Effects of glycosylated hemoglobin on vascular responses in vitro

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Abstract

Vascular responses to endothelium-dependent vasodilators are greatly impaired in vivo, while isolated blood vessels from animals with diabetes mellitus demonstrate less consistent degrees of impairment. Glycation of proteins, such as hemoglobin, has been implicated in the vascular abnormalities associated with diabetes. **Objective:** The purpose of this study was to test the hypothesis that glycosylated hemoglobin is capable of reducing endothelium-dependent vasodilator responses, possibly explaining impaired dilation observed in vivo.

**Methods:** To test this hypothesis, the effect of glycosylated hemoglobin (GH) on vascular responses was studied in several vascular beds, including ventricular microvessels and coronary, mesenteric, femoral, and renal arteries. Coronary arterioles were isolated and mounted between two glass pipettes in a pressurized (30 cmH₂O) organ chamber. Isolated artery segments were studied using a standard isometric ring technique.

**Results:** In ventricular microvessels, 10 nM nGH non-GH and GH both attenuated the relaxation to Ach. A lower concentration, 1 nM nGH or GH, did not alter dilation to Ach. In coronary, femoral, mesenteric and renal artery segments, endothelium-dependent responses were not altered by the presence of 10 or 100 nM nGH or GH.

**Conclusion:** In coronary microvessels, and coronary, femoral, mesenteric and renal arteries, GH is not responsible for the impaired endothelial function associated with diabetes mellitus.

**Keywords:** Glycosylated hemoglobin; Diabetes; Coronary vasculature; Microcirculation; Acetylcholine; Dog, arteries

1. Introduction

Diabetes mellitus is associated with increased cardiovascular morbidity and mortality [1–3]. Reasons for the increased morbidity and mortality are uncertain, but impairment of endothelial function and platelet–endothelium interactions are likely contributing factors [4–7]. In animals and patients with diabetes, endothelial modulation of vascular responses is impaired to endogenous vasodilators such as acetylcholine (Ach) and adenosine [8,9]. Several investigators have observed an impaired response to Ach in vitro which occurred due to endothelial production of vasoconstrictor prostanoids such as TX A₂ and PGH₂ [4,5,7]. Others have suggested that oxygen-derived free radicals may play an important role in the reduced dilation [8,10–12]. Despite these in vitro abnormalities, in vivo vascular responses in diabetes are more often and more severely attenuated than those observed in vitro. The reason for this discrepancy is uncertain.

Since long-term hyperglycemia appears to be the central initiating factor responsible for the development of many of the complications of diabetes [4,9,13], glucose or glucose-derived metabolites may irreversibly modify long-lived extracellular or intracellular macromolecules which can inactivate nitric oxide [14]. Thus, a circulating factor or factors may play a role in endothelial dysfunction associated with diabetes. A recent study by Rodriguez-Manas [15] and colleagues suggests that high levels of glycosylated hemoglobin impair endothelium-mediated vasoreactive responses in rat aorta. This study is consistent with the hypothesis that glycosylated hemoglobin, a factor present in vivo, is responsible for impaired coronary mi-
crovascular responses during hyperglycemia. To test this hypothesis, the effect of glycosylated hemoglobin on endothelium-dependent vasodilation was studied in several vascular beds, including ventricular microvessels and coronary, mesenteric, femoral, and renal conduit arteries.

2. Methods

All protocols were approved by the University of Iowa Animal Care and Use Committee and conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.1. Animals and tissue

Twenty adult mongrel dogs of either sex (20–25 kg) were euthanized with an overdose of sodium pentothal (50 mg/kg). Tissues, including the heart, femoral, mesenteric and renal arteries, were quickly harvested. Hearts and arteries were immediately placed in cold (4°C) , oxygenated (20% O2, 5% CO2 and 75% N2) Krebs’ bicarbonate buffer solution (see solutions) for dissection. Ventricular arterioles and arteries were dissected and trimmed of fat and connective tissue. In some studies of rings, the endothelium was removed by gentle rubbing of the luminal surface with silk suture.

2.2. Isolated microvessels

A standard isolated pressurized arteriole preparation was used to study coronary microvessels [16]. Each end of the arteriole was cannulated with a glass micropipette and attached to independent hydrostatic pressure reservoirs (20 mmHg) under conditions of no flow. The organ chamber was placed on the stage of an inverted microscope. Attached to the microscope were a video camera, a video monitor, and a calibrated video calliper. The organ chamber was connected to a rotary pump which continuously circulated oxygenated Krebs buffer warmed to 37°C. Internal diameters were measured by manually adjusting the video micrometer.

Arterioles were allowed to equilibrate for 30 min at a distending pressure of 20 mmHg. KCl (50 mM) was added to the bath to test constrictor capacity. After washing with fresh buffer, vessels were incubated for 30 min in Krebs buffer alone (control), or Krebs with 1 or 10 nM non-glycosylated human hemoglobin (nGH) or 14% glycosylated human hemoglobin (GH). Endothelin-1 (0.40–8.0 nM) was used to constrict the arterioles to 30–60% of their resting diameter. Cumulative concentration–response relationships were evaluated for acetylcholine (Ach; 10⁻¹⁰ to 10⁻⁴ M) and sodium nitroprusside (SNP; 10⁻¹⁰ to 10⁻⁴ M) by adding the drug directly to the organ bath. The order of the drugs was randomized. The following criteria were required for an acceptable experiment: (1) arterioles could not demonstrate obvious leaks; (2) arterioles had to constrict > 50% to 50 mM KCl, and > 30% to endothelin; and (3) dilate by > 80% to 10⁻⁴ M SNP.

2.3. Isolated vascular rings

Coronary, femoral, mesenteric and renal arteries were studied using a standard isometric ring technique [10,17]. Vascular rings were mounted on two stirrups made by passing stainless steel wires through the vessel lumen. One stirrup was attached to a force transducer, and the other to a micrometer microdrive to allow the vessel to be stretched by known increments. Each vessel apparatus was placed in a 25-ml jacketed organ bath containing Krebs buffer equilibrated at 37°C and aerated with 20% O2, 5% CO2 and 75% N2. Isometric contractions and relaxations were measured on a computer. Rings were individually stretched to the maximum of the length–developed tension relationship by repeated test exposures to 75 mM KCl at increasing vessel diameters.

The vessels were allowed to stabilize 30 min prior to concentration–response curves in either control solution (normal Krebs), or 10 or 100 nM concentrations of nGH or GH solutions. PGF₂α was used to constrict the vessels to 30–50% of their resting tension. After steady–state tension was achieved, a concentration–response curve to Ach (10⁻¹⁰ to 10⁻⁴ M) was performed to evaluate endothelium-dependent relaxation. Vessels were then washed with fresh Krebs, incubated in control or one of 4 hemoglobin solutions, and constricted with PGF₂α. A concentration–response curve to SNP (10⁻¹⁰ to 10⁻⁴ M) was then performed to evaluate smooth muscle vasodilatation. The order of Ach and SNP was randomized. Acceptable experiments met the following criteria: (1) development of > 1 g of tension to PGF₂α; (2) dilate by 75–150% to SNP; (3) denuded vessels must dilate < 30% to Ach (10⁻⁶ M).

2.4. Solutions and drugs

Krebs solution contained (in mM): NaCl 131.5; KCl 5; CaCl₂ 2.5; MgCl₂ 1.2; NaHCO₃ 23.5; KH₂PO₄ 1.2; and glucose 11. Solutions were aerated with 20% O₂, 5% CO₂, and 75% N₂ and maintained at 37°C with pH maintained at 7.4. Human hemoglobin solutions were purchased from Sigma Chemical (St. Louis, MO) and prepared by diluting in distilled H₂O. Glycosylated hemoglobin content was quantified in the clinical pathology laboratory at the University of Iowa. Endothelin-1 was purchased from Calbiochem (San Diego, CA). All solutions and vasoactive agents were prepared fresh on the day of the experiment. Acetylcholine and sodium nitroprusside were purchased from Sigma Chemical.
2.5. Statistical analysis

All concentration–response curves were evaluated for changes in maximal responses and differences at each dose using ANOVA with repeated measures and the Bonferroni correction for multiple comparisons. EC$_{50}$ is defined as the vasodilator concentration that produced 50% of maximal relaxation of the endothelin or PGF$_{2alpha}$ preconstriction. Significance of differences among mean values of resting conditions, maximal effect, and EC$_{50}$ values was assessed with a one-way ANOVA. Data are expressed as mean ± s.e.m. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Coronary microvascular responses

Baseline diameter was 123 ± 12, 105 ± 13, and 117 ± 9 μm for arterioles in control, nGH, and GH groups respectively ($P = n.s.$). Arteriolar responses were preconstricted with similar amounts with endothelin (0.40–8.0 nM). Arteriolar responses were preconstricted to 49 ± 3, 51 ± 4, and 55 ± 2% resting diameter in control, nGH, and GH groups, respectively ($P = n.s.$). Ach produced concentration-related relaxation in canine coronary arterioles. These responses were not affected by incubating the microvessels in 1 nM nGH or GH (Fig. 1, left panel). EC$_{50}$ values for Ach were $-6.3 \pm 0.1$, $-7.0 \pm 0.3$, and $-6.4 \pm 0.1$ for control, 1 nM nGH and 1 nM GH, respectively. The response to Ach in arterioles which were incubated in 10 nM GH was reduced compared to control (Fig. 1, right panel). A similar reduction in dilation to Ach was seen in arterioles incubated with 10 nM nGH (Fig. 1, right panel). EC$_{50}$ values for Ach were $-8.2 \pm 0.3$, $-6.8 \pm 0.3$, and $-6.8 \pm 0.1$ for control, 10 nM nGH and 10 nM GH, respectively ($P < 0.05$).

3.2. Coronary artery responses

Coronary artery segments were taken from the left circumflex and left anterior coronary arteries. Resting tension was 6.5 ± 0.2, 5.7 ± 0.3, 5.5 ± 0.2, and 5.2 ± 0.2 g in control, nGH, GH and denuded groups respectively ($P = n.s.$). Developed tension to PGF$_{2alpha}$ was similar among groups and 3.6 ± 0.3, 3.9 ± 0.4, 4.5 ± 0.5, and 3.5 ± 0.9 g in control, nGH, GH and denuded groups, respectively; $P = n.s.$ In canine coronary arteries preconstricted with PGF$_{2alpha}$, Ach (Fig. 2) and SNP produced concentration-related relaxation. Vessels incubated in 10 or 100 mM GH or nGH did not show an altered response to Ach or SNP. EC$_{50}$ values for Ach and SNP were not altered by the presence of 10 or 100 nM nGH or GH (data not shown). Following endothelial removal, the response to Ach was significantly attenuated in both groups ($^* P < 0.05$).

3.3. Femoral artery responses

Resting tension was 5.8 ± 0.4, 6.0 ± 0.2, 6.1 ± 0.2, and 5.7 ± 0.5 g in control, nGH, GH and denuded groups,
Table 1

<table>
<thead>
<tr>
<th>SNP EC50 values (log [M]) for Ach and SNP and maximal effects (% relaxation) for SNP-induced relaxations for femoral arteries</th>
<th>Control</th>
<th>10 nM nGH</th>
<th>10 nM GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ach</td>
<td>−7.8 ± 0.1</td>
<td>−8.0 ± 0.2</td>
<td>−8.2 ± 0.5</td>
</tr>
<tr>
<td>SNP EC50</td>
<td>−6.7 ± 0.2</td>
<td>−6.4 ± 1.5</td>
<td>−6.4 ± 0.1</td>
</tr>
<tr>
<td>SNP Max</td>
<td>97.9 ± 4.9</td>
<td>102.6 ± 6.8</td>
<td>102.3 ± 11.3</td>
</tr>
</tbody>
</table>

(Number of vessel segments represented for Ach data are the same as in Fig. 3.)

respectively (P = n.s.). All femoral segments constricted similarly to PGF2α (3.7 ± 0.4, 5.7 ± 0.4, and 3.7 ± 1.0 g in nGH, GH and denuded groups, respectively; P = n.s. vs control 5.2 ± 0.7). In canine femoral arteries preconstricted with PGF2α, Ach and SNP (Table 1) produced concentration-related relaxation. In arteries denuded of endothelium, the response to Ach was significantly impaired. Femoral artery response to Ach in the presence of 100 nM nGH and GH is shown in Fig. 3. Vessels incubated in 10 or 100 mM GH or nGH did not show altered responses to Ach or SNP. Endothelium removal did not alter the response of the vessels to SNP. EC50 values are presented in Table 1.

Table 2

<table>
<thead>
<tr>
<th>SNP EC50 values (log [M]) for Ach and SNP and maximal effects (% relaxation) for SNP-induced relaxations for mesenteric arteries</th>
<th>Control</th>
<th>10 nM nGH</th>
<th>10 nM GH</th>
<th>Denuded</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ach</td>
<td>−7.2 ± 0.0</td>
<td>−7.4 ± 0.1</td>
<td>−7.5 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>SNP EC50</td>
<td>−6.8 ± 0.2</td>
<td>−6.7 ± 0.2</td>
<td>−6.3 ± 0.0</td>
<td>−6.5 ± 0.1</td>
</tr>
<tr>
<td>SNP Max</td>
<td>103.2 ± 7.4</td>
<td>95.7 ± 9.3</td>
<td>95.2 ± 13.0</td>
<td>93.0 ± 0.6</td>
</tr>
</tbody>
</table>

(Number of vessel segments represented for Ach data are the same as in Fig. 3.)

3.4. Mesenteric artery responses

Resting tension was 6.1 ± 0.2, 5.8 ± 0.3, 6.1 ± 0.2, and 6.4 ± 0.2 g in control, nGH, GH and denuded groups, respectively. Developed tension to PGF2α was 5.2 ± 0.5, 5.7 ± 0.8, 7.3 ± 0.8, and 4.7 ± 0.9 g in control, nGH, GH and denuded groups, respectively (P = n.s.). Ach (Fig. 3) and SNP (Table 2) produced concentration-related relaxations which were not altered by the presence of 10 or 100 nM GH and nGH. Dilation to Ach was significantly impaired, but the response to SNP was not affected by endothelium removal. EC50’s are presented in Table 2.

3.5. Renal artery responses

In canine renal artery segments, developed tension to PGF2α was 7.1 ± 0.6, 6.3 ± 0.8, and 8.4 ± 1.0 g in control, nGH, and GH groups, respectively (P = n.s.). Preconstricted renal artery segments produced concentration-related relaxation to Ach and SNP. The presence of 100 nM GH and nGH did not alter the response to Ach or SNP (Table 3).

Table 3

<table>
<thead>
<tr>
<th>SNP EC50 values (log [M]) and maximal effects (% relaxation) for Ach and SNP-induced relaxations of renal arteries</th>
<th>Control</th>
<th>100 nM nGH</th>
<th>100 nM GH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ach EC50</td>
<td>−7.0 ± 0.6</td>
<td>−5.7 ± 0.0</td>
<td>−6.8 ± 0.1</td>
</tr>
<tr>
<td>Ach Max</td>
<td>93.2 ± 3.0</td>
<td>92.1 ± 2</td>
<td>82.5 ± 5.0</td>
</tr>
</tbody>
</table>

(Number of vessel segments represented for Ach data are the same as in Fig. 3.)

Fig. 3. Ach-induced relaxation of canine femoral (right panel) and mesenteric (left panel) artery segments in the presence of 100 nM nGH and GH. The arteries were preconstricted with PGF2α. The right panel shows the response of femoral artery segments to Ach in the presence of 100 nM nGH and GH. The left panel shows the response to Ach in the presence of 100 nM nGH and GH. There were no differences in sensitivity or maximal relaxation responses of Ach in the presence of 10 (data not shown) or 100 nGH or GH. In arteries in which the endothelium had been removed, the response to Ach was significantly attenuated in both groups (P < 0.05).
4. Discussion

There are three major findings of the present study. First, glycosylated hemoglobin at concentrations expected in diabetic patients and at concentrations considered supraphysiological for diabetes fails to attenuate conduit artery dilation to Ach in the coronary, mesenteric, femoral or renal vascular beds. Second, the effect of hemoglobin on vascular reactivity varies according to vessel size in the coronary bed. Third, the impaired coronary microvascular responses to Ach in the presence of nGH and GH appears not to be related to glycosylation of hemoglobin. It appears to be an effect that is related to hemoglobin per se, as previously discussed. These results suggest that circulating factors other than glycosylated hemoglobin are responsible for the impaired endothelium-dependent vasodilatation observed in diabetic subjects, in vivo. Our results and conclusions are dependent upon several methodological considerations.

We used standard in vitro preparations to study isolated ventricular coronary microvessels [16] and conduit arteries from the coronary, mesenteric, femoral and renal vascular beds [10,15,17]. These methods have been used by several laboratories, including our own [17]. The concentration of GH we used was high (14%). However, it reflects the degree of glycosylated hemoglobin seen in diabetic subjects [18–20]. In our study, there were no differences in dilation to Ach among vessels treated with nGH or GH (see Figs. 1–3). Furthermore, in coronary, femoral, mesenteric and renal artery segments, endothelium-dependent responses were not altered by the presence of 10 or even 100 nM GH or nGH. In normal subjects, free plasma hemoglobin in the nanomolar concentrations has been reported [21]. Thus, the use of concentrations greater than 100 nM GH would not provide useful physiological information.

Hemoglobin has been used to inhibit endothelium-dependent nitric oxide relaxations in several vascular beds, including coronary arteries [22] and rabbit aorta [23]. Myers et al. [16] have shown concentrations of 10 mM hemoglobin to inhibit Ach relaxation in canine coronary microvessels. Lower concentrations of hemoglobin were not investigated in that study [16]. In our study, both 10 nM nGH and GH attenuated the relaxation to Ach in ventricular microvessels. The effect appears specific for endothelium-dependent responses, since smooth muscle responses were not altered by the presence of nGH or GH.

The results of Rodriguez-Manas et al. [15] contrast with the findings of the current study. Segments of rat aorta which were incubated in 10 nM GH solution for 10 min showed decreased responses to Ach. Our study suggests that even with 10-fold higher concentrations of GH (100 nM), endothelium-dependent responses in canine arteries were not altered. Several potential explanations exist for these results which contrast with those of Rodriguez-Manas. First, in our study a different species of animal was studied. Second, in their isolated aortic ring studies, the organ chambers were aerated with 95% O2 and 5% CO2, while we used a gaseous mixture containing 20% O2, 5% CO2 and 75% N2. It is well established that oxygen-derived free radicals are produced by auto-oxidation of glucose [4,10,11]. These reactive oxygen species may combine with the NO, producing a relatively weak vasodilator, peroxynitrite [24]. Third, agents used to preconstrict the vessels were different (PGF2α vs norepinephrine) [17,25]. Finally, we investigated the reactivity of conduit vessels from the coronary, mesenteric, femoral and renal vascular beds in addition to coronary microvessels, whereas Rodriguez-Manas et al. studied aortic segments. It is possible that responses are different in regional organ blood vessels.

This study was intended to shed light on the discrepancies observed between in vivo and in vitro preparations which evaluate vascular responses in diabetes. Our study fails to provide an explanation for this discrepancy. It is possible that glycation of other circulating proteins within the serum of diabetic individuals may be responsible for the abnormalities in endothelium-dependent vasodilatation observed in vivo in diabetic subjects. Further studies are necessary to clarify this possibility.

In summary, in dog coronary arterioles, and coronary, mesenteric, femoral and renal arteries, GH is not responsible for the impaired endothelium-dependent vasodilatation in response to Ach associated with diabetes mellitus.

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References


