Elevated Glucose Promotes Generation of Endothelium-derived Vasoconstrictor Prostanoids in Rabbit Aorta

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Abstract

The effects of glucose on endothelium-dependent responses and vasoactive prostanoid production were determined by incubating isolated rabbit aortae in control (5.5 or 11 mM) or elevated (44 mM) glucose for 6 h to mimic euglycemic and hyperglycemic conditions. Rings of aortae incubated in elevated glucose, contracted submaximally by phenylephrine, showed significantly decreased endothelium-dependent relaxations induced by acetylcholine compared with the aortae incubated in control glucose. Treatment with indomethacin, a cyclooxygenase inhibitor, or SQ29548, a prostaglandin 
H₂/thromboxane A₂ receptor antagonist, restored acetylcholine relaxations of rings in elevated glucose to normal, while these agents had no effect on the relaxation of rings incubated in control glucose. Aortae incubated with mannose (44 mM) as a hyperosmotic control relaxed to acetylcholine normally. The relaxations in response to A23187 and sodium nitroprusside were not different between rings exposed to control and elevated glucose. Radioimmunoassay measurements showed a significant increase in acetylcholine-stimulated release of thromboxane A₂ and prostaglandin F₂α in aortae with, but not without endothelium incubated with elevated, but not with control glucose. Thus a possible mechanism for endothelium dysfunction in diabetes mellitus is the hyperglycemia-induced increased generation of endothelium-derived vasoconstrictor prostanoids. (J. Clin. Invest. 1990. 85:929–932.) acetylcholine • cyclooxygenase products • endothelium • hyperglycemia

Introduction

The endothelium contributes to the local regulation of vascular smooth muscle function by releasing endothelium-derived relaxing factors (EDRF),¹ prostanoids (PG) and enzymes that activate or degrade vasoactive hormones (1, 2). The integrity and function of the endothelial cell layer are profoundly altered in diabetic animals and man (3). Altered prostanoid production is among the many factors implicated in the pathogenesis of diabetic vascular disease (4–7). Recent evidence indicates that aortic rings from alloxan-induced diabetic rabbits with a mean plasma glucose of 20 mM demonstrate an abnormal cholinergic receptor-mediated endothelium-dependent relaxation. The impaired relaxation is mediated by an increased production of vasoconstrictor prostanoids including thromboxane A₂ by the diabetic endothelium (4). Whether this abnormality results from hyperglycemia or hyperlipidemia associated with the diabetic experimental model is not known. These studies were undertaken to examine the direct effects of an elevated glucose milieu per se, on endothelium-dependent responses and prostanoid production by incubating isolated rabbit aortic rings in control or elevated glucose media. Our results indicate that exposure to an increased glucose concentration for 6 h can impair cholinergic endothelium-dependent relaxations by augmenting the production of vasoconstrictor prostanoids from the endothelium.

Methods

The abdominal aorta was dissected from male New Zealand white rabbits (2.2–2.5 kg) killed by exsanguination after anesthesia with pentobartal sodium (30 mg/kg i.v.) and anticoagulation with heparin sodium (150 U/kg i.v.). The adhering perivascular tissue was carefully removed. Rings of aortae (5 mm long) were suspended from strain gauges for measurement of isometric circumferential force. The rings were placed in organ baths (25 ml) filled with physiological salt solution (PSS) of the following composition (in mM): NaCl 118.3, KCl 4.7, MgSO₄ 0.6, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25.0, and calcium ethylenediamine tetraacetate acid 0.026. The solutions were maintained at 37°C and gassed with 95% O₂–5% CO₂ to maintain pH at 7.4. Length of the smooth muscle was increased stepwise over 90 min to adjust basal tension to 6 g. This was found to be optimal for contraction by testing repeated contractions to potassium (80 mM). Thereafter, length was not altered. Aortic rings were then incubated in 5.5, 11, or 44 mM glucose for 6 h. Mannose (44 mM) was used as a hyperosmotic control. After the 6-h incubation the arteries were contracted with phenylephrine to 40–50% of their maximal contraction induced by potassium (120 mM). When the contraction stabilized the response to acetylcholine, A23187, and sodium nitroprusside were obtained by increasing bath concentration in half-log cumulative increments. Inhibitors were present during the 6-h incubations and during subsequent concentration-responses.

Radioimmunoassay. Segments of aortae (2.5 cm) were incubated in PSS and gently bubbled with 95% O₂–5% CO₂ at 37°C for 6 h in control (11 mM) or elevated (44 mM) glucose. The PSS was changed every hour. Segments were prepared in which the endothelium was left intact or removed mechanically by gently rolling the segment on wet filter paper using forceps inserted into the lumen. At the end of the 6-h period the segments were incubated in PSS (1 ml) sequentially in the absence and presence of acetylcholine (10⁻⁶ M) for 30 min each. The tissues were blotted dry and weighed. The incubates were frozen at −80°C until analyzed. Radioimmunoassays were used to quantify the release of thromboxane B₂ (the stable hydrolytic product of thromboxane A₂), 6-keto-PGF₁α (the stable hydrolytic product of prostacy-
clin), PGE$_2$, and PGF$_2$ in the incubation buffers. Radioimmunoas-
says were performed using specific antisera of thromboxane B$_2$,
PGE$_2$, and PGF$_2$ (courtesy of Dr. Lawrence Levine, Brandeis Uni-
versity, Waltham, MA), 6-keto-PGF$_{1\alpha}$, (Biomol Research Laboratories,
Inc., Plymouth, PA), tritiated standards (Dupont-NEN, Boston, MA),
and unlabeled standards (UpJohn Co., Kalamazoo, MI; 8). Standard
curves contained an equal volume of PSS to that being assayed and all
dilutions were made with PSS. Cross-reactivity with other measured
prostanoids was < 5%. The limits of sensitivity for the radioimmuno-
assay with the experimental conditions described for thromboxane B$_2$,
6-keto-PGF$_{1\alpha}$, and PGF$_2$ were 1 pg/ml and for PGE$_2$ was 10 pg/ml.
Standard curves performed with the addition of glucose (44 mM) were
identical to those performed in control glucose.

Drugs: The pharmacological agents used were the following: ace-
tylylcholine chloride, calcium ionophore A23187, indomethacin, man
nose, phenylephrine, and sodium nitroprusside (Sigma Chemical Co.,
St. Louis, MO), dazmegrel (Pfizer Inc., Groton, CT), ibuprofen and
meclomenamate (Biomol Research Laboratories, Inc.), and SQ29548, a
gift from Squibb Pharmaceuticals (Princeton, NJ). Concentrations
were expressed as final molar bath concentrations. Unless otherwise
specified, drugs were dissolved in distilled water such that volumes of
0.1 ml were added to the organ bath. A23187 was prepared in ethanol
(95%). Indomethacin was prepared in 2% Na$_2$CO$_3$ immediately
before use. Stock solutions of SQ29548 were made in 95% ethanol and further
dilutions were made in PSS. Ibuprofen and meclofenamate were pre-
pared in 0.1 N NaOH.

Data analysis. Maximal relaxation obtained in response to each
concentration of agonist is expressed as percent change in the level of
tone induced by phenylephrine. The IC$_{50}$ was estimated graphically as
the concentration causing 50% relaxation of the induced tone. Data are
expressed as means±SE. Statistical evaluation of the data was made
using repeated measures of analysis of variance for concentration-re-
sponse curves or Student’s t test for paired comparisons of responses of
rings or release of prostanoids from arterial segments from the same
animal. P values < 0.05 were regarded as significant. In all experi-
m ents, n equals the number of rabbits from which rings were taken.

Results

Endothelium-dependent relaxations. Rings of aortae with in-
tact endothelium incubated with 5.5, 11, or 44 mM for 6 h
were contracted with phenylephrine (concentration, $-\log M$:
6.5±0.5, n = 5; 6.8±0.2, n = 10; and 6.7±0.1, n = 10; respec-
tively) which caused similar contractions of 7.9±0.8, 7.7±0.4,
and 7.2±0.4 g, respectively. The rings were then exposed to
increasing concentrations of acetylcholine (10$^{-10}$–10$^{-4}$ M). The
relaxations induced by acetylcholine were significantly de-
creased in aortic rings incubated with elevated (44 mM) glucose
compared with those in control (5.5 and 11 mM) glucose
(IC$_{50}$-$-\log M$: 6.4±0.2 (n = 10) vs. 7.2±0.1 (n = 5) and 7.2±0.1
(n = 10), respectively, P < 0.05, Figs. 1, 2). The relaxations
caused by acetylcholine were not significantly different be-
tween rings incubated in 5.5 and 11 mM glucose. Relaxations
caused by acetylcholine (3×10$^{-7}$–10$^{-4}$ M) were followed by
recontractions of aortae incubated in elevated, but not in con-
trol glucose (Fig. 1). Aortae incubated with mannose (44 mM)
for 6 h relaxed to acetylcholine normally (IC$_{50}$-$-\log M$: 7.2±0.1, n = 4). Aortae incubated in elevated glucose con-
traced with PGF$_2$ was not stimulated by phenylephrine showed similar
impaired acetylcholine-induced relaxations (data not shown).

Treatment with indomethacin, meclofenamate or ibupro-
fen (10$^{-5}$ M) restored acetylcholine relaxations of rings incu-
bated with elevated glucose (IC$_{50}$-$-\log M$: 7.3±0.1, n = 6,
7.2±0.1, n = 4 and 7.2±0.1, n = 4, respectively), such that the
relaxations did not differ statistically from those observed in
rings incubated in control glucose. Similarly, treatment with
SQ29548 (3×10$^{-6}$ M) restored acetylcholine relaxations of
rings incubated in elevated glucose to normal (IC$_{50}$-$-\log M$: 7.0±0.1, n = 4). Neither the cyclooxygenase inhibitors nor
SQ29548 had a significant effect on the relaxation to acetyl-
choline of rings incubated with control glucose (Fig. 2). In
rings of aortae incubated in elevated glucose, treatment with
dazmegrel (3×10$^{-6}$ M) did not significantly affect the abnor-
mal relaxations caused by acetylcholine (IC$_{50}$-$-\log M$: 6.2±0.2, n = 3).

The relaxations caused by A23187 (10$^{-5}$–3×10$^{-6}$ M) were
not significantly different between rings incubated in control
(11 mM) or elevated (44 mM) glucose. The maximal relaxa-
tion caused by A23187 (3×10$^{-6}$ M) was 34±5.6 vs.
37±5.4%, respectively, (n = 5).

Endothelium-independent relaxations. The relaxations
cau sed by sodium nitroprusside (10$^{-6}$–10$^{-5}$ M) were not signif-
ificantly different between rings incubated in control (11 mM)
or elevated (44 mM) glucose (IC$_{50}$-$-\log M$: 8.0±0.2 vs.
8.0±0.1, respectively, n = 5).

Prostanoid production. Under basal conditions or in the
presence of acetylcholine (10$^{-6}$ M), the production of the
prostanoids, thromboxane B$_2$, 6-keto-PGF$_{1\alpha}$, PGF$_2$ and
PGE$_2$, was significantly greater in segments with, than in seg-

Figure 1. Tracings of rings of aortae contracted with phenylephrine (PHE) and then exposed to half-molar increases in concentration of acetylcholine. Aortae incubated for 6 h in control glucose (11 mM) show normal relaxations (A), but those incubated in elevated glucose (44 mM) show decreased relaxations to acetylcholine (B). Treatment with SQ29548 (3×10$^{-6}$ M) normalized the abnormal responses in aortae incubated in elevated glucose (C). Aortae incubated in mannose (44 mM), to serve as a hypotonic control, relaxed to acetyl-
choline normally (D).
Figure 2. (A) Comparison of maximal relaxations induced by each concentration of acetylcholine of aortic rings incubated with control glucose (5.5 or 11 mM; n = 5, 10, respectively) and elevated glucose (44 mM, n = 10). Relaxations were significantly less in aorta incubated with elevated glucose compared with those of aorta in control glucose (ANOVA P < 0.05). (B and C): Effects of indomethacin (10−6 M, n = 6) and SQ29548 (3 × 10−6 M, n = 4), respectively, on relaxations induced by acetylcholine in aortic rings incubated in control (11 mM) and elevated (44 mM) glucose. Indomethacin or SQ29548 restored acetylcholine-induced relaxations in aortic rings incubated in elevated glucose such that the relaxations were not significantly different from those in control glucose. Neither inhibitor affected the response of aorta in control glucose. Values are shown as means±SE.

ments without endothelium (Table I). There was no significant difference in the production of any of the prostanooids in the presence of control (11 mM) or elevated (44 mM) glucose under basal conditions, in the presence or absence of endothelium. Acetylcholine (10−6 M) significantly increased all of the measured prostanooids in the presence of normal or elevated glucose in segments with endothelium, as well as increased 6-keto-PGF1α production in segments without endothelium. In the presence of acetylcholine, the production of thromboxane B2 and PGF2α by segments with, but not without endothelium was significantly greater in the presence of elevated glucose compared with control, whereas that of 6-keto-PGF1α and PGF2α was not significantly affected.

In the presence of dazmegrel (3 × 10−6 M), the release of thromboxane B2 from aortae incubated in elevated glucose under basal conditions or in the presence of acetylcholine (10−6 M) was significantly inhibited (1.8±0.3 and 2.2±0.3 pg/mg per 30 min, respectively, n = 3).

Discussion

The present experiments performed after exposure to condi-
tions that mimic pronounced hyperglycemia demonstrate impair-
ment of endothelium-dependent acetylcholine-induced relax-
lation by stimulated production of endothelium-derived va-
soconstrictor factor(s). The abnormal relaxations observed after incubating with elevated glucose for 6 h was a time-de-
pendent effect because incubation for 2 or 3 h in elevated glucose caused a less pronounced abnormality (unpublished observations). The alterations caused by elevated glucose are not due to a hyperosmotic effect because the same concentra-
tion of mannose had no effect on the relaxations induced by acetylcholine. The cyclooxygenase inhibitors, indomethacin, meclofenamate, and ibuprofen, restored acetylcholine-induced relaxations suggesting that the inhibition was mediated by a cyclooxygenase product produced in the presence of elevated glucose. The restoration of acetylcholine-induced relax-
ation by the vasoconstrictor prostaglandin receptor antagonist, SQ29548 (9), is consistent with mediation by prostaglandin endoperoxides or their derivatives, including thromboxane A2 and PGF2α.

A role for these vasoconstrictor prostaglandins is further supported by radioimmunoassay measurements. In these experiments elevated glucose induced rapid alterations in arachidonate metabolites produced during stimulation with ace-
tylcholine yielding increased amounts of thromboxane A2 and PGF2α. Cholinergic agents have been shown to also stimulate prostacyclin and PGE2 synthesis by the endothelium of rabbit aorta (10, 11). It is less likely that prostacyclin or PGE2 contribute to the abnormal relaxation to acetylcholine because the stimulated release of these prostanooids was independent of glucose concentration in the medium, and both are less potent vasoconstrictors of the rabbit aorta compared with the thromboxane A2 mimetic, U46619, or PGF2α (4).

Experiments with pharmacological antagonists as well as radioimmunoassay measurements point to vasoconstrictor prostaglandins including thromboxane A2 and PGF2α as the mediators of the abnormal acetylcholine response. A major role for thromboxane A2 in the abnormal acetylcholine-medi-
ated relaxation is less likely as suggested by the failure of the thromboxane synthase inhibitor, dazmegrel (12), to correct the

| Table I. Basal and Acetylcholine-stimulated Release of Immunoreactive Prostanoids from Aortic Segments with and without Endothelium Incubated in Control and Elevated Glucose |
|---|---|---|---|---|
| Glucose: Control | Eleved | Control | Eleved |
| Thromboxane B2 | | | | |
| Basal | 8.5±1.1† | 8.2±1.4‡ | 5.7±0.7 | 4.3±0.3 |
| Acetylcholine | 14±1.8‡ | 29±4.3×‡ | 6.0±1.0 | 7.6±1.4 |
| PGF2α | | | | |
| Basal | 61±6.2† | 94±18‡ | 49±7.2 | 56±10 |
| Acetylcholine | 152±26‡ | 193±29‡ | 65±12 | 79±10 |
| 6-keto-PGF1α | | | | |
| Basal | 201±67† | 273±54‡ | 53±4.8 | 38±3.8 |
| Acetylcholine | 937±164‡ | 963±88‡ | 146±37§ | 156±44§ |
| PGE2 | | | | |
| Basal | 99±22‡ | 101±27† | 52±22 | 56±18 |
| Acetylcholine | 219±40° | 215±52′ | 76±23 | 83±5.3 |

Values are expressed as means±SE (pg/mg tissue per 30 min). The weights of the rabbit aortae used for control and elevated glucose incubations were 44±5.1 and 49±6.8 mg (with endothelium, n = 6) and 46±3.1 and 44±3.8 (without endothelium, n = 3), respectively. * Indicate significant difference between prostanooid production in control and elevated glucose. † Indicate significantly greater release from segments with endothelium compared to that from those without endothelium. ‡ Indicate significant increase caused by acetylcholine (10−6 M) compared with basal.

High Glucose Promotes Endothelium-derived Prostanoid Vasoconstrictors 931
response. Increased production of prostaglandin endoperoxides could cause the abnormal acetylcholine response of aorta exposed to elevated glucose because the vasoconstriction which they cause is blocked by SQ29548 (9) and their formation is not prevented by dazmegrel (12). The preferential synthesis of more potent vasoconstrictor endoperoxide-derived prostanoids may also favor their direct role in the impaired relaxation to acetylcholine.

The relaxations induced by sodium nitroprusside, an endothelium-independent vasodilator which relaxes smooth muscle by a mechanism similar to that of EDRF (13), as well as that to the calcium ionophore A23187, a non-receptor-mediated endothelium-dependent vasodilator, were not different between aortae in control or elevated glucose. This suggests that the release, or responsiveness of the smooth muscle to EDRF is not altered by elevated glucose. A major finding in this study is that elevated glucose enhances release of vasoconstrictor prostanoids following cholinergic stimulation, and the endothelium is its source. This is supported by the normal basal release of prostanoids in aortae with endothelium and by the normalization of acetylcholine-stimulated prostaglandin production after removal of the endothelium.

The present findings complement the findings in isolated aortae of alloxa-induced diabetic rabbits, which showed impaired endothelium-dependent relaxations induced by acetylcholine that were corrected by cyclooxygenase inhibition or SQ29548 and were associated with increased thromboxane A2 production (4). Others have reported that endothelium-dependent relaxations to acetylcholine are impaired in aortae of streptozotocin-induced diabetic rat and in the spontaneously diabetic BB Wistar rat (14-16). Additionally, decreased endothelium-dependent relaxations to acetylcholine have been reported in isolated penile corpus cavernosum tissue of impotent diabetic men (17). Thus, the present studies suggest that by varying the glucose concentration in the medium, a useful in vitro model is achieved for studying the changes in endothelial cell vasodilator function as well as prostanoid production seen in diabetic animals and man. In experimental diabetes, hyperlipidemia and elevated cholesterol similar to that seen in diabetic patients have been reported to increase platelet thromboxane A2 generation (18, 19). The present study provides evidence that elevated plasma glucose per se may be a primary factor for the increased production of vasoconstrictor prostanoids by the endothelium. The observation that glucose can readily contribute to changes in the function of the endothelium by inducing generation of vasoconstrictor prostanoids in response to cholinergic stimulation suggests that production of these prostanoids during hyperglycemia may contribute to vascular complications in diabetes mellitus.

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