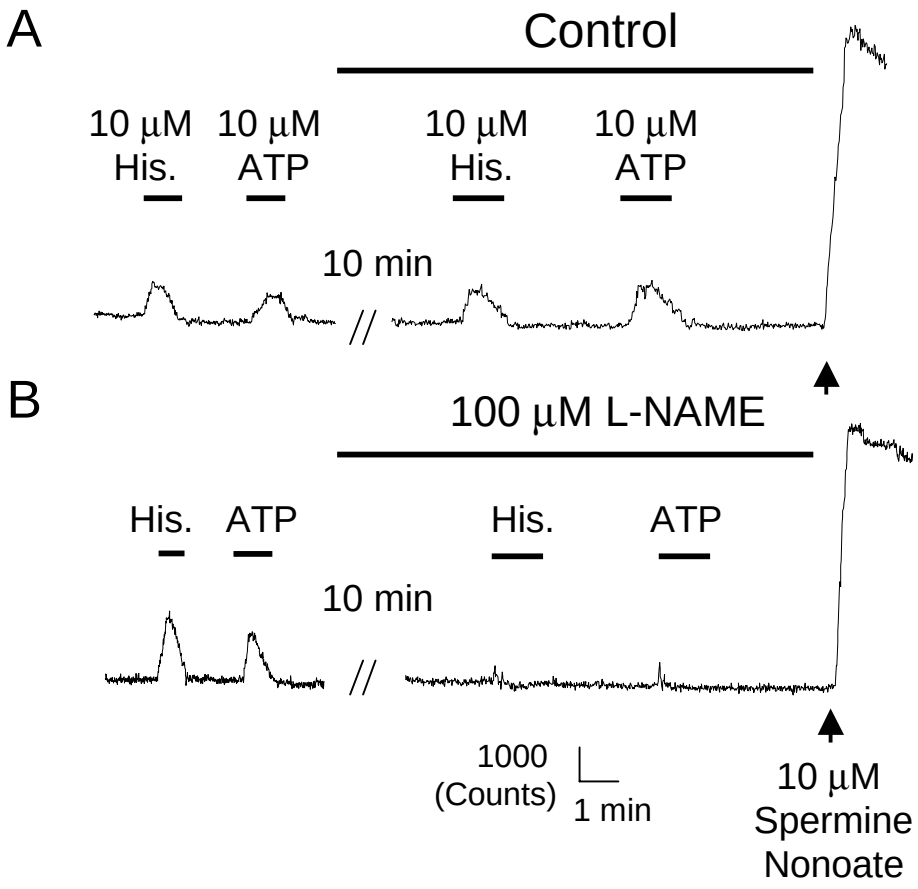

Figure 1

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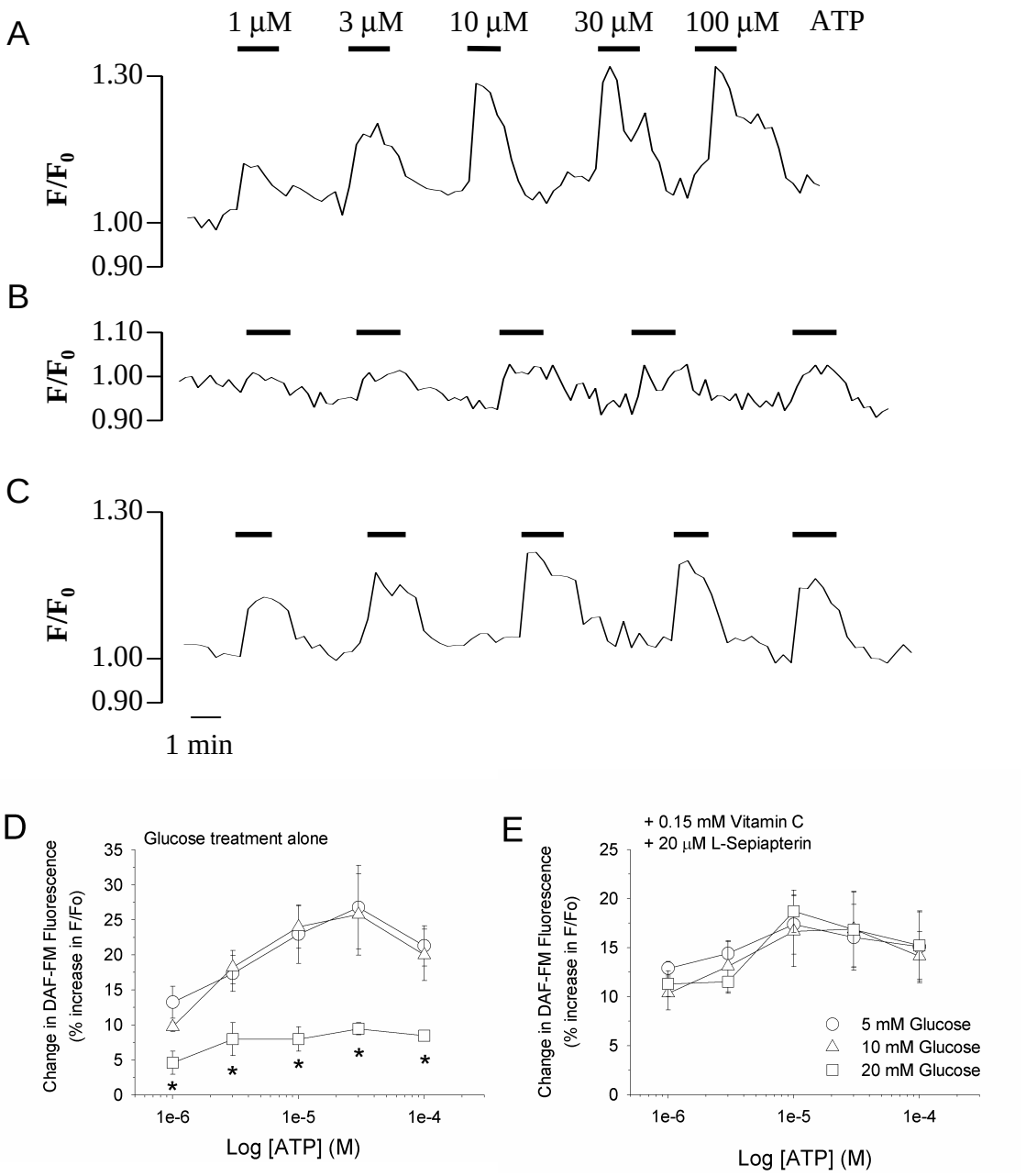

Figure 2

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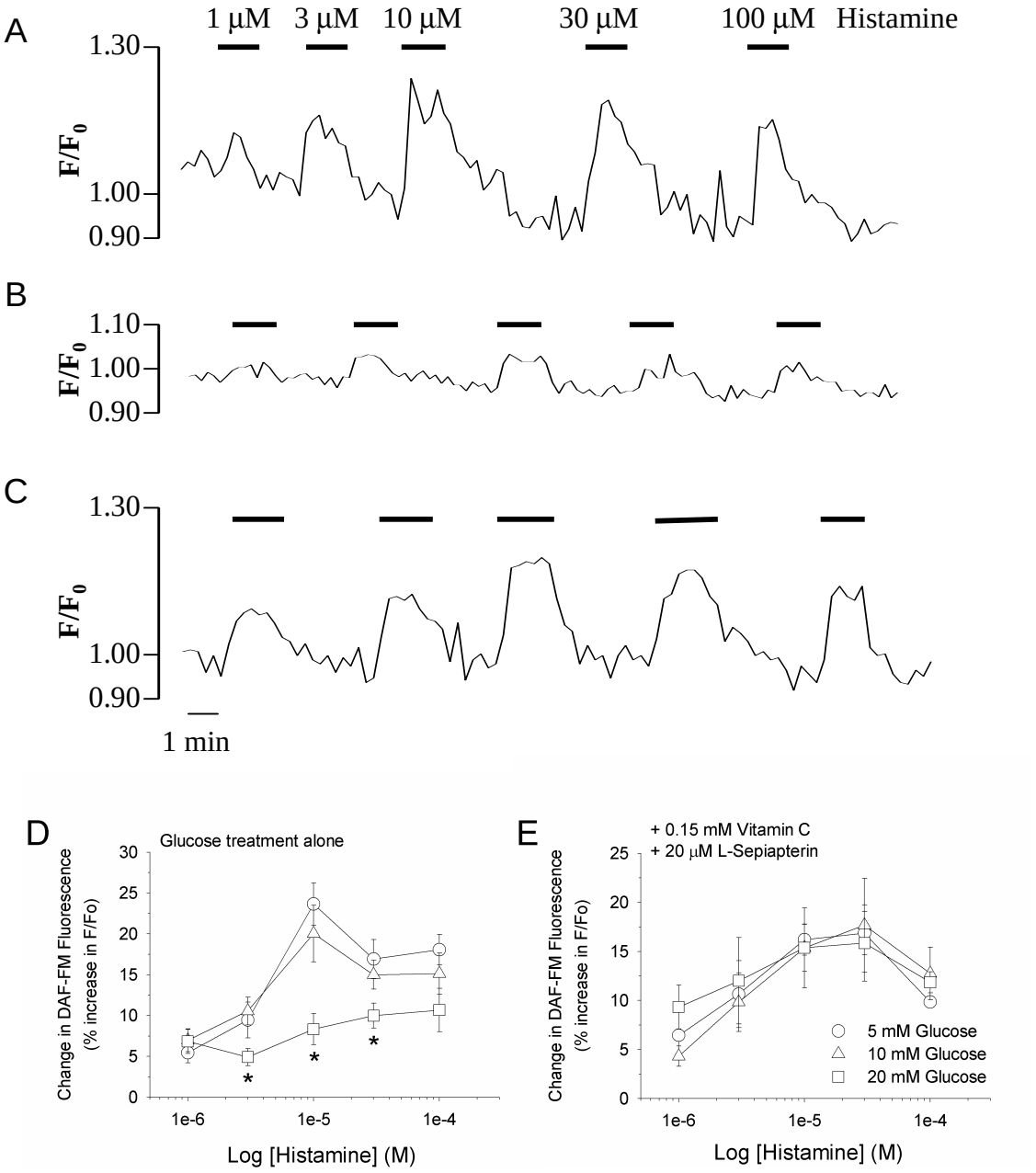

Figure 3

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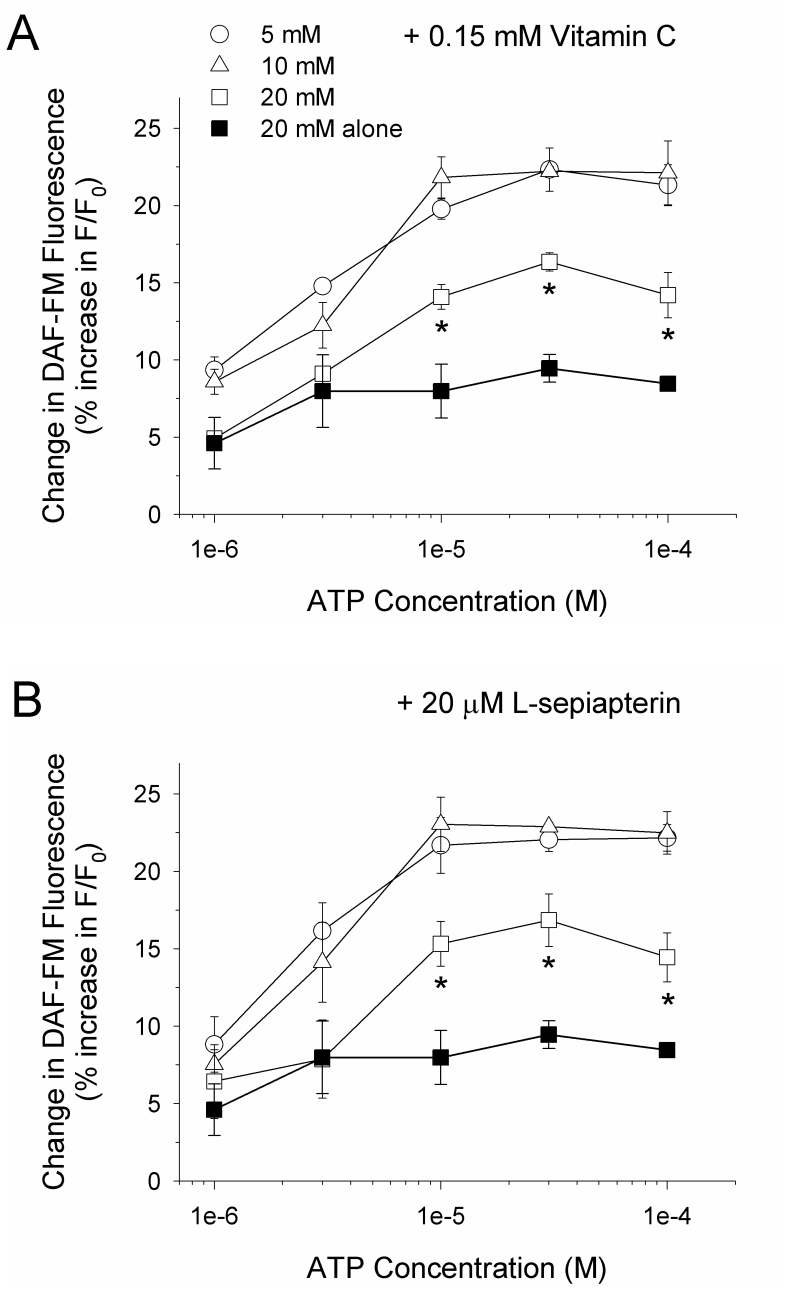

Figure 4

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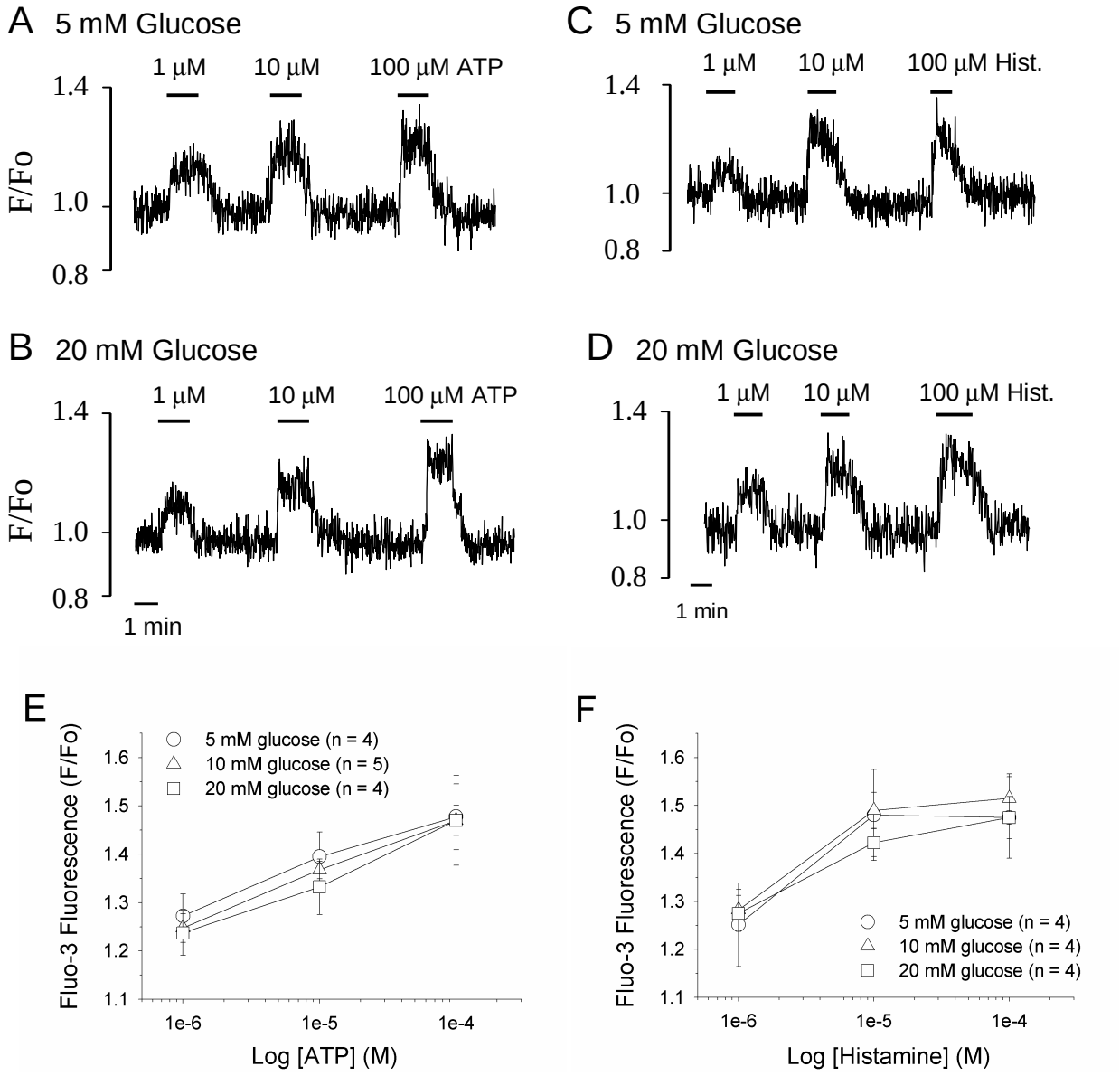

Figure 5

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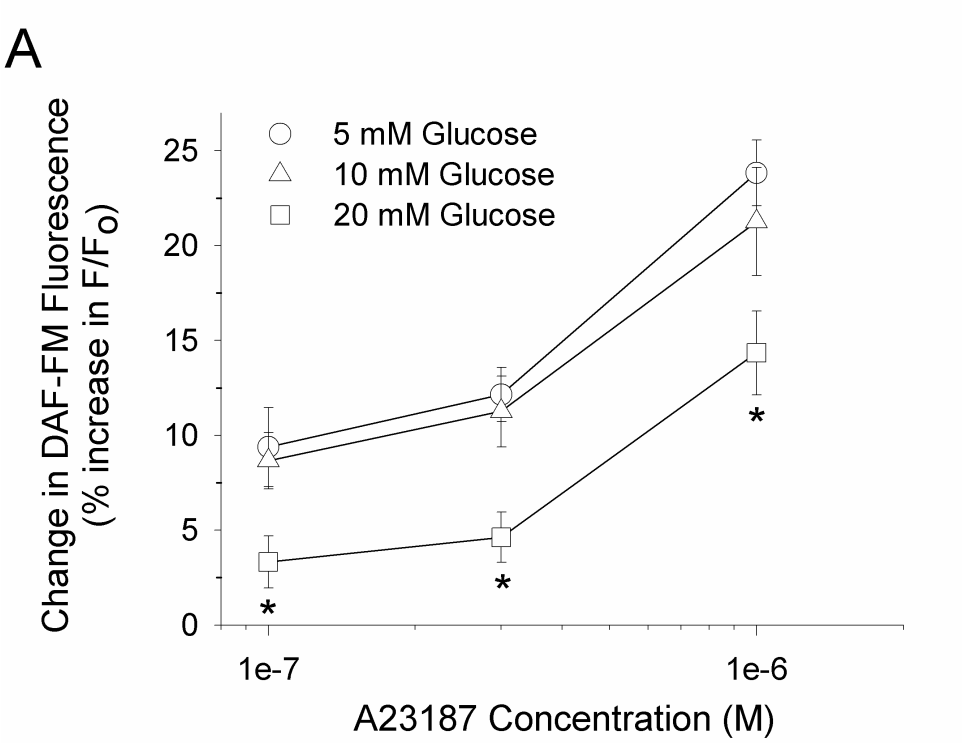

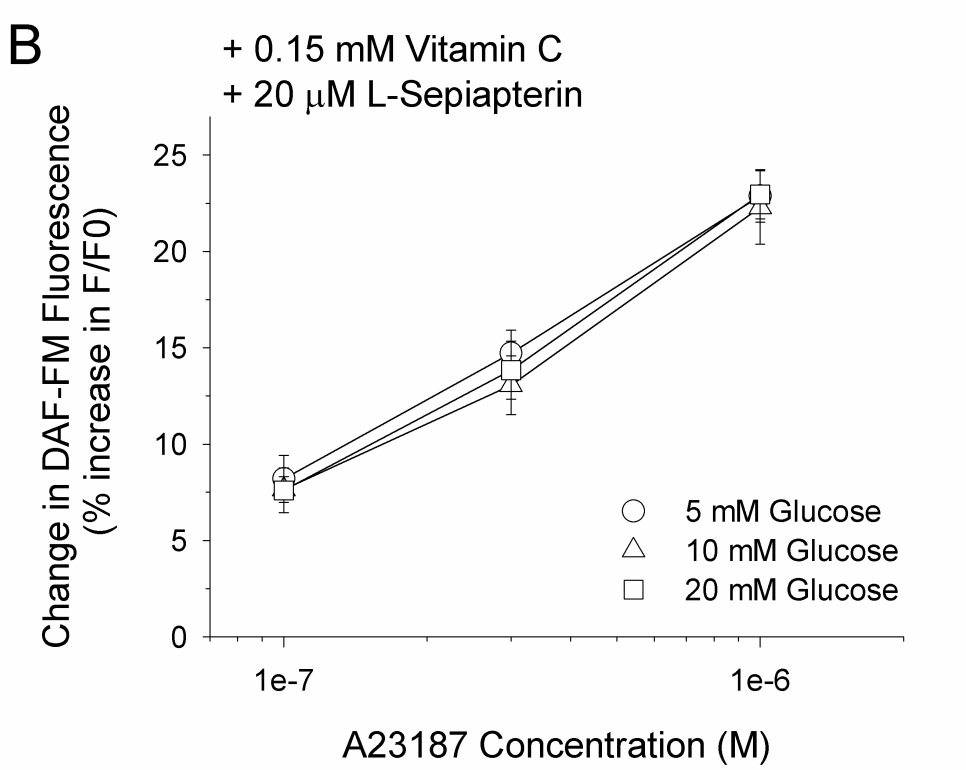

Figure 6

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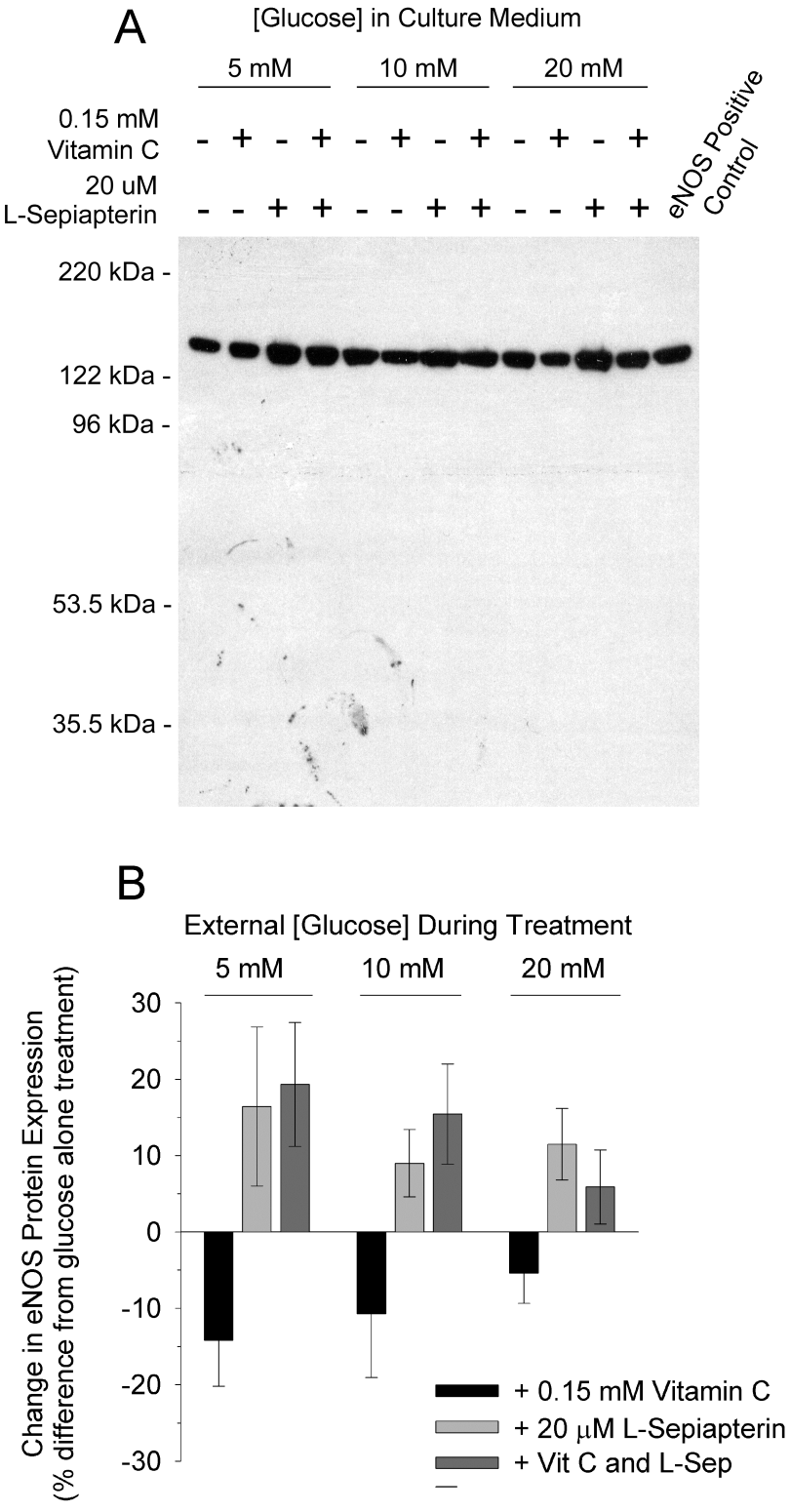

Figure 7

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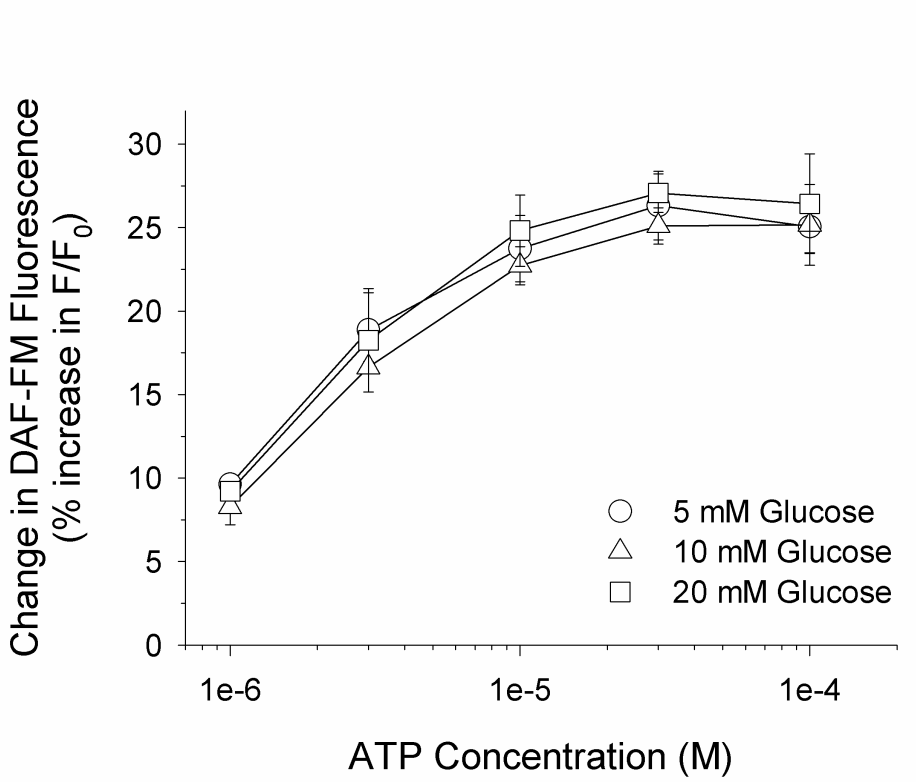

Figure 8