Arteriolar nitric oxide concentration is decreased during hyperglycemia-induced βII PKC activation

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Bohlen, H. Glenn, and Geoffrey P. Nase. Arteriolar nitric oxide concentration is decreased during hyperglycemia-induced βII PKC activation. Am J Physiol Heart Circ Physiol 280: H621–H627, 2001.—βII protein kinase C (βPKC) is activated during acute and chronic hyperglycemia and may alter endothelial cell function. We determined whether blockade of βPKC protected in vivo endothelial formation of NO, as measured with NO-sensitive microelectrodes in the rat intestinal vasculature. NaCl hyperosmolarity, a specific endothelial stimulus to increase NO formation, caused 20% arteriolar vasodilatation and 30% increase in NO concentration ([NO]). After topical 300 mg/dl hyperglycemia for 45 min, both responses were all but abolished. In comparison, pretreatment with LY-333531, a specific βPKC inhibitor, maintained vasodilation and [NO] responses to NaCl hyperosmolarity after hyperglycemia. The βPKC inhibitor alone had no significant effects on resting diameter or [NO] or their responses to NaCl hyperosmolarity. In separate rats, after topical hyperglycemia had suppressed dilation to ACh, LY-333531 restored 70% of the dilatory response. These data demonstrated that activation of βPKC during acute hyperglycemia depressed in vivo endothelial formation of NO at rest and during stimulation. This abnormality can be minimized by inhibition of βPKC before hyperglycemia and can be substantially reversed by PKC inhibition after hyperglycemia-induced abnormalities have occurred.

protein kinase C; arteriole

SUPPRESSION OF ENDOTHELIAL nitric oxide (NO) formation is presumed to be a major microvascular regulatory problem during diabetes mellitus. Most of what is known about this abnormality during hyperglycemia is derived from bioassay of depressed endothelium-dependent relaxation of in vivo and in vitro arterial vessels, decreased production of NO by-products in tissue culture, and suppressed expression of constitutive endothelial NO synthase (eNOS) in endothelial cells (3, 8, 19, 20, 22, 28). We recently showed, using NO-sensitive microelectrodes, that acute hyperglycemia (300 mg/dl) decreased the in vivo periarteriolar resting NO concentration ([NO]) and abolished ACh-stimulated NO formation (19). These studies were performed in the rat spinotrapezius muscle microvasculature. Similar abnormalities of NO physiology, as judged by vasodilatory responses, have been found in the skeletal muscle vasculature of humans exposed to acute and chronic hyperglycemia (17, 28). In all bioassay studies of vascular performance referenced thus far, the primary deficiency is very likely decreased NO availability. The reactivity of vascular smooth muscle to exogenous NO consistently was essentially normal, even in studies of prolonged diabetic hyperglycemia (3, 4, 14, 20, 22).

Impairment of endothelial cells by hyperglycemia at ≥300 mg/dl is known to occur within hours in in vitro studies (26, 29). In some in vivo studies of normal animals (19, 20, 23, 28), hyperglycemia impaired endothelial NO function within 15–60 min. This brief onset of altered in vivo regulation indicated an abnormality of acute regulation or a cellular injury, rather than an initial genomic alteration. Possible mechanisms to explain the rapid disturbance of microvascular function during acute hyperglycemia include oxidant destruction of NO (3), increased eicosanoid metabolism (3, 26), and activation of endothelial βII protein kinase C (βPKC) in response to hyperglycemia (10, 11, 21, 24). Increased βPKC activity may depress eNOS and thereby lower NO generation by endothelial cells (15, 16). Simultaneously, PKC activation will likely stimulate phospholipase C to increase membrane lipid-catabolism to form diacylglycerol, a precursor of arachidonic acid for eicosanoid formation, and the associated increased generation of oxygen radicals that interact with NO. Consistent with these proposals are the observations that long-term suppression of βPKC lessened abnormalities of microvascular cells during chronic diabetic hyperglycemia (7, 8, 13, 21). Furthermore, during acute hyperglycemia, nonspecific inhibitors of PKC improved endothelium-mediated vasodilation in the cerebral circulation (20).

The key issue of this study was to determine whether suppression of βPKC preceding acute hyperglycemia would protect the in vivo endothelial formation of NO. Our secondary issue was to ascertain whether βPKC inhibition after hyperglycemic impairment would restore endothelium-dependent vasodilation. Our concern is that multiple deleterious mechanisms occur
simultaneously during hyperglycemia such that altered regulation and cellular damage are present. While compromised regulation may be amenable to rapid improvement, cellular damage of endothelial processes might limit the improvement of NO physiology. Rather than use bioassay of arteriolar dilation as our primary means to evaluate NO physiology, we measured the NO concentration with microelectrodes on the outer surface of in vivo intestinal arterioles in the normal rat. The results of these studies allowed us to confirm the hypothesis that acute activation of βPKC in endothelial cells by hyperglycemia suppressed NO formation, and this deficit could be substantially prevented as well as reversed by appropriate PKC inhibition.

METHODS

The anesthetic and surgical procedures were approved by the Laboratory Animal Research Committee of the Indiana University Medical School. Adult Sprague-Dawley male rats (400–500 g; Harlan Industries, Indianapolis, IN) were given thiopental sodium (200 mg/kg; Abbott Laboratories, Chicago, IL) subcutaneously in four locations over the lower back and each thigh. This injection approach necessitated twice the dosage required for intraperitoneal injection to induce anesthesia. However, subcutaneous administration avoided any possibility of intestinal damage by the hypodermic needle or exposure to the anesthetic solution. With this route of anesthetic administration, the anesthetic effect was very prolonged, and supplemental anesthetic was rarely required. On reaching surgical-depth anesthesia, the trachea was cannulated to maintain a patent airway, and the right femoral artery was cannulated to measure arterial pressure. The animal was given normal saline (0.5 ml 100 g body wt−1) to compensate for fluid loss by urine formation and mechanical ventilation. All animals were ventilated at a rate of 70 breaths/min, the typical ventilation frequency of conscious rats, and a tidal volume indicated from the Harvard Apparatus nomogram for small animals. In addition, the tidal volume was then adjusted up or down in small increments until the end-tidal CO2 tension was <40 mmHg (SC-219 CO2 monitor, Pyron, Menomonee, WI). With the fluid replacement and ventilatory support, the arterial pressure was virtually constant for 4–5 h after completion of surgery. If the mean arterial pressure decreased by >15 mmHg from the beginning of the experiment, data collection was stopped. The small intestine was prepared for observation with a standard technique (1) that required a midline abdominal incision of 1.5–2 cm. The jejunal region of the bowel was located, and an 8- to 10-cm loop was exteriorized into a basal heated pool of saline and covered with plastic wrap (Saran Wrap, Dow Chemical, Indianapolis, IN). The bowel wall was dried of the tissue. With this technique, no nerves or vascular supply to the intestinal wall are severed or injured. During catherization, the bowel wall was kept wet at all times with saline. The bowel contents were evacuated, and small threads were tied to the edges of the bowel incisions. The bowel was then draped with the mucosal surface downward over a translucent pedestal and held in place by the threads. A fluid chamber was lowered over the bowel and into the support device. A 4–5 ml/min flow of bicarbonate-buffered physiological solution (1) was passed through the chamber after being heated to 37.5 ± 0.5°C. The support device was internally heated to 37.5 ± 0.5°C with heated circulating water. The physiological solution was equilibrated with 5% O2, 5% CO2, 90% N2, and the fluid lines were protected from equilibration with the atmosphere until the fluid entered the stainless steel heating and tissue support system.

The arterioles were observed with a closed-circuit television camera (model XC-77, Hammamatsu) coupled to a computerized digitizing and image analysis system (Image 1, Universal Imaging, West Chester, PA). Images were stored in digital format, and dimensions of the vessels were measured with the virtual caliper of the image analysis system. Linear dimensions were calibrated in the x- and y-dimensions with a stage micrometer marked in 10- and 100-μm units. The nanomolar [NO] was measured with an adaptation of the polarographic technique for gold-plated, recessed-tip glass microelectrodes, as developed by Buerk et al. (6) and used in our past studies (2, 19). The microelectrodes were sharpened to an 8- to 10-μm OD at the base of the sharpened region. Each electrode had a tip recess of 10–20 μm beyond the sharpened region, and the recess was coated with Nafion (Aldrich, Milwaukee, WI). The Nafion coating decreased the random electrical noise of the microelectrodes and essentially eliminated the interference of nitrate, ascorbic acid, tyrosine, and norepinephrine at physiological concentrations with measurements of NO (2, 6, 19). The microelectrodes were polarized at +0.8 V relative to a carbon fiber reference electrode (World Precision Instruments, Sarasota, FL), and the current generated was measured with an electrometer (model 610B, Keithley, Cleveland, OH). A calibration curve was obtained on the morning of each experiment by measurement of the current at 0, ~600, and ~1,200 nM NO. Each batch of calibration gas was slightly different in composition, but the actual concentration was known in every case. Each microelectrode was found to have a linear current-[NO] relationship. We only used microelectrodes that generated ≥1 nA/1,000 nM NO. This sensitivity translated to an ~1-mV increase in output of the electrometer for each nM elevation in [NO] or 80–120 mV above baseline for typical perivascular [NO]. Uncertainties caused by tissue motion, electronic noise, electrode drift characteristics, and very slow declines in microelectrode sensitivity over time limit the resolution of the microelectrodes during in vivo measurements to ~10 nM NO. The slow electrical drift of the microelectrode during experiments was compensated by calculation of an interpolated baseline over time during the individual measurements. Before and immediately after each tissue measurement, the current generated by the microelectrode 500 μm above the tissue surface was used as the “0” nM reference. These data were used to calculate the rate of electronic drift and an interpolated baseline (0 nM) for any given time. The typical duration of a tissue or perivascular measurement was 10 min, and over such time frames, worse-case drift of the baseline would be ~2% of the typical resting [NO] on the surface of arterioles. The reference 0 nM equivalent output voltage and the calibration output voltage-[NO] relationship were used to calculate tissue [NO].

During perivascular measurements, the ideal micropipette penetration was the microelectrode shaft nearly parallel to the arteriole, with the sharpened microelectrode tip appearing to touch the arteriolar wall. If the microelectrode tip was pulled away from the vessel wall, the [NO] decreased dramatically and approached the tissue background [NO] of 100–150 nM at ~50–100 μm from the vessel wall. Our goal in all measurements was to achieve the highest possible [NO] for a given vessel, and we moved the micropipette tip as required to maintain close contact of the vessel wall and microelectrode tip. However, if the microelectrode tip pene-
trated the arteriolar wall, we often found a large and usually transient increase in the [NO]. This was not an artifact of the intestinal microvasculature, because we have observed transient increases in [NO] after penetration of arterioles in the skeletal muscle microvasculature (19).

Protocols

Protocol 1: effect of βPKC inhibitor treatment before hyperglycemia-induced endothelial dysfunction. The animals were divided into two groups: a control group (untreated) and a group that was to be exposed to the endothelial βPKC inhibitor LY-333531 (treated). In both groups of animals, the inner arteriolar diameter and [NO] of first-order arterioles were measured during several different conditions: 1) before PKC treatment, during resting conditions and after endothelial stimulation with NaCl hyperosmolality, 2) after PKC treatment, during resting conditions and after endothelial stimulation with NaCl hyperosmolality, and 3) after PKC treatment and hyperglycemia, during resting conditions and after endothelial cell stimulation with NaCl hyperosmolality. The only difference between the groups was that the treated group was exposed to 20 nM LY-333531 in the bathing fluid for 45 min before the commencement of hyperglycemia.

As a physiological stimulus to increase endothelial generation of NO, we used NaCl hyperosmolality. In the rat intestine, NaCl hyperosmolality is developed during mucosal nutrient absorption, such that the major resistance arterioles of the submucosa are exposed to hypertonic lymph and venular blood returning from the intestinal mucosa (5). For practical purposes, 360 mosM represented the maximum osmolarity of submucosal tissue during near-maximal rates of glucose absorption. Furthermore, we have demonstrated a concentration-dependent relationship of NaCl hyperosmolality to arteriolar dilation and periarteriolar [NO] with a maximal response near 360–380 mosM (2). Comparable mannitol hyperosmolality generated little additional NO above the resting state, and vasodilation was minor. For the first part of the experimental protocol, the resting [NO] and inner arteriolar diameter were measured in both groups of animals before and after bathing fluid osmolality was increased to 360 mosM; this was achieved by topical superfusion of NaCl for 15 min.

After restoration of isotonic conditions, one group was exposed to 20 nM LY-333531 (treated) in the bathing fluid for 45 min and the other group was left untreated. LY-333531 is a specific inhibitor of endothelial βPKC, which is also the isozyme of PKC most increased in diabetic animals and endothelial cells raised in hyperglycemic culture media (10, 11, 21, 24). The 20 nM concentration of LY-333531 was based on studies by Ishii et al. (13). They found that this plasma concentration of LY-333531 minimized microvascular abnormalities in insulin-dependent diabetic rats for many weeks. In addition, our own evaluation indicated that a topical concentration of 10 nM was less effective and a concentration of 5 nM was not effective. Since the drug was topically exposed to just the intestinal wall for 45 min, a lower intravascular concentration or longer topical exposure time may be as or even more effective for acute studies. After treatment with the βPKC inhibitor, inner arteriolar diameter and [NO] measurements were repeated during resting conditions and after endothelial stimulation to NaCl hyperosmolality.

After restoration of isotonic conditions, both groups of rats were exposed to topical 300 mg/dl d-glucose for 45 min. To maintain an isotonic bath media, isotonic glucose solution (5,000 mg/dl) was added to isotonic bathing media. Under these conditions, acute hyperglycemia has been previously shown to result in endothelial cell dysfunction in this vascular bed (3), as well as the cerebral (20) and skeletal muscle (19) microvasculatures. We have found 300 mg/dl d-glucose to be as detrimental to endothelium-dependent vasodilation as 500 mg/dl d-glucose (3, 19). After hyperglycemia, diameter and [NO] were measured during resting conditions and after NaCl hyperosmolality.

Protocol 2: effect of βPKC inhibitor treatment after hyperglycemia-induced endothelial dysfunction. To test receptor-mediated impairments of NO generation, ACh was applied to the wall of individual in vivo arterioles; nitroprusside was used to test endothelium-independent dilation. The inner diameter of arterioles was measured at rest and during microiontophoretic application of ACh and sodium nitroprusside to the exterior of the vessel wall. ACh was used as a receptor-mediated stimulus for NO formation, and sodium nitroprusside decomposed to release NO, as verified by measurement of increased [NO] in tissue when nitroprusside was topically exposed to the tissue. The microvasculature was then exposed to topical 300 mg/dl d-glucose-physiological saline for 45 min, and both dose-response curves were repeated. Thereafter, the tissue was exposed to topical 20 μM LY-333531 for 45 min, and both dose-response curves were repeated. The total time for the experiment was ~2.5 h.

Statistics

All statistical tests were performed with SigmaStat software (Jandel Scientific Software, San Rafael, CA). For the tests using hyperosmolality and LY-333531, diameter and [NO] were remeasured multiple times at the same vascular site, and multiple dosages or treatments were used. Therefore, two-way repeated-measures ANOVA was used to determine whether significant effects occurred. Where significant effects were indicated, pairwise comparisons with Tukey's test were performed. For tests with just the effects of hyperglycemia on responses to NaCl hypertonicity, one-way repeated-measures ANOVA was used. For the tests of post-treatment with LY-333531 after hyperglycemia, the Friedman repeated-measures ANOVA on ranks was used. This approach was necessary because the data were normally distributed but the individual subgroups had unequal variances. Significance of changes was accepted at P ≤ 0.05.

RESULTS

Figures 1 and 2 present the percentage of control [NO] and inner arteriolar diameter data collected during the pretreatment protocol. There were eight rats in each group of animals, and only one arteriole was studied per animal. The mean resting [NO] and inner diameter of the untreated arterioles were 432 ± 76 nM and 82.5 ± 4.8 μm compared with 408 ± 39 nM and 77.1 ± 13.6 μm before treatment with LY-333531. In both groups of animals before application of LY-333531, the 360 mosM NaCl solution caused a 30–35% increase in [NO] (Fig. 1), and the arterioles dilated ~20% (Fig. 2). Vessels of this type routinely are capable of ~50% maximum dilation with sodium nitroprusside (Fig. 3). After exposure of the treated arterioles to LY-333531 for 45 min, neither resting diameter of the arterioles nor [NO] was significantly altered. After the drug exposure, the dilation of treated arterioles to 360 mosM NaCl solution was equivalent to that before treatment with LY-333531 (Fig. 2). In addition, the increase in [NO] in response to hypertonic
NaCl solution after treatment was equivalent to that of the same vessels before drug exposure (Fig. 1).

Hyperglycemia caused significant constriction of the arterioles in untreated tissues but did not significantly constrict arterioles in treated tissues (Fig. 2). In the untreated and treated groups of arterioles, hyperglycemia was associated with substantial reduction in [NO] (Fig. 1). In the untreated group, [NO] was 61.8 ± 6.1% of control; in the treated group, it was 73.5 ± 3.6% of control. Inasmuch as the NO-sensitive microelectrodes were not responsive to the drug or the glucose concentration used, the reduction in [NO] represented an actual decline in the [NO] in the periarteriolar space.

Exposure of the untreated arterioles to hypertonic NaCl solution after hyperglycemia did not significantly change the [NO], which was 60.5 ± 8.1% of control during the period of hypertonic exposure. By comparison, there was a 25% increase (relative to initial normal [NO]) in [NO] during hyperosmolarity in the drug-treated arterioles (Fig. 1). This relative increase in [NO] in the treated group was equivalent to that before hyperglycemia before and after drug exposure. During hypertonic exposure, the arterioles in the treated group dilated by ~22% to a diameter equivalent to that before hyperglycemia when just the drug was present. Although arterioles of the untreated group dilated 12% (relative to original control diameter) during hypertonic exposure, the vessel diameter was 20% smaller than that during their normal response to hyperosmolarity.

![Fig. 1. Periarteriolar nitric oxide (NO) concentration ([NO]) in 2 groups of 8 animals. The untreated (control) group was exposed to 300 mg/dl hyperglycemia; the treated group was exposed to LY-333532 before hyperglycemia. In both groups, topical 360 mosM NaCl was used to stimulate NO production, which increased ~30%. The protein kinase C (PKC) inhibitor LY-333531 did not alter resting or hyperosmolarity-stimulated [NO]. After 300 mg/dl hyperglycemia, resting [NO] decreased in both groups, with a significantly larger decline in untreated arterioles. During 300 mosM NaCl after hyperglycemia, untreated arterioles were unable to increase their [NO], but treated arterioles generated a normal increase in [NO]. *Significantly different from untreated resting state (P ≤ 0.05); †significantly different from treated resting state (P ≤ 0.05).](http://ajpheart.physiology.org/)

![Fig. 2. Inner arteriolar diameters of the same vessels presented in Fig. 1. In both groups, topical 360 mosM NaCl increased arteriolar diameter by ~20%. The PKC inhibitor LY-333531 did not alter resting or hyperosmolarity-stimulated diameter. After 300 mg/dl hyperglycemia, resting diameter was decreased to 95% of control in the treated group and decreased to 89% of control in untreated arterioles. During 300 mosM NaCl after hyperglycemia, untreated arterioles were able to increase their diameter to 102% of the original control diameter for a dilation of 13%. In comparison, treated arterioles dilated to 117% of the original control diameter for a 22% dilation. *Significantly different from untreated resting state (P ≤ 0.05); †significantly different from treated resting state (P ≤ 0.05).](http://ajpheart.physiology.org/)
2. *Significantly different from untreated resting state (*P* per rat); animals are different from those used for data in Figs. 1 and influenced. Data are based on 8 arterioles studied in 4 rats (2 vessels dilation during ACh application; responses to nitroprusside were not PKC then restored the resting diameter and improved the vasodilation to ACh, but not nitroprusside. Blockade of hyperglycemia. Hyperglycemia caused vasoconstriction and decreased vasodilation to ACh, but not nitroprusside. Blockade of βPKC then restored the resting diameter and improved the vasodilation during ACh application; responses to nitroprusside were not influenced. Data are based on 8 arterioles studied in 4 rats (2 vessels per rat); animals are different from those used for data in Figs. 1 and 2. *Significantly different from untreated resting state (*P* ≤ 0.05); **significantly different from treated resting state (*P* ≤ 0.05).

Figure 3 presents the data for a separate group of four rats in which eight vessels (2 per rat) were studied. All vessel diameters are referenced to their initial resting diameter. In these animals, the dilatory responses to ACh and nitroprusside were compared at rest, after 300 mg/dl glucose exposure, and after 45 min of treatment with LY-333531. Diameter of the arterioles was 78.9 ± 2 μm at rest, 65.9 ± 1.2 μm after hyperglycemia, and 74.3 ± 2.3 μm after PKC blockade in the presence of hyperglycemia. Hyperglycemia caused vasoconstriction and decreased vasodilation to ACh, but not nitroprusside. Blockade of βPKC then restored the resting diameter and improved the vasodilation during ACh application; responses to nitroprusside were not influenced. Data are based on 8 arterioles studied in 4 rats (2 vessels per rat); animals are different from those used for data in Figs. 1 and 2. *Significantly different from untreated resting state (*P* ≤ 0.05); **significantly different from treated resting state (*P* ≤ 0.05).

Figure 3 presents the data for a separate group of four rats in which eight vessels (2 per rat) were studied. All vessel diameters are referenced to their initial resting diameter. In these animals, the dilatory responses to ACh and nitroprusside were measured under normal conditions, after 45 min of 300 mg/dl hyperglycemia, and after 45 min of treatment with LY-333531. Diameter of the arterioles was 78.9 ± 2 μm at rest, 65.9 ± 1.2 μm after hyperglycemia, and 74.3 ± 2.3 μm after PKC blockade in the presence of hyperglycemia. Hyperglycemia caused vasoconstriction and decreased vasodilation to ACh, but not nitroprusside. Blockade of βPKC then restored the resting diameter and improved the vasodilation during ACh application; responses to nitroprusside were not influenced. Data are based on 8 arterioles studied in 4 rats (2 vessels per rat); animals are different from those used for data in Figs. 1 and 2. *Significantly different from untreated resting state (*P* ≤ 0.05); **significantly different from treated resting state (*P* ≤ 0.05).

**DISCUSSION**

In prior studies (3, 20, 27, 28) of impaired vascular regulation after acute hyperglycemia, the typical result was suppressed endothelium-dependent vasodilation with minor changes in cGMP-mediated relaxation of vascular muscle. In some in vivo studies, hyperglycemia was also associated with sustained vasoconstriction (3, 19). From these various studies, it was reasonable to assume that hyperglycemia reduced the bioavailability of NO at rest and during endothelial stimulation. Direct measurements of [NO] in the intestinal microvasculature in the present study and those in the skeletal muscle vasculature in a recent study (19) proved that these earlier assumptions were correct. In addition, the data in the present study provide direct evidence of the magnitude of the NO impairment. In intestinal tissues without the benefit of PKC inhibition (untreated group), hyperglycemia caused a ~40% decline in the resting [NO] within 45 min (Fig. 2) and a sustained vasoconstriction (Figs. 2 and 3). Even with the protection of LY-333531, there was a 24% decline in resting [NO] of the intestinal arterioles after hyperglycemia. However, in the treated group of arterioles, there was no vasoconstriction after hyperglycemia, and the vessels could immediately increase the [NO] when appropriately challenged. These observations suggest that the eNOS mechanism is protected when PKC activation is inhibited during hyperglycemia. We suspect the decline in [NO] at rest in treated tissues occurred for reasons unrelated to the ability of endothelial cells to generate NO. Inasmuch as the microvascular response to nitroprusside was normal with and without the protection of LY-333531 after hyperglycemia (Fig. 3), a change in sensitivity of the vascular muscle to NO did not occur. This observation explained how the arterioles could dilate normally to the near-normal relative increase in [NO] in treated tissues during NaCl hyperosmolarity (Figs. 2 and 3).

We used NaCl hyperosmolarity to test functional stimulation of NO formation, because it is a major endothelium-dependent vasodilator mechanism used by the intestine during absorptive hyperemia (2). When the NO generation mechanism is compromised, vasodilation is suppressed by approximately two-thirds. This explains in part why control arterioles could respond partially to NaCl hyperosmolarity after hyperglycemia (Fig. 3), a change in sensitivity of the vascular muscle to NO did not occur. This observation explained how the arterioles could dilate normally to the near-normal relative increase in [NO] in treated tissues during NaCl hyperosmolarity (Figs. 2 and 3).

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There is ample evidence in the literature that PKC activation in endothelial cells is increased during acute and chronic hyperglycemia (10, 12, 24). Furthermore, PKC inhibition during chronic hyperglycemia in vitro and in vivo conditions lessens microvascular permeability abnormalities (10, 11, 13). Our study adds new information by demonstrating that the ability of endothelial cells to generate NO during acute hyperglycemia can be spared after appropriate PKC inhibition. Part of our justification for studying acute hyperglycemia, rather than a chronic model of sustained hyperglycemia, is that the vast majority of human diabetic patients experience episodic hyperglycemia of a few hours duration rather than sustained hyperglycemia. On the basis of our results, increased endothelial PKC activation was a major mechanism to decrease [NO] at rest and during physiological stimulation of endothelial cells with NaCl hyperosmolality and ACh. As mentioned earlier, without protection against βPKC activation, hyperglycemia strongly suppressed the resting [NO], vasoconstriction occurred, and the increase in [NO] in response to NaCl hyperosmolality was suppressed (Figs. 1 and 2). By comparison, pretreatment to inhibit PKC activity before hyperglycemia allowed the resting diameter to be maintained, caused less suppression of the resting [NO], and allowed a normal-magnitude increase in [NO] and vessel diameter from the resting to the stimulated state (Figs. 1 and 2). This combination of positive effects leads us to speculate that vasoconstriction during hyperglycemia can be avoided with appropriate PKC blockade. Inasmuch as our results are based on actual measurement of the perivascular [NO] rather than just bioassay of changes in microvascular diameter, we are confident that βPKC inhibition during hyperglycemia protected NO formation within the arteriolar wall.

Testfamariam and Cohen (25, 26) demonstrated that, during in vitro conditions, oxidant injury and increased eicosanoid production were partially responsible for decreased endothelium-dependent dilation during acute hyperglycemia. We (3) confirmed these observations for in vivo conditions using the intestinal vasculature. Blockade of eicosanoid metabolism and scavengers of oxidant species minimized the suppression of endothelium-dependent vasodilation after 2 h of acute 500 mg/dl hyperglycemia. However, in these prior studies (3), we found that suppression of oxidant injury or eicosanoid formation after hyperglycemia was ineffective to restore endothelium-dependent vasodilation in vivo preparations. By comparison, as shown in Fig. 3, suppression of βPKC activity after endothelium-dependent dilation had been compromised was associated with ~75% recovery of the endothelium-dependent vasodilation. Mayhan and Patel (20) also found similar recovery of endothelial vasodilation in the cerebral vasculature exposed to acute hyperglycemia and then posttreated with less specific PKC inhibitors. Therefore, PKC activation during acute hyperglycemia can be substantially reversed and, thereby, substantially restores NO-dependent vasodilation in the cerebral and intestinal microvasculatures.

One of our concerns was whether hyperglycemia simply inhibited NO formation or in some way damaged the ability of eNOS to function. In Fig 3 the diameters of the arterioles during a given stimulus with ACh after hyperglycemia were smaller than during normal vasodilation at equivalent dosages. However, the vessels were actually starting their dilatory responses from a smaller resting diameter after hyperglycemia and failed to reach their normal diameter when challenged with ACh, despite well-developed vasodilation. By comparison, direct suppression of eNOS with arginine analogs has entirely different effects. NO synthesis blockade with analogs of L-arginine in the spinotrapezius muscle (18) and intestinal vasculatures (9) strongly prevented vasodilation to ACh, and substantial constriction occurred at rest. The posttreatment with the βPKC inhibitor restored the resting diameter to 94% of the original normal resting diameter and improved the relative increase in diameter for each current dosage of ACh. While βPKC inhibition did not perfectly restore endothelium-dependent dilation, it clearly made a major improvement. This improvement was not caused by altered sensitivity of the vascular smooth muscle to NO during hyperglycemia, because dilatatory responses to sodium nitroprusside were completely normal (Fig. 3). These various observations lead us to conclude that hyperglycemia, presumably acting through PKC, was inhibiting eNOS rather than damaging the enzymatic production of NO. Therefore, the reductions in endothelium-dependent vasodilation during studies of acute hyperglycemia, or perhaps even during the early stages of diabetes mellitus, appear to be a problem of endothelial regulation rather than serious cellular damage of the NO production mechanism.

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