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

YAOXIAN DING, NOSRATOLA D. VAZIRI, RICHARD COULSON, VAIJINATH S. KAMANNA, AND DAEYOUNG D. ROH
Division of Nephrology and Hypertension, Department of Medicine, University of California, Irvine, California 92697

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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 physiological 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.

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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 10⁻⁸ 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).

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). Similarly, 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
found between eNOS protein abundance and NO pro-

glucose concentrations. 

teraction and was seen with both normal- and high-
enon was independent of extracellular glucose concen-

production by endothelial cells (Fig. 4). This phenom-

action of insulin on both eNOS expression and NO 

system, it virtually abrogated the upregulatory 

tion depressed the stimulatory action of insulin on the 

in cells exposed to high-glucose than in those exposed 
to normal-glucose levels. Thus high-glucose concentra-
tion depressed the stimulatory action of insulin on the 

NO system in coronary endothelial cells (Fig. 2). 

Effect of glucagon. Addition of glucagon at either 

or M concentration failed to alter eNOS 
protein expression in endothelial cells maintained in 
either normal- or high-glucose media. Similarly, gluca-
gon had no effect on NO production by endothelial cells 
under either condition (Fig. 3). 

Effect of glucagon on insulin action. Although gluca-
gon alone had no discernible effect on the endothelial 
NO system, it virtually abrogated the upregulatory 
action of insulin on both eNOS expression and NO 
production by endothelial cells (Fig. 4). This phenom-

was independent of extracellular glucose concen-

tration and was seen with both normal- and high-
glucose concentrations. 

Correlation. A significant direct correlation was 

found between eNOS protein abundance and NO pro-
duction in all experiments ($r = 0.803, P < 0.01$). How-
ever, glucose concentration showed an inverse correla-
tion 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 im-
pairs endothelial function in humans and experimen-
tal animals (13, 34). This can be due to impaired 
production of endothelium-derived NO, enhanced inacti-
vation 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 sim-
ulated hyperglycemia for 5 days (13). These observa-
tions support the contribution of enhanced NO inactiva-
tion in the pathogenesis of endothelial dysfunction 
associated with chronic hyperglycemia. In addition to 
inactivating NO, oxygen free radicals can promote gen-
eration of vasocontractible 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 sim-
ulated hyperglycemia, insulin, and glucagon on the NO 
system in cultured endothelial cells. 

Exposure to simulated hyperglycemia for 7 days re-
sulted in a significant downregulation of eNOS expres-
sion as well as basal and bradykinin-stimulated NO 
production in cultured human coronary endothelial 
cells. The downregulatory effect of high glucose concen-
tration 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 dif-
ferently. In fact, studies conducted by Cosentino et al. 
(13) with cultured aorta endothelial cells revealed in-
creased 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 sim-
ulated 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 endothel-
ial 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, re-
spectively). 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-

Fig. 4. A: representative Western blots and group data depicting eNOS protein abundance in endothelial cells incubated for 24 h in the absence of insulin and glucagon (CTL) and in the presence of equimolar concentrations of insulin plus glucagon ($10^{-8}$ M and $10^{-7}$ M). The 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.
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 hyperglucagonemia, 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


