Effect of Elevated Glucose on Cyclic GMP and Eicosanoids Produced by Porcine Aortic Endothelium

Robert M. Weisbrod, Michael L. Brown, and Richard A. Cohen

The short-term effects of elevated glucose on cyclic GMP (cGMP) and eicosanoid production in pig aortic endothelial cell monolayers was determined by incubating cells in 5.5 mM or 44 mM glucose for 6 hours. Bradykinin- or A23187-stimulated cGMP production was significantly reduced in cells incubated in 44 mM glucose compared with 5.5 mM glucose. Stimulation of cGMP levels with exogenously added nitric oxide (NO) was also decreased to a similar extent in cells exposed to 44 mM glucose. These data suggest that NO production stimulated by bradykinin or A23187 was unchanged by elevated glucose. Assayed eicosanoids, including 6-ketoprostaglandin (PG) F₁α, PGE₂, PGF₂α, and 15(S)-hydroxy-(5Z, 8Z, 11Z, 13E)-eicosatetraenoic acid, stimulated by bradykinin or A23187, were increased in cells exposed to 44 mM glucose. These eicosanoid products formed from exogenously added arachidonic acid did not differ between cells incubated in 5.5 mM or 44 mM glucose. Hyperosmolar concentrations of mannose or sucrose had no effect on cGMP levels but did mimic the effect of elevated glucose on eicosanoid production. These data suggest that hyperglycemia in diabetes may interfere with NO-induced guanylate cyclase activation but not NO production in the endothelium and that increased phospholipase activity, secondary to hyperosmolarity, may account for elevated eicosanoid levels. (Arteriosclerosis and Thrombosis 1993;13:915–923)

KEY WORDS • pig aortic endothelial cells • nitric oxide • cyclic GMP • eicosanoids • glucose • diabetes mellitus

The endothelium is an important source of vasoactive substances that regulate vascular tone. Endothelium-derived relaxing factor (EDRF), which has been identified as nitric oxide (NO), stimulates smooth muscle guanylate cyclase to produce cyclic GMP (cGMP) and initiate relaxation. Eicosanoids also produced in the endothelium may act as vasodilators or vasoconstrictors, depending on the vascular bed and species. Alterations in the production or action of these substances may explain abnormal endothelium-dependent vasomotor responses in several vascular diseases, including diabetes mellitus, hypertension, and hypercholesterolemia.

Studies on diabetic rabbit and rat models and human vascular tissue have shown decreased endothelium-dependent relaxations. It is possible that the abnormal relaxation seen in diabetes is due to decreased levels of NO, increased vasoconstrictor eicosanoids, or a combination of both. Tesfamariam et al have shown that acetylcholine-induced relaxations of the aorta are reduced in the alloxan-induced diabetic rabbit and that there is a development of dose-dependent contractions that occurs concomitantly with increased production of vasoconstrictor eicosanoids. Since the abnormal relaxations are restored and the contractions are blocked by cyclooxygenase inhibitors or the prostaglandin (PG) H₂/thromboxane A₂ receptor antagonist SQ29548, increased activity of the cyclooxygenase pathway has been implicated in the abnormal vasomotor response. Alternatively, Abiru et al have suggested that the abnormal relaxation of diabetic arteries is due to decreased NO production, because decreased cGMP levels were measured in diabetic rabbit aorta.

The abnormal production of vasoactive substances by the endothelium that is observed in diabetic rabbit aorta is believed to be directly caused by exposure of the endothelial cells to hyperglycemia, because following a 6-hour exposure to 44 mM glucose, normal rabbit aortic rings show reduced endothelium-dependent relaxation and increased eicosanoid production quite similar to those seen in the diabetic rabbit aorta. To better understand the mechanism by which elevated glucose level alters the function of the endothelium in intact arteries in the short-term, the present studies were designed to determine the effects of exposure of cultured endothelial cells for 6 hours to elevated glucose levels under conditions similar to those used for the studies of intact arteries. Pig aortic endothelial cells (PAECs) are an apt model with which to study the...
effects of elevated glucose levels, as Brown et al. have demonstrated that exposure of PAECs to elevated glucose levels for several days causes a decreased A23187-induced release of arachidonic acid but an increased production of 15(S)-hydroxy-(5Z, 8Z, 11Z, 13E)-eicosatetraenoic acid (15-HETE). In the present studies NO was assessed by measuring the accumulation of endothelial cell cGMP that occurs during NO production. Eicosanoids were measured by radioimmunoassay. A preliminary report of some of these observations has been made.

Methods

Cell Culture

Pig thoracic aortas were obtained from a local abattoir and were aseptically prepared by removing the perivascular connective tissue and opening longitudinally to expose the luminal surface. The PAECs were obtained by scraping the luminal surface once with a razor blade. They were transferred to a 100-mm-diameter Petri dish (Costar, Cambridge, Mass.) containing 10 mL medium and dispersed by pipetting up and down several times. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco No. 430-1600, Grand Island, N.Y.) supplemented with 3.7 g/L NaHCO3, 10% fetal bovine serum (FBS; Hazelton or Sigma), 100 units/mL penicillin, and 100 µg/mL streptomycin (GIBCO). The media were changed every 2-3 days. Cell cultures were maintained in a humidified incubator at 37°C in the presence of 5% CO2 to keep the media saturated with ethyl ether and concentrated by lyophilization. The residue was then resuspended in 0.1 M sodium acetate buffer, and cGMP levels were quantified after acetylation with a commercially available cGMP radioimmunoassay kit (Biomedical Technologies Inc.). The protein pellet was solubilized by resuspending in 250 µL of 38.5 mM mannose or sucrose was added to 5.5 mM glucose as a hyperosmotic control. In other experiments superoxide dismutase (SOD; 150 units/mL) was present throughout the 6-hour incubation. The last 30 minutes of the incubation was done in the presence of 3-isobutyl-1-methylxanthine (IBMX; 10−4 M), a phosphodiesterase inhibitor. In a few studies, IBMX was excluded. Bradykinin (10−7 M) or the calcium ionophore A23187 (10−4 M) was applied for 1- and 10-minute periods. In initial studies measurements were also done at 0.5, 2, and 30 minutes and excluded a shift in the peak levels of cGMP stimulated by bradykinin in elevated glucose. Values of cGMP (mean ± SEM fmol/mg, three experiments, n = 9) at 0, 0.5, 1, 2, 10, and 30 minutes after addition of bradykinin (10−7 M) were 16±2.3, 424±84, 697±158, 862±148, 255±67, and 30±4.6 for 5.5 mM glucose and 14±2.3, 368±95, 602±137, 820±160, 315±70, and 40±9.3 for 44 mM glucose, respectively. Captopril (10−6 M) was added 30 minutes before the addition of bradykinin to prevent breakdown by angiotensin converting enzyme.

cGMP Radioimmunoassay

At the end of agonist stimulation, the PSS was immediately removed for eicosanoid measurement, and 0.6 mL of 6% trichloroacetic acid was added to the cell monolayer. Cells were detached with a rubber policeman and placed in a 1.5 mL Eppendorf vial. The cells were washed with another 0.4 mL of 6% trichloroacetic acid that was combined with the first aliquot for a total volume of 1 mL. The vial was centrifuged at 12,000 rpm to separate denatured proteins. The supernatant was decanted by washing four times with 4 volumes of water-saturated ethyl ether and concentrated by lyophilization. The residue was then resuspended in 0.1 M sodium acetate buffer, and cGMP levels were quantified after acetylation with a commercially available cGMP radioimmunoassay kit (Biomedical Technologies Inc.). The protein pellet was solubilized by resuspending in 250 µL of 38.5 mM mannose or sucrose was added to 5.5 mM glucose as a hyperosmotic control. In other experiments superoxide dismutase (SOD; 150 units/mL) was present throughout the 6-hour incubation. The last 30 minutes of the incubation was done in the presence of 3-isobutyl-1-methylxanthine (IBMX; 10−4 M), a phosphodiesterase inhibitor. In a few studies, IBMX was excluded. Bradykinin (10−7 M) or the calcium ionophore A23187 (10−4 M) was applied for 1- and 10-minute periods. In initial studies measurements were also done at 0.5, 2, and 30 minutes and excluded a shift in the peak levels of cGMP stimulated by bradykinin in elevated glucose. Values of cGMP (mean ± SEM fmol/mg, three experiments, n = 9) at 0, 0.5, 1, 2, 10, and 30 minutes after addition of bradykinin (10−7 M) were 16±2.3, 424±84, 697±158, 862±148, 255±67, and 30±4.6 for 5.5 mM glucose and 14±2.3, 368±95, 602±137, 820±160, 315±70, and 40±9.3 for 44 mM glucose, respectively. Captopril (10−6 M) was added 30 minutes before the addition of bradykinin to prevent breakdown by angiotensin converting enzyme.

Eicosanoid Radioimmunoassay

At the end of agonist stimulation, the 2 mL PSS on the cells was immediately removed, combined with 50 µL of 6% trichloroacetic acid, and frozen (−70°C) until analysis. Radioimmunoassay was used to quantify the levels of 6-keto-PGFlα (the stable hydrolytic product of prostacyclin), PGE2, PGFM, and thromboxane B2 (TXB2, the stable hydrolytic product of thromboxane A2), and 15-HETE in the PSS. Radioimmunoassays were performed by using specific antisera for 6-keto-PGF1α, PGE2, PGFM, TXB2, and 15-HETE (Advanced Magnetics, Cambridge, Mass.), tritiated standards (DuPont–New England Nuclear, Boston), and unlabeled standards (UpJohn, Kalamazoo, Mich.). Cross-reactivity with other measured eicosanoids was ≤5%. The limits of sensitivity of the radioimmunoassay were 1 pg/mL for 6-keto-PGF1α, PGFM, TXB2, and 15-HETE, and 10 pg/mL for PGE2. Results are expressed as nanograms eicosanoid per well.

Drugs

Agents used were bradykinin, A23187, ATP, arachidonic acid, indomethacin, IBMX, N-nitro-l-arginine (NNA), SOD (Sigma Chemical Co., St. Louis, Mo.), and NO (Matheson Gas, Gloucester, Mass.). Concentrations are expressed as final molar concentrations. Indomethacin was prepared in 2% Na2CO3 immediately before use. A23187 was prepared in 95% ethanol.
IBMX was prepared in dimethyl sulfoxide. NO was prepared in water (4°C) in air-tight Vacutainer tubes (Becton Dickinson, Rutherford, N.J.) that were purged of O₂ by vacuum for 20 minutes and bubbling with N₂ for 20 minutes. NO gas was bubbled in 8 mL water for 10 minutes to yield a saturated solution (approximately 10⁻³ M) and was serially diluted 1:10 so that a 100-fold dilution of the NO solution occurred when it was added to the cells. NO solutions were made up immediately before addition to the cells.

Data Analysis

Data are expressed as mean±SEM. Statistical evaluation of the data was achieved using Student's t test for paired comparisons between cells incubated in 5.5 mM or 44 mM glucose PSS from the same plate or with unpaired comparisons between drug treatments in different plates. A value of p<0.05 was considered significant. In all experiments, n represents the number of 9.6-cm² wells used. On any one day, from one to three replicates of each experimental condition were performed.

Results

cGMP Levels

After 30 minutes of equilibration in the presence of IBMX, basal cGMP levels in PAECs exposed to 5.5 mM glucose were 13.7±3.6 fmol/µg. Bradykinin (10⁻⁷ M) increased cGMP to 348.9±51.2 fmol/µg at 1 minute, and values had decreased to 51.1±9.7 after 10 minutes (Figure 1). Basal levels of cGMP were not significantly different in PAECs exposed to 44 mM glucose for 6 hours (12.2±2.4 fmol/µg). cGMP levels at 1 minute after bradykinin stimulation were significantly decreased in cells incubated in 44 mM glucose compared with cells incubated in 5.5 mM glucose, but at 10 minutes there was no significant difference (Figure 1).

In cells incubated in 5.5 mM glucose for 6 hours, A23187- (10⁻⁶ M) stimulated cGMP levels at 1 minute were similar to those after addition of bradykinin, but cGMP increased further at 10 minutes (Figure 1). In PAECs exposed to 44 mM glucose for 6 hours, A23187-stimulated levels of cGMP were significantly less than control cells at 1 and 10 minutes (Figure 1). ATP (10⁻⁵ M) also stimulated cGMP levels at 1 minute (Figure 2) and returned to near basal levels at 10 minutes (data not shown). Although the average ATP-stimulated cGMP levels were less in cells exposed for 6 hours to 44 mM glucose, the differences were not significant (Figure 1).

Exogenous NO (10⁻⁶ M) stimulated cGMP levels maximally at 1 minute, and levels decreased at 10 minutes (data not shown). Levels of cGMP 1 minute after NO stimulation were significantly less in cells exposed to 44 mM glucose for 6 hours compared with those exposed to 5.5 mM glucose (Figure 2).

In the presence of SOD (150 units/mL; n=6) added throughout the 6-hour period, elevated glucose still caused a statistically significant decrease in cGMP levels stimulated by bradykinin (10⁻⁷ M); Figure 2).

Eicosanoid Production

In unstimulated cells, 6-keto-PGF₁α levels were unchanged over the 30-minute period measured, and there was no significant difference in basal levels of 6-keto-PGF₁α between cells incubated in 5.5 mM or 44 mM glucose (Figure 3). 6-Keto-PGF₁α levels were stimulated by bradykinin (10⁻⁷ M), peaking at 10 minutes and unchanged at elevated levels at 30 minutes (Figure 3). The other eicosanoids measured showed similar time courses to that shown for 6-keto-PGF₁α (data not shown); in further studies, therefore, eicosanoids were unchanged (4.2±0.8 fmol/µg), but A23187-stimulated levels were significantly less at 1 and 10 minutes (14±0.7 and 6.1±1.3 fmol/µg, respectively; n=3) compared with 5.5 mM glucose.

FIGURE 1. Line graphs showing (panel A) bradykinin-(10⁻⁷ M) or (panel B) A23187-(10⁻⁶ M) stimulated production of cyclic GMP (cGMP) in pig aortic endothelial cells incubated in 5.5 mM (○) or 44 mM (●) glucose physiological salt solution for 6 hours. Values are expressed as mean±SEM; n=20 wells for bradykinin and n=26 for A23187. From one to three replicates were performed on any one day. *Significant difference in cGMP production between 5.5 mM and 44 mM glucose.

FIGURE 2. Line graphs showing (panel A) bradykinin-(10⁻⁷ M) or (panel B) A23187-(10⁻⁶ M) stimulated production of 6-keto-PGF₁α (6-keto-PGF₁α) in pig aortic endothelial cells incubated in 5.5 mM (○) or 44 mM (●) glucose physiological salt solution for 6 hours. Values are expressed as mean±SEM; n=20 wells for bradykinin and n=26 for A23187. From one to three replicates were performed on any one day. *Significant difference in 6-keto-PGF₁α production between 5.5 mM and 44 mM glucose.
Figure 2. Bar graph showing basal (n=26), ATP- (10^{-5} M; n=4), A23187- (10^{-6} M; n=26), bradykinin- (Brk; 10^{-7} M; n=20), Brk with superoxide dismutase- (SOD; 150 units/mL; n=6), and nitric oxide- (NO; 10^{-6} M; n=13) stimulated production of cyclic GMP (cGMP) after 1 minute in pig aortic endothelial cells incubated in 5.5 mM or 44 mM glucose physiological salt solution for 6 hours. Values are expressed as mean±SEM. n represents the number of wells; from one to three replicates were performed on any one day. *Significant difference in cGMP production between 5.5 mM and 44 mM glucose.

Figure 3. Line graph showing basal and bradykinin- (10^{-7} M) stimulated production of 6-ketoprostaglandin F_{1alpha} (6-k- PGF_{1alpha}), PGE_{2}, and 15-HETE in pig aortic endothelial cells incubated in 5.5 mM or 44 mM glucose compared with 5.5 mM glucose (Figure 4). The time course of eicosanoid production after addition of A23187 (10^{-6} M) was similar to that after addition of bradykinin (data not shown). After 10 minutes of stimulation with A23187, levels of PGE_{2} and PGF_{2alpha} were significantly increased in cells exposed to 44 mM glucose, but the levels of the other eicosanoids measured were not significantly different (Figure 4). TXB_{2} levels did not increase after stimulation with bradykinin or A23187, and there was no significant difference between the levels produced by cells exposed to either 5.5 or 44 mM glucose.

Figure 4. Bar graphs showing (panel A) bradykinin- (10^{-7} M; 10 minutes; n=37) and (panel B) A23187- (10^{-6} M; 10 minutes; n=23) stimulated 6-ketoprostaglandin F_{1alpha} (6-k- PGF_{1alpha}), PGE_{2}, PGF_{2alpha}, thromboxane B_{2} (TXB_{2}), and 15(S)-hydroxy-(5Z, 8Z, 11Z, 13E)-eicosatetraenoic acid (15-HETE) production in pig aortic endothelial cells incubated in 5.5 mM or 44 mM glucose physiological salt solution for 6 hours. Values are expressed as mean±SEM. *Significant difference in eicosanoid or 15-HETE levels between 5.5 mM and 44 mM glucose. Note that the right y axis has a different scale for TXB_{2} and 15-HETE.

Effects of Hyperosmolarity

Supplementing 5.5 mM glucose for 6 hours with mannose (38.5 mM) or sucrose (38.5 mM) to achieve equivalent osmolarity to 44 mM glucose had no significant effect on cGMP levels under basal conditions or at 1 or 30 minutes after agonist stimulation. Ten minutes after bradykinin stimulation, levels of 6-keto-PGF_{1alpha}, PGE_{2}, and 15-HETE were significantly increased in cells exposed to 44 mM glucose compared with 5.5 mM glucose (Figure 4). The time course of eicosanoid production after addition of A23187 (10^{-6} M) was similar to that after addition of bradykinin (data not shown). After 10 minutes of stimulation with A23187, levels of PGE_{2} and PGF_{2alpha} were significantly increased in cells exposed to 44 mM glucose, but the levels of the other eicosanoids measured were not significantly different (Figure 4). TXB_{2} levels did not increase after stimulation with bradykinin or A23187, and there was no significant difference between the levels produced by cells exposed to either 5.5 or 44 mM glucose.

Arachidonic acid (10^{-6} and 10^{-3} M) stimulated production of eicosanoids at 10 minutes to levels similar to those caused by bradykinin or A23187 (Figure 5). There were no apparent differences in the levels of eicosanoids produced from exogenous arachidonic acid in cells exposed to 44 mM glucose compared with 5.5 mM glucose (Figure 5).
physiological salt solution for 6 hours. Values are expressed as mean±SEM; n=4 wells performed on four separate days. Note that the right y axis has a different scale for TxB2 and 15-HETE.

FIGURE 5. Bar graphs showing arachidonic acid—(panel A, 10^-6 M; panel B, 10^-5 M; both concentrations for 10 minutes) stimulated 6-ketoprostaglandin F1α (6-k-PGF1α), PGE2, PGF2α, thromboxane B2 (TxB2), and 15(S)-hydroxy-(5Z, 8Z, 11Z, 13E)-eicosatetraenoic acid (15-HETE) production in pig aortic endothelial cells incubated in 5.5 or 44 mM glucose physiological salt solution for 6 hours. Values are expressed as mean±SEM; n=4 wells performed on four separate days. Note that the right y axis has a different scale for TxB2 and 15-HETE.

10 minutes after bradykinin stimulation (Figure 6). Exogenous NO—(10^-6 M) stimulated levels of cGMP were also not significantly affected by mannose (Figure 6).

Levels of eicosanoids under basal conditions were unaffected by exposure to hyperosmolar mannose or sucrose for 6 hours compared with 5.5 mM glucose (data not shown). Bradykinin—(10^-7 M) stimulated levels of eicosanoids after 10 minutes were significantly elevated in cells exposed to elevated mannose or sucrose compared with 5.5 mM glucose (Figure 6).

Interactions Between cGMP and Eicosanoids

Treatment with indomethacin (10^-6 M) markedly inhibited production of all prostanoids as well as 15-HETE (Table 1), but had no apparent effect on basal or A23187-stimulated eicosanoid levels (Table 1). IBRX (10^-4 M), which markedly increased cGMP levels at 1 or 10 minutes after stimulation with A23187, had no significant effect on levels of eicosanoids produced by PAECs (Table 1).

Discussion

PAECs have been used by others as a model to study the release of endothelium-derived vasoactive factors including NO and eicosanoids. White and Martin have studied production of NO and 6-keto-PGF1α simultaneously by methods similar to those used here. Intracellular levels of cGMP in the endothelial cells were used in this study as an indicator of NO production. This technique is validated by the fact that the NO synthase inhibitor NNA prevented the agonist-induced rise in cGMP, as previously reported with another NO synthase inhibitor. Measurement of the product of guanylate cyclase provides an assay that depends not only on NO production but also on guanylate cyclase stimulation and phosphodiesterase activity. Differences in guanylate cyclase sensitivity and phosphodiesterase activity may be inferred if any changes occur in cGMP levels in response to exogenous NO. The phosphodiesterase inhibitor IBMX was used to enhance the levels of cGMP and to exclude the contribution of phosphodiesterase to the differences observed.

The eicosanoids measured by radioimmunoassay in this study demonstrate that these endothelial cells produce large amounts of PGE2 and PGF2α in addition to 6-keto-PGF1α. PAECs also produce 15-HETE. In this study the release of 15-HETE was reduced significantly by indomethacin, which suggests, along with other studies, that it can arise as a result of cyclooxygenase activity. Thromboxane A2 levels did not significantly increase after agonist stimulation, raising doubt as to whether thromboxane A2 is made enzymatically in significant amounts by these cells. This is also suggested by the fact that TxB2 was the only metabolite that did not increase after the addition of exogenous arachidonic acid (Figure 5). Nevertheless, the levels detected were reduced by indomethacin (Table 1), and it is unlikely that the low levels represent cross-reactivity with other eicosanoids because an increase was not observed with elevated glucose, as seen for the bulk of eicosanoids.

IBMX greatly enhanced the levels of cGMP but did not influence the measurements of released eicosanoids (Table 1). In fact, entirely preventing the rise in cGMP with NNA did not influence eicosanoid release (Table 1). Also, inhibiting eicosanoid production with indomethacin had no apparent effect on cGMP levels (Table 2). These studies indicate that although NO and eicosanoids are released by the agonists simultaneously, there is no apparent influence of either product on the release of the other, as suggested previously.

Effect of Elevated Glucose on cGMP

A 6-hour exposure to elevated glucose significantly decreased agonist-stimulated cGMP levels; the lower levels were noted during the peak stimulation by bradykinin but persisted throughout the stimulation by A23187. The difference caused by glucose is unlikely to be due to a change in phosphodiesterase activity because the difference was noted both in the presence and stimulated increases in cGMP levels (Table 2). NNA had no apparent effect on A23187-stimulated eicosanoid levels (Table 1). IBMX (10^-4 M), which markedly increased cGMP levels at 1 or 10 minutes after stimulation with A23187, had no significant effect on levels of eicosanoids produced by PAECs (Table 1).
FIGURE 6. Bar graphs showing cyclic GMP (cGMP) (panels A and B) and eicosanoid (panels C and D) production in pig aortic endothelial cells incubated for 6 hours in 5.5 mM glucose physiological salt solution supplemented with 38.5 mM mannose (panels A and C) or 38.5 mM sucrose (panels B and D). cGMP levels were measured in cells under basal conditions and after stimulation with bradykinin (Brk) (10⁻⁷ M) for 1 or 10 minutes and with nitric oxide (NO) (10⁻⁶ M) for 1 minute. Eicosanoids were measured after stimulation with bradykinin (10⁻⁷ M) for 10 minutes. Values are expressed as mean±SEM; n=6-12 for mannose and n=3 for sucrose. Sucrose data were obtained on one day, and experiments were performed on cells from a different primary culture than those on which mannose was tested. *Significant difference in eicosanoid production between cells incubated in normal glucose and in elevated mannose or sucrose. Note that the right y axis has a different scale for thromboxane B₂ (TxB₂) and 15(S)-hydroxy-(5Z, 8Z, 11Z, 13E)-eicosatetraenoic acid (15-HETE) in panel C. nd, Not detectable; k, keto; PG, prostaglandin.

absence of IBMX. The fact that exogenous NO-stimulated levels were decreased similarly to those elicited by the agonists indicates that the effect of elevated glucose on the cGMP rise in response to the agonists is not due to decreased NO production but rather to impaired stimulation of PAEC guanylate cyclase. This result may imply that agonist-induced, receptor-mediated transmembrane signaling, which leads to a rise in intracellular calcium levels and activation of NO synthase, is also normal in those PAECs exposed to elevated glucose.

Superoxide anion interferes with guanylate cyclase stimulation by NO, so that increased levels of the free radical in elevated glucose could explain the decrease in cyclic nucleotide observed. This is less likely, given that SOD did not prevent the decrease in cGMP. The effect of elevated glucose on guanylate cyclase stimulation was not due to hyperosmolarity, because increasing the osmolarity to the same levels with mannose or sucrose had no significant effect on the bradykinin- or NO-stimulated levels of cGMP.

Effect of Elevated Glucose on Eicosanoid Production

Although basal levels of eicosanoids were unaffected by exposure to elevated glucose, there was a generalized increase when production was stimulated by either bradykinin or A23187 in cells exposed to elevated glucose. The effect of elevated glucose on the response to the two agonists was similar, with the notable exception of 15-HETE, which was increased when cells exposed to elevated glucose were stimulated with bradykinin but not when they were stimulated with A23187. The increased eicosanoid production caused by expo-
sure to elevated glucose apparently depends on the release of endogenous arachidonic acid substrate, because the production following the addition of exogenous substrate was unaffected. This suggests that the increased production in response to both agonists depends on altered phospholipase activity. These results differ from the effect of culturing PAECs for two passages in 15.6 mM glucose, in which an overall decrease in arachidonate metabolites were produced, although interestingly, 15-HETE released in response to the two agonists was increased. This is commensurate with previous reports that have suggested altered arachidonic acid availability and release in other tissues or cells exposed to elevated glucose. The fact that cGMP levels in response to both bradykinin and A23187 were decreased by glucose also suggests that the increase observed in arachidonate metabolites is specific for that pathway, rather than a generalized increase in activation of cell signaling pathways. Interestingly, unlike the decreased NO responsiveness, the increase observed in eicosanoids after exposure to elevated glucose was apparently due to hyperosmolarity, as eicosanoids were also increased after exposure to elevated mannose and sucrose.

In contrast to our findings that glucose increased bradykinin-stimulated prostacyclin, measured as 6-keto-

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<th>TABLE 1. Effects of Cyclooxygenase, NO Synthase, and Phosphodiesterase Inhibition on A23187-Stimulated Eicosanoid Formation</th>
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NO, nitric oxide; PG, prostaglandin; TxB<sub>2</sub>, thromboxane B<sub>2</sub>; 15-HETE, 15(5)-hydroxy-(5Z, 8Z, 11Z, 13E)-eicosatetraenoic acid; nd, not detectable; –, in the absence of; +, in the presence of. Data are expressed as mean±SEM in nanograms per well; n=4, 2, and 9 for indomethacin, N-nitro-L-arginine (NNA), and 3-isobutyl-1-methylxanthine (IBMX), respectively. Cells used in each of the three treatments are from different primary cultures. Pig aortic endothelial cells were incubated for 6 hours in physiological salt solution containing 5.5 mM or 44 mM glucose. Indomethacin was present throughout the 6-hour incubation, and NNA and IBMX were added during the last 30 minutes. Eicosanoid levels were measured after stimulation with A23187 (10<sup>-6</sup> M) for 10 minutes.

*Indomethacin significantly decreased all eicosanoids; NNA or IBMX had no apparent effect.

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<th>TABLE 2. Effects of Cyclooxygenase or NO Synthase Inhibition on A23187-Stimulated cGMP Formation in Pig Aortic Endothelial Cells</th>
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NO, nitric oxide; cGMP, cyclic GMP. Data are expressed as mean±SEM in femtomoles cGMP per microgram protein; n=2-5 for indomethacin and 1-2 for N-nitro-L-arginine (NNA). After a 6-hour incubation in physiological salt solution containing 5.5 mM or 44 mM glucose, pig aortic endothelial cells were equilibrated for 30 minutes with 3-isobutyl-1-methylxanthine (10<sup>-4</sup> M). Indomethacin was present throughout the 6-hour incubation and NNA was added during the last 30 minutes. cGMP levels were measured in unstimulated cells (basal) and after 1 and 10 minutes of A23187 (10<sup>-6</sup> M). Indomethacin had no apparent effect on cGMP levels, whereas NNA abolished A23187-stimulated increases in cGMP levels.
PGF<sub>2α</sub>, others have found that diabetic human and animal vascular tissue and endothelial cells cultured in elevated glucose produce less prostacyclin (see References 22 and 23). In our own studies, the aortas of 6-week-old diabetic rabbits<sup>5</sup> and those of normal rabbits in incubated for 6 hours in elevated glucose<sup>11</sup> showed no significant change in this eicosanoid, although others, notably PGF<sub>2α</sub> and thromboxane A<sub>2</sub> were increased. Ono et al<sup>23</sup> found that cultured bovine endothelial cells grown for five to eight passages in elevated glucose had markedly decreased prostacyclin production. In addition to differences in species, the different results could be due to several factors. In cultured endothelial cells it has been demonstrated that arachidonic acid content can decrease as much as 66% over the first four passages, and this can markedly decrease eicosanoid production.<sup>44</sup> This factor alone could explain the difference found in our cells, which were grown for only two passages. Also, tissues from diabetics and cells grown for several passages with serum, unlike our cells, which underwent a brief exposure to elevated glucose in PSS, are exposed to insulin, which can itself influence eicosanoid release.<sup>45</sup> Several studies, including our own, have suggested that elevated glucose interferes with prostacyclin production at the level of phospholipase A<sub>2</sub>.<sup>6,26</sup> Whether elevated glucose causes an increase or decrease in eicosanoids, perhaps the most important factor is the duration of exposure, because the decreases observed have occurred over a duration of days to weeks. The present study suggests that the early and perhaps most direct result of elevated glucose is an increased release of endothelial cell eicosanoids, including prostacyclin.

**Comparisons With Blood Vessels Exposed to Elevated Glucose**

Aortas isolated from diabetic rabbits that have plasma glucose values averaging 20 mM or normal aortas exposed to 44 mM glucose for 6 hours release increased amounts of eicosanoids when stimulated by the endothelium-dependent agonist acetylcholine and demonstrate decreased relaxation.<sup>5,11</sup> The increased eicosanoid release was ascribed to the endothelial cells because it was eliminated when the endothelium was removed. Although not all studies agree,<sup>27-29</sup> abnormal endothelium-dependent relaxations in diabetes have been observed in the rat aorta,<sup>9,20-33</sup> rabbit aorta,<sup>5,8,11</sup> pig and dog coronary artery,<sup>24</sup> and human penile corpus cavernosum.<sup>10</sup> The reversal by cyclooxygenase inhibitors of reduced endothelium-dependent relaxations of rabbit aorta exposed to elevated glucose suggests that eicosanoids produced by the endothelium cause contraction that offsets the relaxation mediated by NO. Therefore, reduced NO production need not be invoked to explain the impaired endothelium-dependent relaxation, of which it is the primary mediator.<sup>5,11</sup> This possibility is further supported by the apparent lack of any effect on NO release with increased eicosanoid production in PAECs exposed to elevated glucose.<sup>37</sup>

The identity of the endothelium-derived eicosanoid vasoconstrictor is not known with certainty. Recent studies have suggested that the prostaglandin end products themselves, although they can all contract the rabbit aorta when applied exogenously,<sup>2</sup> may not be released in sufficient quantities by the endothelium of the isolated rabbit aorta to contract the smooth muscle. Under normal-glucose conditions relatively large amounts of eicosanoids are produced, but there is no effect of cyclooxygenase blockade on endothelium-dependent relaxations.<sup>11</sup> Also, aldose reductase inhibitors<sup>35</sup> or free-radical scavengers<sup>36</sup> both normalize the impaired endothelium-dependent relaxation of the diabetic rabbit aorta without affecting the increased production of eicosanoid metabolites. These findings suggest that the metabolism of glucose by the aldose reductase pathway may give rise to free radicals<sup>37,38</sup> that can interfere with endothelium-dependent relaxation. Furthermore, superoxide anion was shown to be a key element in the contraction caused by exogenous prostaglandin endoperoxide (PGH<sub>2</sub>) but not the prostaglandin end products.<sup>39</sup> A generalized increase in eicosanoid metabolites would be compatible, nevertheless, with an increase in PGH<sub>2</sub> formation by cyclooxygenase, which also generates free radicals.<sup>40,41</sup> It has been shown that the levels of PGH<sub>2</sub> produced in the rabbit aortic endothelium are sufficient to contract the smooth muscle.<sup>42</sup> It would appear then that the coincident production of free radicals and PGH<sub>2</sub> may account for the endothelium-derived vasoconstrictor in blood vessels exposed to elevated glucose. Of interest is the fact that exposure of the rabbit aorta to hyperosmolar mannose does not impair endothelium-dependent relaxation as does glucose,<sup>11</sup> which suggests that the metabolism of glucose is required for free-radical production, whereas the exposure to hyperosmolarity increases eicosanoid production.

In conclusion, various studies have shown that diabetes and exposure to elevated glucose can alter the levels of vasoactive substances, such as eicosanoids, released from the endothelium of blood vessels. These studies in PAECs may provide a model system with which to study the elaboration of vasoactive substances from endothelium exposed to elevated glucose.

**References**


Effect of elevated glucose on cyclic GMP and eicosanoids produced by porcine aortic endothelium.

R M Weisbrod, M L Brown and R A Cohen

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