Effects of simulated hyperglycemia, insulin, and glucagon on endothelial nitric oxide synthase expression

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Ding, Yaoxian, Nosratola D. Vaziri, Richard Coulson, Vaijinath S. Kamanna, and Daeyoung D. Roh. Effects of simulated hyperglycemia, insulin, and glucagon on endothelial nitric oxide synthase expression. Am J Physiol Endocrinol Metab 279: E11–E17, 2000.—Diabetes is associated with endothelial dysfunction and increased risk of hypertension, cardiovascular disease, and renal complications. Earlier studies have revealed that hyperglycemia impairs nitric oxide (NO) production and diabetes causes endothelial dysfunction in humans and experimental animals. This study was designed to test the effects of altered concentrations of glucose, insulin, and glucagon, the principal variables in types I and II diabetes, on NO production and endothelial NO synthase (eNOS) expression in cultured human coronary endothelial cells. Cultured endothelial cells were incubated in the presence of glucose at either normal (5.6 mM) or high (25 mM) concentrations for 7 days. The rates of basal and bradykinin-stimulated NO production (nitrate + nitrite) and eNOS protein expression (Western blot) were then determined at the basal condition and in the presence of insulin (10^{-8} and 10^{-7} M), glucagon (10^{-8} and 10^{-7} M), or both. Incubation with a high-glucose concentration for 7 days significantly downregulated, whereas insulin significantly upregulated, basal and bradykinin-stimulated NO production and eNOS expression in cultured endothelial cells. The stimulatory action of insulin was mitigated by high-glucose concentration and abolished by cotreatment of cells with glucagon. Thus hyperglycemia, insulinopenia, and hyperglucagonemia, which frequently coexist in diabetes, can work in concert to suppress NO production by human coronary artery endothelial cells.

endothelial cells; diabetes; coronary artery; hypertension; arteriosclerosis; endothelium-derived relaxation

NITRIC OXIDE (NO) is an endogenous modulator with diverse biological functions. It is the most potent endogenous vasodilator and, as such, plays an important role in the regulation of renal and systemic vascular resistance (20, 37, 48). In addition, by inhibiting tubular Na^+ reabsorption, NO promotes natriuresis (7, 16, 28, 35, 40). Thus constitutively produced NO plays an important role in blood pressure homeostasis. The physiological importance of NO in the regulation of blood pressure is evidenced by the fact that pharmaco-logical inhibition of NO synthases leads to severe hypertension, vascular injury, and glomerulosclerosis in experimental animals (50). Moreover, endothelial NO synthase (eNOS) knockout mice exhibit hypertension (32), thus providing further support for the importance of NO in the regulation of blood pressure. In addition to being a potent vasodilator and a natriuretic agent, NO inhibits platelet and leukocyte adhesion, cell migration and proliferation, and matrix accumulation (1, 30, 36). Because these events are intimately involved in the pathogenesis of arteriosclerosis, atherosclerosis, and glomerulosclerosis, their inhibition by NO protects cardiovascular and renal function. Occurrence of these lesions with NO inhibitors in experimental animals strongly supports this contention.

Diabetes is one of the major risk factors for ischemic cardiovascular complications and the leading cause of end-stage renal disease (19, 45). Advanced diabetes is frequently complicated by hypertension, premature arteriosclerosis, and endothelial dysfunction, suggesting depressed NO availability (8, 22, 39, 55). The effect of diabetes on NO metabolism is controversial. Several studies have demonstrated impaired endothelium-dependent vasorelaxation in diabetic humans (18, 33, 41, 54, 58) and experimental animals (4, 15, 43, 49). In contrast, a number of other studies have found no discernible impairment of endothelium-dependent vasodilation (6, 9, 31, 38, 53). In fact, several recent studies have revealed increased NO production in diabetes (11–14, 24–27, 42, 62). However, avid inactivation of NO by reactive oxygen species has been shown to reduce its bioavailability in diabetes (10–13, 23, 56, 57). Support for the latter contention comes from several studies demonstrating improvement of endothelial dysfunction with antioxidant therapy in diabetes (4, 13, 15, 43, 58). Accordingly, endothelial dysfunction can occur as a result of increased NO inactivation by reactive oxygen species despite enhanced NO production. It should be noted that most of the available studies reporting endothelial dysfunction have evaluated humans and animals with long-term diabetes as...
opposed to exploring the direct effects of altered glucose or glucose regulatory hormones.

The untreated type I diabetes is characterized by hyperglycemia and insulinopenia (51). Treatment of type I diabetes with intermittent insulin administration leads to variable glycemia control. In addition, subcutaneously administered insulin exposes the systemic vasculature to higher levels of insulin than those occurring normally when insulin is released by the pancreas into the hepatic portal venous circulation (47). Type II diabetes is marked by insulin resistance and, as such, hyperglycemia is coupled with elevated insulin levels in these subjects (44). Administration of oral hypoglycemic agents or exogenous insulin further raises insulin levels in patients with type II diabetes. In advanced stages of type II diabetes, exhaustion of insulin-producing β-cells leads to insulinopenia and exogenous insulin dependence. In addition to insulin deficiency or resistance, diabetes is frequently associated with increased production of glucagon, which is a potent insulin antagonist (21, 60).

The present study was designed to explore the effects of the principal variables in diabetes, namely, glucose concentration, insulin level, and the insulin antagonist glucagon on endothelial NOS protein expression and NO production in cultured human coronary endothelial cells.

METHODS

Cell culture. Human coronary artery endothelial cells (Bio-whittaker, San Diego, CA) were cultured in a manner that was precisely the same as that described in our previous studies (61). Cells obtained on the third and fourth passages were used.

Study protocol. Cultured endothelial cells were incubated in the medium containing glucose at either a high (25 mM) or normal (5.6 mM) concentration for 7 days. Subsets of the cells were then treated with insulin at either 10⁻⁵ M or 10⁻⁷ M alone, with glucagon at either 10⁻⁷ M or 10⁻⁸ M alone, or with insulin plus glucagon for 24 h. Cells treated with inactive vehicle served as controls. Both insulin and glucagon were purchased from Sigma Chemical (St. Louis, MO). At the conclusion of the 24-h treatment period, cells were harvested and processed for measurement of eNOS protein abundance by Western blot analysis, and the medium was used for determination of total nitrate plus nitrite (NOx). On each occasion, cell viability was determined by Trypan blue exclusion test and optical inspection and was found to be >95%.

In a separate set of experiments, subsets of cells exposed to normal- or high-glucose concentrations for 7 days were treated with 10⁻⁶ M bradykinin or inactive vehicle for 1 h. The supernatant was then harvested for NOx measurement, and cells were collected for total protein determination. The choice of the given bradykinin concentration was based on our preliminary experiments, which showed a dose-dependent increase in NOx production within a 10⁻⁵ to 10⁻⁷ M concentration range in this system.

Measurement of NO production. NO production was determined from the NOx recovered in the culture medium. NOx was quantified by use of the purge system of the Sievers NO Analyzer (model 270 B NOA, Sievers Instruments, Boulder, CO). The amount of NOx produced was normalized against total cellular protein. The procedures involved in this assay have been described in detail in our earlier studies (17).

Measurement of eNOS protein. Endothelial cells were processed for determination of eNOS protein abundance by Western analysis, as described in our earlier studies (17). Briefly, cells were washed with PBS and extracted directly into the sample buffer (2% SDS, 10% glycerol, 0.0025% bromphenol, and 63 mmol/l Tris·HCl, pH 6.8), and total protein was determined by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Fifty micrograms of cell lysate protein were size-fractionated on 4–12% Tris-glycine gel at 130 V for 3 h. In preliminary experiments, the given protein concentrations were found to fall within the linear range of detection for our Western blot technique. After electrophoresis, proteins were transferred onto Hybond enhanced chemiluminescence (ECL) membrane at 400 mA for 120 min by use of the Novex transfer system (Novex, San Diego, CA). The membrane was prehybridized in 10 ml of buffer A (10 mM Tris·HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, and 10% nonfat milk powder) for 1 h and then hybridized for an additional 1 h in the same buffer containing 10 μl of the anti-eNOS monoclonal antibody (Transduction Laboratories, Lexington, KY) at 1:1,000 dilution. Thereafter, the membrane was washed for 30 min in a shaking bath, with the wash buffer (buffer A without nonfat milk) changed every 5 min before a 1-h incubation in buffer A plus goat anti-mouse IgG-horseradish peroxidase at the final titer of 1:1,000. Experiments were carried out at room temperature. The washes were repeated before the membrane was developed with a light-emitting nonradioactive method using ECL reagent (Amersham Life Science, Arlington Heights, IL). The membrane was then subjected to autoradiography for 1–5 min. The autoluminographs were scanned with a laser densitometer (model PD1211, Molecular Dynamics, Sunnyvale, CA) to determine the relative optical densities of the bands. In all instances, the membranes were stained with Ponceau stain before prehybridization to verify the uniformity of protein load and transfer efficiency across the test samples.

Data analysis. Analysis of variance (ANOVA), regression analysis, and Student’s t-test were used in evaluation of the data, which are presented as means ± SEM. P values <0.05 were considered significant.

RESULTS

Effect of glucose concentration. Exposure to high-glucose concentration for 7 days resulted in a significant downregulation of eNOS expression in cultured human coronary artery endothelial cells. This was accompanied by a marked reduction in basal NO production as discerned from NOx recovered in the extracellular medium (Fig. 1). Stimulation with 10⁻⁶ M bradykinin for 1 h resulted in an expected rise in NOx generation in cells exposed to normal glucose concentration (0.047 ± 0.01 vs. 0.072 ± 0.01 nmol/μg protein, P < 0.02, vehicle vs. bradykinin-treated cells). Similar, bradykinin augmented NOx production in cells exposed to simulated hyperglycemia (0.031 ± 0.003 vs. 0.054 ± 0.007 nmol/μg protein, P < 0.001). However, both basal and bradykinin-stimulated NOx productions were significantly lower in cells exposed to simulated hyperglycemia than their counterparts kept in the medium containing normal glucose concentration (P < 0.03 for both).

Effect of insulin. Addition of insulin at 10⁻⁸ and 10⁻⁷ M to endothelial cells, maintained in a medium with normal-glucose level, led to a concentration-dependent
A significant direct correlation was found between eNOS protein abundance and NO production in all experiments ($r = 0.803, P < 0.01$). However, glucose concentration showed an inverse correlation with eNOS expression ($r = -0.816, P < 0.01$) and NO production ($r = -0.787, P < 0.01$).

**DISCUSSION**

Several earlier studies have revealed that both short- and long-term exposure to hyperglycemia impairs endothelial function in humans and experimental animals (13, 34). This can be due to impaired production of endothelium-derived NO, enhanced inactivation of NO, or increased release of endothelium-derived vasoconstrictive factors. Earlier studies have demonstrated a marked increase in production of the superoxide radical (O$_2^-$), which is an avid scavenger of NO, in cultured aorta endothelial cells exposed to simulated hyperglycemia for 5 days (13). These observations support the contribution of enhanced NO inactivation in the pathogenesis of endothelial dysfunction associated with chronic hyperglycemia. In addition to inactivating NO, oxygen free radicals can promote generation of vasoconstrictive prostanoids (12, 42, 56, 57). Thus elevated glucose concentration can contribute to endothelial dysfunction through an enhanced oxygen free radical-mediated NO inactivation and increased generation of vasoconstrictive prostanoids. The present study was carried out to explore the direct effects of simulated hyperglycemia, insulin, and glucagon on the NO system in cultured endothelial cells.

Exposure to simulated hyperglycemia for 7 days resulted in a significant downregulation of eNOS expression as well as basal and bradykinin-stimulated NO production in cultured human coronary endothelial cells. The downregulatory effect of high glucose concentration was seen at different levels of insulin in the culture media. On the basis of these observations, it appears that hyperglycemia can contribute to coronary artery endothelial cell dysfunction at a wide range of insulin levels. We caution against extension of these findings in human coronary artery endothelial cells to endothelial cells of other origins that may behave differently. In fact, studies conducted by Cosentino et al. (13) with cultured aorta endothelial cells revealed increased NO production and eNOS expression in cells exposed for 5 days to a high-glucose concentration. The reason for the observed difference in the effect of simulated hyperglycemia on the NO system in the two studies is not entirely clear. It should be noted that Cosentino et al. used aorta endothelial cells, whereas the present study employed coronary artery endothelial cells. In addition, glucose concentration used to simulate hyperglycemia (22.2 mmol/l) and duration exposure (5 days) in the former study (13) were less than those employed here (25 mmol/l and 7 days, respectively). Moreover, cells up to passage 6 were used in the former study, whereas only cells from the third and fourth passages were used here. It should be noted that increased NO production in aorta endothelial cells reported by Cosentino et al. was coupled with an even greater increase in generation of superoxide, which is
Fig. 2. Representative Western blots and group data depicting eNOS protein abundance in endothelial cells incubated for 24 h in the absence of insulin (CTL) or in the presence of insulin at $10^{-8}$ M and $10^{-7}$ M concentrations. Experiments were conducted in media containing normal (5.6 M)- and high (25 M)-glucose concentrations. B: NO production rates obtained with the corresponding experiments. Data represent means ± SE of ≥4 separate experiments. *$P < 0.05$ vs. other groups; **$P < 0.01$ vs. control group.

Fig. 3. A: representative Western blots and group data depicting eNOS protein abundance in endothelial cells incubated for 24 h in the absence of glucagon (CTL) or in the presence of glucagon at $10^{-8}$ M and $10^{-7}$ M concentrations. Experiments were conducted in media containing normal (5.6 M)- and high (25 M)-glucose concentrations. B: NO production rates obtained with the corresponding experiments. Data represent means ± SE of ≥4 separate experiments.
known to inactivate NO. The imbalance between NO production and superoxide generation shown in the latter study can clearly contribute to endothelial dysfunction associated with high glucose levels (15).

Incubation with insulin at $10^{-8}$ and $10^{-7}$ M for 24 h resulted in a dose-dependent upregulation of eNOS expression and NO production by cultured coronary endothelial cells. It should be noted that, on each occasion, the stimulatory action of insulin on the NO system was dampened by high-glucose concentration. Thus it appears that insulinopenia, and perhaps insulin resistance, may contribute to coronary artery endothelial dysfunction, compounding the adverse effect of hyperglycemia. In a recent study, Kawaguchi et al. (34) demonstrated that administration of insulin for 4 wk to diabetic obese Zucker rats ameliorated hypertension and raised plasma concentration and urinary excretion of NO metabolites, suggesting enhanced NO production. Similarly, Zeng and Quon (63) have shown increased NO production with insulin. The results of the present in vitro studies are consistent with the findings of in vivo studies reported by Kawaguchi et al. and Zeng and Quon. A number of previous studies have demonstrated a reduction in blood pressure with insulin therapy in diabetic humans (2, 3, 5). Enhanced NO production and decreased NO inactivation by superoxide with insulin replacement and amelioration of hyperglycemia may play a part in this process. Further studies are needed to explore this possibility.

Glucagon is a peptide hormone produced by the $\alpha$-cells of the pancreas. By promoting glycogenolysis and gluconeogenesis from amino acids, glycerol, and pyruvate, glucagon raises glucose concentration. Thus glucagon serves as a natural insulin antagonist. Glucagon levels have been shown to be elevated in both type I and type II diabetes mellitus (21, 60). Therefore, we sought to explore the possible effect of glucagon on the NO system in coronary endothelial cells. This study revealed that glucagon, per se, has no discernible effect on either eNOS expression or NO production in isolated human coronary endothelial cells. However, glucagon virtually abolished the stimulatory effect of insulin on eNOS protein expression and NO production in these cells. These observations revealed another anti-insulin action of glucagon beyond its known effects on glucose metabolism. Moreover, the results suggest that the associated hyperglucagonemia may potentially contribute to coronary artery endothelial dysfunction in diabetes. The inhibitory action of glucagon on insulin-mediated upregulation of eNOS expression and NO production in endothelial cells shown here parallels the results of earlier studies showing that glucagon mitigated the cytokine-mediated induction of inducible NOS in cultured hepatocytes (29, 52). Therefore, the downregulatory action of glucagon on the NO system is not limited to eNOS. Interestingly, glucagon has been shown to cause a fast-acting renal vasodilation in rat in vivo and vasorelaxation in dog arterial and venous preparations in vitro (46, 59). This effect was cAMP mediated and was present in endothelium-denuded as well as endothelium-bearing preparations. It should be noted that the fast-acting direct vasodila-
tory action of glucagon shown in the latter studies is distinct from its late effect on eNOS expression via inhibition of insulin’s action shown here.

In conclusion, simulated hyperglycemia resulted in a significant downregulation of eNOS expression and NO production by cultured human coronary endothelial cells. In contrast, insulin caused a dose-dependent upregulation of eNOS expression and NO production in this system. The stimulatory action of insulin was mitigated by simulated hyperglycemia and was abrogated by glucagon administration. Thus hyperglycemia, insulinopenia, and hyperglycagonemia, which frequently coexist in diabetes, can suppress the NO system in coronary endothelial cells in vitro. Caution should be exercised in extending the present in vitro findings to the highly complex clinical setting in vivo.

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REFERENCES