Elevated glucose impairs cAMP-mediated dilation by reducing Kv channel activity in rat small coronary smooth muscle cells

Hongwei Li, Qiang Chai, David D. Gutterman, and Yanping Liu

Departments of Internal Medicine and Cardiovascular Center, Medical College of Wisconsin, and Zablocki Veterans Affairs Medical Center, Milwaukee, Wisconsin 53226

Submitted 14 March 2003; accepted in final form 20 May 2003

Li, Hongwei, Qiang Chai, David D. Gutterman, and Yanping Liu. Elevated glucose impairs cAMP-mediated dilation by reducing Kv channel activity in rat small coronary smooth muscle cells. Am J Physiol Heart Circ Physiol 285:H1213–H1219, 2003. First published May 22, 2003; 10.1152/ajpheart.00226.2003.—Hyperglycemia impairs endothelium-dependent vasodilation. In this study, we examined the effect of high glucose (HG) on vascular smooth muscle function. Rat small coronary arteries were freshly isolated or incubated for 24 h with normal glucose (NG; 5.5 mmol/l) or HG (23 mmol/l). In freshly isolated arteries, dilation to isoproterenol (Iso) was reduced in HG cells. These results suggest that HG impairs cAMP-mediated vasodilator mechanisms observed in large coronary arteries also extend to small coronary arteries. We next examined whether vasodilation to isoproterenol (Iso), a pathophysiologically relevant stimulus that operates through a cAMP mechanism, is impaired in coronary arterioles exposed to HG. We also examined whether the mechanism of the impairment can be explained solely by reduced activity of Kv channels or whether other components of the intracellular signal transduction pathway are involved.

We tested the hypothesis that hyperglycemia reduces cAMP-mediated dilation and that the mechanism involves suppression of cAMP production and Kv channel function.

MATERIALS AND METHODS
Preparation of rat small coronary arteries. Seven-week-old male Sprague-Dawley rats (Harlan; Madison, WI) were anesthetized with pentobarbital sodium (60 mg/kg ip). RSCAs were dissected from the ventricle and incubated in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin for 24 h at 37°C. Dulbecco’s modified Eagle’s medium was supplemented with either 5.5 mmol/l d-glucose (normal glucose; NG) or 23 mmol/l d-glucose, or HG; 5.5 mmol/l d-glucose plus 17.5 mmol/l L-glucose (LG) was used for osmotic control. After incubation, vessels were prepared for videomicroscopic studies, patch clamp, and cAMP measurement. Some RSCAs were used freshly after dissection.

All rats were housed in an animal care facility at the Medical College of Wisconsin, which is approved by the American Association for the Accreditation of Laboratory Animal Care, and all protocols were approved by the Animal Care Committee at the Medical College of Wisconsin.

Videomicroscopy. Vessels were prepared as described previously (20). Briefly, RSCAs were placed into an organ chamber filled with physiological salt solution containing (in mmol/l) 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2...
were measured in vessels exposed to NG, LG, or HG. Vessel diameters were measured 3–5 min after adding each dose of vasodilator. K+ channel blockers, 4-aminopyridine (4-AP) and iberiotoxin (IbTX) + 4-AP were sequentially added to the vessel chamber and incubated for 15–20 min before dose-response curves were initiated. At the end of each experiment, vessels were maximally dilated with a Ca2+-free solution and the percent dilation to agonists was normalized to this maximal diameter. In some vessels, the endothelium was denuded with air (16). The efficacy of denudation was verified by showing 1) failure to dilate to 1 μmol/l acetylcholine; 2) preservation of vascular smooth muscle constriction to U-46619 or presence of prominent myogenic tone; and 3) similar dilation to removal of calcium in denuded and non-denuded vessels.

**cAMP measurement.** The vessels were exposed NG or HG for 24 h as described above or freshly isolated. RSCAs were placed into Krebs solution containing 100 μmol/l 3-isobutyl-1-methyloxanthine, a cAMP phosphodiesterase inhibitor, at 37°C for 20 min. Freshly isolated vessels were then divided into the following groups: group 1, no treatment, served as cAMP baseline control; group 2, no incubation; group 3, incubated with NG for 24 h; and group 4, incubated with HG for 24 h. After incubation, vessels in groups 2–4 were treated with forskolin (10 μmol/l) for 10 min at 37°C. All RSCAs were transferred to ice-cold 6% (wt/vol) trichloroacetic acid, snap frozen with liquid nitrogen, and stored at −80°C. RSCAs were thawed, homogenized, and centrifuged (2,000 g, 4°C) for 15 min at 4°C. Supernatants were collected and extracted three times with four volumes of ethyl ether. The samples were then treated with a vacuum concentrator. cAMP level was determined with the competitive protein binding assay with the use of an Amersham cAMP assay kit (26). The production of cAMP was normalized to total protein content.

**Patch-clamp recording of K+ currents.** Enzymatic isolation of single VSMCs was performed according to published methods (17). Whole cell patch-clamp recordings were obtained with the use of standard patch protocols and instrumentation as previously described (20). Briefly, the K+ current families were generated by stepwise 10-mV depolarizing pulses (400-ms duration, 5-s intervals) from a holding potential of −60 mV in cells dialyzed with 100 nmol/l ionized Ca2+. Seal resistance was 2–10 GΩ. Peak current elicited at a single membrane potential was defined as the average of five sample points encompassing the maximal current point. In a single cell, BKca and Kv currents were defined as the difference between outward current recorded in drug-free bath solution and after superfusion with 100 nmol/l IbTX, a BKca channel blocker (17), or 4-AP, a K+ channel blocker (25). Trials were performed in triplicate and averaged to estimate peak current amplitudes (picoamperes per picofarad) to normalize for cellular membrane area (18). For each cell, 10-mV hyperpolarizing steps were averaged to account for capacitance and leak compensation values.

**Statistical analysis.** All data are expressed as means ± SE. Percent dilation was calculated as the change from control internal diameter to maximal diameter measured in the presence of a Ca2+-free solution. Data from videomicroscopy, patch clamp, and cAMP measurements were compared with the use of one-way repeated-measures ANOVA to detect differences between the two conditions (control group vs. experimental group) according to their dose-response measures. When test statistics for the main ANOVA test were significant (P < 0.05), a corollary Newman-Keuls test was performed to detect the differences between individual doses.

**RESULTS**

**Effect of K+ channel blockers on Iso- and forskolin-induced dilation.** Figure 1 illustrates that Iso and forskolin elicit vasodilation of RSCA in a dose-dependent fashion. The maximal dilation to Iso (1 μmol/l) was reduced by 3 mmol/l 4-AP (44 ± 10% vs. 77 ± 4%, P < 0.05 vs. control) and further reduced by 100 nmol/l IbTX (17 ± 2%, n = 6, P < 0.05 vs. 4-AP) in the presence of 4-AP (Fig. 1A), indicating that K+ and BKca channels play an important role in Iso-induced dilation. 4-AP abolished dilation to forskolin (1 μmol/l): 73 ± 9% vs. −3 ± 17, n = 6; P < 0.05), suggesting that the cAMP-mediated dilation in these vessels is predominantly regulated by K+ channels.

**Effect of HG on cAMP-mediated dilation.** Dilations to Iso and forskolin were compared among vessels incubated with NG, LG, and HG. Similar dilation to Iso (Fig. 2A) and forskolin (Fig. 2B) was observed in ves-
sels incubated with NG and LG. In contrast, maximal dilations to both Iso and forskolin in RSCAs exposed to HG were reduced (Iso: 41 ± 5 vs. 70 ± 6%, forskolin: 40 ± 4 vs. 75 ± 4%, n = 6 each group, P < 0.05 vs. NG). To confirm these observations, dilation to papaverine, an endothelium-independent cAMP-dependent dilator, was also reduced in RSCA that was exposed to HG compared with NG (Fig. 2C). These results suggest that HG impairs dilation mediated by cAMP. This impairment was not due to an osmotic effect.

Impaired cAMP-mediated dilation is in VSMCs. To examine the potential role for endothelial involvement, we compared Iso dilation in the presence and absence of endothelium. Endothelial denudation did not reduce dilation to Iso (Fig. 3). Furthermore, the reduction in Iso-induced dilation in presence of HG was similar in intact and denuded vessels. Thus impaired cAMP-mediated dilation in presence of HG can be explained by vascular changes independent of the endothelium.

Effect of HG on cAMP production. To explore the mechanisms by which elevated levels of glucose impair cAMP-mediated dilation, we first examined whether HG altered cAMP production stimulated by forskolin. As illustrated in Fig. 4, the forskolin-induced increase in cellular cAMP was similar in freshly isolated vessels and vessels incubated in either NG or HG for 24 h (n = 10, P = not significant) indicating that the impaired dilation to Iso and forskolin in vessels exposed to HG is not likely due to reduced formation of cAMP.

Effect of HG on cAMP-mediated K⁺ channel function. Because dilation to Iso is mediated by both 4-AP and IbTX-sensitive mechanisms, we determined whether the reduced dilation to Iso involves impairment of Kᵦ or BKCa channel function. Iso dose-response curves were performed in vessels incubated with NG or HG in the absence and presence of IbTX or 4-AP. Calculated IbTX and 4-AP-sensitive components of Iso-induced dilation, which reflect BKCa and Kᵦ channel function, are summarized in Fig. 5 (n = 6, each group). Both IbTX- (Fig. 5A) and 4-AP (Fig. 5B)-sensitive components of Iso-induced dilation were reduced in HG vessels compared with NG vessels. However, the reduction of 4-AP-sensitive component in HG RSCAs was greater than for the IbTX-sensitive component.
Effect of HG on cAMP-mediated K⁺ channel currents. Figure 6A shows sample traces of whole cell K⁺ currents generated by 10-mV incremental depolarizing steps from −60 to +60 mV in VSMCs from RSCAs exposed to NG or HG for 24 h. Whole cell K⁺ currents were suppressed in cells from arteries exposed to HG. Iso (10 μmol/l) enhanced K⁺ currents in cell from RSCAs incubated with NG but had less effect on cell from arteries exposed to HG. Capacitances for NG and HG cells were 7 and 8 pF, respectively. Figure 6B summarizes current-voltage relationships of K⁺ current density in VSMCs isolated from RSCAs incubated in NG or HG. Iso increased peak K⁺ current densities in cells from arteries exposed to NG from 34 ± 5 to 51 ± 10 pA/pF (n = 6, P < 0.05). However, Iso had little effect on K⁺ current density in cells from arteries incubated with HG (22 ± 4 vs. 27 ± 3 pA/pF) (Fig. 6C). IbTX-sensitive and 4-AP-sensitive K⁺ current densities were compared in cells from arteries exposed to NG and HG media. In HG cells, IbTX-sensitive K⁺ current density (Fig. 7C) tended to decrease, but this did not reach statistical significance (n = 6, P = not significant). However, 4-AP-sensitive current densities were significantly reduced in HG cells (Fig. 7D) (n = 7, P < 0.05), supporting the functional studies.

DISCUSSION

The key novel findings of this study are threefold. First, in addition to the well-established observations of endothelial dysfunction in diabetes, the present study demonstrates that cAMP-mediated endothelium-independent dilation is reduced in RSCA exposed to HG. This suggests that alterations in VSMC func-
tion during hyperglycemia contribute to the impaired dilation. This conclusion is based on data from complementary videomicroscopic studies in vessels denuded of endothelium and patch clamp analyses in VSMCs. Second, the reduced cAMP-mediated dilation in HG vessels was not due to a change in osmolarity or cAMP production. Third, reduced opening of vascular smooth muscle Kv channels plays a primary role in the reduced cAMP-mediated dilation in vessels exposed to HG. These data suggest that an important component of reduced coronary vasodilation in diabetes stems from reduced opening of Kv channels in the vascular smooth muscle.

Effect of HG on cAMP-mediated dilation. Impaired endothelium-dependent dilation occurs after exposure to elevated glucose either in vitro (elevated glucose in the media) (28, 29) or in vivo (diabetes) (9, 10). The liberation of O$_2^{-}$ in response to elevations in glucose reduces NO bioavailability (24) by multiple mechanisms, including a decrease in activity of cGMP (1, 30). The present study is the first to demonstrate that cAMP-mediated endothelium-independent dilation is diminished after short-term exposure of RSCAs to HG. This conclusion is supported by several studies. In patch-clamp studies, Iso failed to increase whole cell K$^+$ currents in VSMCs from arteries exposed to HG. However, in isolated vessels, the endothelium could confound interpretation of results if agonists act on it, but because the responses were similar with endothelium intact or denuded, this suggests that the endothelium is not involved in the impaired cAMP-mediated dilation in HG treated vessels. Furthermore, reduced dilation to Iso in the presence of HG is independent of VSMC receptor activation because the responses to forskolin and papaverine, both of which act distally to surface adrenoreceptors are also impaired. Finally, generation of cAMP is not altered in vessels exposed to HG indicating the reduced dilation likely originates downstream from cAMP.

Differential response of K$^+$ channels to HG. We have previously demonstrated that elevated glucose impairs Kv channel activity by generation of superoxide (20). In this study, we show the functional consequence of this impairment; namely that dilation to Iso and forskolin are reduced. These agonists mediate dilation by activating adenylyl cyclase and opening Kv channels. BKCa channels also participate in Iso-induced dilation. Under the same pathophysiological circumstance, the reduction in the IbTX-inhibitable component of the response to Iso in HG vessels was less than the reduction in the 4-AP-sensitive portion. We have previously shown that HG-induced reductions in Kv channel function are the result of heightened levels of superoxide (20). In a separate study, we (19) showed in human coronary arterioles that BKCa channel function is more resistant to superoxide. This may explain the differential response to HG in the current study and is consistent with the study by Najibi and Cohen (23), which showed that BKCa channels can compensate for the loss of NO in hypercholesterolemia, another condition where oxidative stress is enhanced. However, in the present study, the IbTX-sensitive dilation did not com-
pensate for loss of the \( K_v \) component. In fact, there was a tendency toward a reduction in the IbTX-sensitive component in HG vessels. One possible explanation is that in diabetes peroxynitrite formation has been described (33). Peroxynitrite directly inhibits BK\(_{Ca} \) channel activity in coronary arterioles (19) and cerebral small arteries (7). Although we (20) have demonstrated a prominent increase in superoxide production in this model (which is responsible for the reduced \( K_v \) channel response), simultaneous production of limited amounts of peroxynitrite during exposure to HG might be expected to lower the activity of BK\(_{Ca} \) channels as well. Alternatively, 24 h of incubation may not be sufficient for the development of compensation in this model. Examination of vessels from diabetic animals may help clarify this possibility. The results from this study suggest that HG impairs cAMP-mediated dilation primarily by reducing \( K_v \) channel function.

**HG and cAMP production.** Our data showed that HG had no effect on production of cAMP stimulated by forskolin in rat small coronary arteries. Similar findings were reported by Yoshida et al. (32), in which decreased dilation to isoproterenol was observed in pulmonary arteries with unchanged cAMP levels after lung transplantation where oxidative stress was enhanced. The direct effect of \( O_2^- \), generated by hypoxanthine and xanthine oxidase, on cAMP and cGMP production was examined in cultured aortic smooth muscle cells from Wistar-Kyoto or spontaneously hypertensive rats (30). \( O_2^- \) did not affect cAMP levels, whereas it significantly reduced cGMP levels. This finding was also observed in cultured mesenteric smooth muscle cells (31).

**Study limitations.** In the present study, we have demonstrated that reduced \( K_v \) channel function partially contributes to the impaired Iso-mediated dilation induced by HG. However, it is possible that other factors, resulting from glucose incubation, contribute to the reduced Iso-mediated dilation including endothelial factors or changes in receptor binding or signaling. However, because dilation to forskolin was similarly impaired and cAMP levels were unchanged by HG, it is likely that these changes are downstream from the adenylyl cyclase cascade.

It has been reported that HG increases protein kinase C (PKC) activity (15, 27). In rabbit portal vein smooth muscle cells, PKC inhibits 4-AP-sensitive delayed-rectifier \( K^- \) currents (2). Although we did not directly examine the role of PKC in impaired cAMP-mediated dilation in the present study, the increased PKC activity by elevation of glucose might contribute to the reduced \( K_v \) channel function during hyperglycemia in the coronary circulation.

One would expect that the resting tone of RSCAs exposed to HG would increase due to the reduced \( K_v \) channel function. However, the resting diameters in HG vessels are similar compared with NG vessels. One possible explanation relates to other vasodilator mediators in HG vessels. ROS levels are elevated in HG and production of \( H_2O_2 \) generated from \( O_2^- \) is likely. \( H_2O_2 \) dilates and hyperpolarizes human coronary arterioles (21). The dilator effect of \( H_2O_2 \) may counteract with vasoconstriction by reduced \( K_v \) channel activity.

Koh et al. (14) showed that hyperosmotic stress stimulates several regulatory proteins of signal transduction cascade, which may in turn alter vasomotor function. In the present study, L-glucose was used as an osmotic control. Dilator responses to either Iso or forskolin were similar in vessels treated with NG or LG suggesting the reduced dilator response is not a result of osmolarity changes in the tissue.

In summary, although it is known that diabetes and hyperglycemia impair endothelium-dependent vasodilation, the effect of glucose on dilator mechanisms independent of endothelium is not well understood. In the present study, we determined that dilation to Iso and forskolin is reduced in vessels exposed to HG for 24 h with no change in cAMP. The 4-AP but not the IbTX-sensitive component to Iso-induced dilation was diminished in HG vessels. These results provide a functionally important correlate to our previous finding that hyperglycemia reduces \( K_v \) channel activity due to elevations in \( O_2^- \) (18). We now show that HG impairs cAMP-mediated dilation to Iso due primarily to a reduction in \( K_v \) channel activity.

We appreciate the expert statistical assistance of Dr. Fausto Loberiza.

**DISCLOSURES**

This study was supported by National Heart, Lung, and Blood Institute Grant RO1 HL–59238 and by a Veterans Administration Merit Award (to D. D. Gutterman). Y. Liu is a recipient of a Scientist Development Grant from the American Heart Association.

**REFERENCES**


