**Interference of Glycosylated Human Hemoglobin With Endothelium-Dependent Responses**

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**Background.** Hypertension and other vascular diseases are more prevalent in diabetic patients than in the general population. In humans and in several animal models of diabetes, a disturbance of endothelium-dependent responses has been shown. Oxyhemoglobin is one of the most known modulators of these endothelium-dependent responses. We postulate that high levels of plasmatic glycosylated hemoglobin, a frequent profile in diabetic patients, may be the cause of the disturbance in endothelium-dependent relaxation and/or contraction.

**Methods and Results.** Endothelium-dependent responses to acetylcholine and several α-adrenergic agonists (norepinephrine, methoxamine, and clonidine) were tested in segments of rat aorta. Experiments were carried out in control segments and in those preincubated with several concentrations of nonglycosylated, low-glycosylated (7.3%), and high-glycosylated (14%) human hemoglobin. Low concentrations of high-glycosylated human hemoglobin (1 to 100 nmol/L) but not of low- or nonglycosylated hemoglobin, inhibited endothelium-dependent relaxation caused by acetylcholine in intact vessels. The same effect was observed on relaxations caused by nitric oxide in denuded ones. High-glycosylated human hemoglobin (10 nmol/L) induced an increase in norepinephrine-evoked contraction in intact vessels; this latter effect was also shown in vessels contracted with methoxamine but not with clonidine. Deendothelialization of the vascular segments blunted these effects of high-glycosylated human hemoglobin.

**Conclusions.** High glycosylation of human hemoglobin impairs endothelium-mediated vasoactive responses and may play a pathophysiologigal role in producing hypertension and vascular diseases in diabetic patients. (Circulation. 1993;88[part 1]:2111-2116.)

**KEY WORDS** • diabetes • hemoglobin • endothelium

Hypertension, diabetes, and vascular diseases are very prevalent entities that have some common physiopathologic features. The presence of hypertension in diabetic patients markedly worsens their morbidity and mortality.1 and the absence of hypertension is a usual finding in long-term survivors of diabetes.2 Despite these facts, data about their interactions remain unclear and controversial.2 One of the likely explanations for this deleterious association is the endothelial dysfunction. Endothelial dysfunction has been shown to take place in those entities,3-5 so some authors have claimed a pathologic role of this vascular component in the development of hypertension in diabetic patients.6 Among other substances, hemoglobin exerts a modulatory role on endothelial function by promoting a decrease in the endothelium-dependent vasorelaxant responses and an increase in the contractile ones, which leads to enhancement of vascular tone.7,8

On the other hand, it is well known that diabetic patients exhibit higher levels of glycosylated hemoglobin than do nondiabetics. Protein glycosylation has been proposed as a pathogenic mechanism producing long-term diabetic effects.9 Moreover, recent articles have connected the percentage of glycosylated hemoglobin with the generation of advanced glycosylation end-products formed spontaneously from a post-Amadori modification of hemoglobin10 as well as with increased capillary pressure in diabetic patients.11,12

Our hypothesis is that glycosylated hemoglobin in the pathologic range may exert a more powerful effect on endothelium-dependent responses than nonglycosylated or low-glycosylated hemoglobin. This effect could be observed at lower concentrations, promoting an increase in vascular tone. This increased vascular tone will contribute to the pathogenesis of hypertension and other vascular diseases in diabetic patients.

**Methods**

Male Sprague-Dawley rats (300 to 450 g) were anesthetized with sodium pentobarbital (70 mg/kg IP) and killed by bleeding. From then on, the aorta was carefully removed, placed into a Petri dish containing Krebs-Henseleit solution (KHS) at 4°C, and divided into cylindrical segments of 4 to 5 mm in length. For isometric tension recording, each segment was set up in an organ bath according to the method of Nielsen and Owman.11 The organ bath contained 5 mL KHS at 37°C.
continuously bubbled with a 95% O₂–5% CO₂ mixture, which gave 7.4 pH. Two horizontally arranged stainless-steel pins were passed through the lumen of the vascular cylinder. One pin was fixed to the organ bath wall, while the other one was connected vertically to a strain gauge for isometric tension recording. The isometric contraction was recorded through a force-displacement transducer (FT03C, Grass, Quincy, Mass) connected to a Grass model 7D polygraph. The segments were subjected to a tension of 1.5g (optimal resting tension), which was readjusted every 15 minutes during a 90-minute equilibration period before drug administration. At the beginning of the experiments, the vessels were exposed to 75 mmol/L K⁺ to check their functional integrity. After a washout period, to confirm the presence and functionality of vascular endothelium, each segment was contracted with 10 nmol/L norepinephrine (NE). Once a stable plateau was reached, a concentration-response curve to acetylcholine (ACh; 0.01 to 10 μmol/L) was performed. Segments with relaxant responses to 10 μmol/L ACh of >50% were considered with endothelium. To analyze the endothelium-dependent effects of the drugs, some segments were previously deendothelialized by treating them with saponin (0.3 mg/mL KHS oxygenated at 37°C) for 15 minutes.14 To test the effect of glycosylation of hemoglobin on the responses to ACh, NE, methoxamine, clonidine, and nitric oxide (NO), the segments were preincubated with different concentrations of nonglycosylated human oxyhemoglobin (nGHH), low-glycosylated human oxyhemoglobin (7.3% LGHH), or high-glycosylated human oxyhemoglobin (14% HGHH) for 10 minutes.

The composition of KHS (mmol/L) was NaCl 115, CaCl₂ 25, KCl 4.6, KH₂PO₄ 1.2, MgSO₄, 7H₂O 1.2, NaHCO₃ 25, glucose 11.1, and Na₂EDTA 0.03. Drugs used were NE hydrochloride, ACh chloride, methoxamine hydrochloride, clonidine hydrochloride, human hemoglobin, glycohemoglobin A₁ control-E (14% glycosylation), and control-N (7.3% glycosylation) (all of them from Sigma, St Louis, Mo) and NO (Sociedad Española de Oxígeno, Madrid, Spain). Drug solutions were made in distilled water except NE, which was prepared in saline (0.9% NaCl)–ascorbic acid (0.01% w/v), and the stock solutions were kept at −20°C. All tested hemoglobins were prepared by reduction of commercial compounds with sodium dithionite, and were subsequently dialyzed and stored in vials at −70°C. Concentration of oxyhemoglobin was determined spectrophotometrically.8 NO was prepared from a saturated gas solution in deoxygenated distilled water at room temperature. Because of the variability of vascular responses, the experiments were systematically paired, and the data were normalized as established in every legend. The results are expressed as mean±SEM. Deviations from the mean regarding the respective paired controls were statistically analyzed using ANOVA. As significance was obtained, two-tailed t test was used as a post-ANOVA test for establishing differences along the curve response. A value of P<0.05 was considered significant.

Results

In basal conditions, the three types of oxyhemoglobin (nGHH, LGHH, and HGHH) at concentrations used in this study (1 nmol/L to 1 μmol/L) did not exert any effect on the tone of segments of rat aorta. This absence of effect was not modified by the removal of endothelium (results not shown).

Effect of nGHH, LGHH, and HGHH on ACh-Induced Relaxation

Preincubation with HGHH at concentrations ranged from 1 nmol/L to 1 μmol/L induced a decrease in relaxant responses elicited by cumulative concentrations of ACh in vessels with endothelium (Fig 1). By contrast, nGHH at concentrations ranging from 1 nmol/L to 100 nmol/L did not produce a modification of those relaxant responses (Figs 1A, B, and C), but at 1 μmol/L nGHH inhibited ACh-evoked vasodilation (Fig 1D). LGHH (10 nmol/L to 1 μmol/L) induced an effect similar to that observed with nGHH. LGHH produced an inhibitory effect on ACh-induced endothelium-dependent relaxation only at the highest concentration (1 μmol/L); lower ones lacked effect (Fig 2).

Effect of nGHH, LGHH, and HGHH on NO-Induced Relaxation

HGHH (10 nmol/L) but not nGHH or LGHH at the same concentration diminished NO-induced concentration-dependent relaxations in deendothelialized vessels precontracted with 10 nmol/L NE (Figs 3A and B).

Effect of nGHH, LGHH, and HGHH on NE-Induced Contraction

Preincubation with HGHH (10 nmol/L) increased the maximum response to NE without modifying its potency (Fig 4A). On the other hand, preincubation with either 10 nmol/L nGHH or 10 nmol/L LGHH (Figs 4A and B) did not exert an effect on this NE-induced contraction. Vascular deendothelialization increased the responses to NE. These latter responses in deendothelialized segments were not modified by preincubation of vascular segments with HGHH or LGHH at the same concentration (10 nmol/L) (Fig 5).

Effect of nGHH, LGHH, and HGHH on Responses Induced by α-Adrenergic Agonists

Methoxamine (α₁-agonist) and clonidine (α₂-agonist) induced contractile responses in intact rat aortas. HGHH (10 nmol/L) increased the responses evoked by methoxamine but not those induced by clonidine (Fig 6).

Discussion

Vascular diseases, including hypertension, are the most burdening consequences of diabetes. The role that endothelium may play in the development of those complications is thought to be a clue to the physiopathologic linkage between those entities.9 It is well known that a high percentage of plasmatic glycosylated hemoglobin is a frequent profile in diabetic patients with poor disease control. On the other hand, hemoglobin is a potent modulator of endothelial-dependent responses.8 So, we thought that HGHH may contribute to the impaired endothelium-dependent responses in diabetics. In this study, we demonstrate that HGHH, a classic feature of poorly-controlled diabetes, inhibits endothelium-dependent relaxation caused by ACh at lower concentrations than those needed of nGHH to produce such an inhibition. We also analyzed the percentage of glycosylation that impairs the endothelium-dependent
vasodilation. For this purpose, we tested the effect of hemoglobin when it is glycosylated at a normal percentage (7.3%). LGHH, at similar lower concentrations than those used with HGHH (<100 nmol/L), does not modify ACh-evoked relaxations. A concentration of 1 μmol/L is necessary to show some inhibitory effect; the same concentration is needed when using nGHH. These results suggest that the decreases in vasodilatory responses are depending on the percentage of glycosylation because LGHH produces an effect similar to those seen with nGHH. The experiments that elucidate the mechanism by which HGHH impairs endothelium-dependent vasodilations suggest that NO is involved. Indeed, in denuded vessels, preincubation with 10 nmol/l HGHH but not with an equal concentration of nGHH or LGHH decreased the vasodilatory responses induced by NO. These results agree with those reported by other authors, and confirm the assumption of an impairment of NO-mediated effects in diabetic animals.

Several studies have analyzed the role of endothelial function in the pathologic vasoactive responses in diabetic animals. Results are controversial depending on the type of agonist, animal species and strains, diabetes animal model, time of hyperglycemia, and mechanisms involved. Those studies have been carried out on vascular segments from diabetic animals in vitro or in different vascular beds in diabetic animals in vivo. However, our experimental approach allowed us to test a single effect on vascular segments from nondiabetic animals, thereby excluding other possible alterations that could be present in vessels from diabetic animals. It is noteworthy that except in the WBN/Kob diabetic rat model, the impairment of endothelium-dependent responses has been shown only when hyperglycemia had been developed, disregarding the diabetes model that is used. Furthermore, in animals treated with insulin, this impairment of endothelium-dependent vasodilations is blunted, suggesting that a hyperglycemia-linked event plays a role in determining all those impaired responses. Interestingly, these impairments in animal models of diabetes appear to be time dependent. They become present between 21 and 30 days since the development of hyperglycemia but not at early stages, reaching their plateau at 2 to 3 months. This time dependency is consistent with the involvement of a plasmatic high-glycosylated hemoglobin because glycosylated hemoglobin takes from 6 to 13 weeks to reach its steady state.

The mechanism mediating the effect of HGHH on endothelium-dependent NO-mediated vasorelaxant re-

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**Figure 1.** Plots of effects of several concentrations of high- (14%) and nonglycosylated human oxyhemoglobin (HGHH and nGHH, respectively) on the vasorelaxant responses to acetylcholine (ACh). Data are expressed as mean ± SEM of the percentage of a previous contraction elicited by 10 nmol/L norepinephrine (100% = 1109±186 mg, 1066±178 mg, and 998±205 mg for control, nGHH, and HGHH, respectively) in segments of rat aorta. Three to nine animals were used for each set of experiments. Number of segments used are in parentheses. *P<0.05; **P<0.01.
Three substances, elicited responses has not yet been elucidated, but generation of advanced glycosylation end-products (AGEs) may be involved. Thus, it has been shown that plasmatic levels of these substances, formed from the reaction of glucose with hemoglobin, exhibit a highly significant correlation with HbA1c levels in diabetic patients. Moreover, AGEs inactivate NO in vitro and inhibit NO-mediated vasodilation in vivo. This latter effect is time dependent; it reaches its plateau after 2 months of chronic hyperglycemia and is prevented by inhibition of advanced glycosylation using aminoguanidine. Buca et al suggest that those effects are linked to advanced glycosylation of vessel wall proteins, especially collagen modification, but hemoglobin glycosylation may also be involved in producing that impairment of endothelium-dependent vasodilation. Indeed, HGGH may be one of the most important factors involved; in our experimental approach, vessel wall proteins were not glycosylated but endothelium-mediated responses were blunted in the presence of HGGH. Although the buffer used in our experimental conditions may modify the permeability of the endothelium by causing loss of the glycocalix, thereby enhancing the presence of hemoglobin in the

Fig 2. Plots of effects of several concentrations of low-(7.3%) and nonglycosylated human oxyhemoglobin (LGHH and nGHH, respectively) on the vasorelaxant responses to acetylcholine (ACh). Data are expressed as mean±SEM of the percentage of a previous contraction elicited by 10 nmol/L norepinephrine (100%=1014±203 mg, 1121±172 mg, and 1172±205 mg for control, nGHH, and LGHH, respectively) in segments of rat aorta. Three to seven animals were used for each set of experiments. Number of segments used are in parentheses. *P<.05.

Fig 3. Plots of effect of 10 nmol/L high-(A) and low-glycosylated human oxyhemoglobin (B) (HGGH and LGHH) on the responses elicited by nitric oxide (NO) in deendothelialized rat aortic segments. Data are expressed as mean±SEM of the percentage of a previous contraction elicited by 10 nmol/L norepinephrine (100%=1216±139 mg, 1307±281 mg, 1167±179 mg, and 1222±98 mg for control, nGHH, HGHH, and LGHH, respectively). Four animals were used for each set of experiments. Number of segments used are in parentheses. *P<.05; **P<.01.

Fig 4. Plots of effect of 10 nmol/L high- (A) and low-glycosylated human oxyhemoglobin (B) (HGGH and LGHH) on the responses elicited by norepinephrine (NE) in segments with endothelium. Data are expressed as mean±SEM of the percentage of contraction induced by cumulative concentrations of NE. 100% is the contraction induced by 75 mmol/L K+ [2489±201 mg, 2429±129 mg, 2443±188 mg, and 2274±213 mg for control, nGHH, HGHH, and HGGH, respectively]. Five animals were used for each set of experiments. Number of segments used are in parentheses. *P<.05.
vascular wall, it is unlikely that this phenomenon could interfere with the results. In fact, the buffer was the same for all the experiments that were paired. Moreover, it is well known that hemoglobin penetrates the vascular wall and inactivates NO in the extracellular space. On the other hand, several proteins apparently originating from human plasma, including high molecular weight glycoproteins, have been identified in the wall of normal human arteries. So, the effect of HGHH would not be restricted to inhibiting circulating NO but also NO into the vascular wall.

HGHH not only diminished endothelium-dependent vasodilation but also increased NE-induced contractions. This effect was not observed when vessels were preincubated with either nGHH or LGHH, and it was abolished by deendothelialization. Those increases in maximal responses were also observed in vessels contracted with the α₁-adrenergic agonist methoxamine but not in those contracted with the α₂-adrenergic agonist clonidine, suggesting an α₁-adrenoceptor mediation of the above exposed effect. An increased responsiveness to α-adrenergic agonists in diabetic animals has been shown by many other authors, and several mechanisms have been proposed. Once again, these increased responses are time dependent and prevented or reversed by treating diabetic animals with insulin. Our results in aorta from nondiabetic rats raise the possibility of a role for HGHH in producing this increased responsiveness by NO blockade. The absence of an effect of HGHH on clonidine-induced responses may be due to (1) the contraction evoked by clonidine, which was almost undetectable in control vessels, so it was very difficult to appreciate any effect of HGHH on α₁-stimulated NO release, and/or (2) the rat aorta, which possesses an important basal NO release, and due to the blocking of the NO released by stimulation of α₁-adrenoceptors, the effect of HGHH will not occur. In contrast, the enhanced response of methoxamine could be explained by an effect of HGHH on basal NO release. A role for endothelial α₁-adrenoceptors in these methoxamine-induced responses can likely be excluded because this type of receptor is distributed scarcely in the endothelium of rat aorta. Similar increased responses to NE and methoxamine, but not to clonidine, have been shown in nondeendothelialized vessels from diabetic rat aortas. Although we have not excluded the release of a contractile endothelial substance, found by others in diabetic rabbit aorta, it seems unlikely because HOHH did not induce an effect on basal vascular tone in vessels with endothelium at the concentrations used in these experiments. The same is true for a direct effect on vascular smooth muscle; the addition of cumulative concentrations of HGHH did not induce a change in vascular resting tone in denuded vessels, also discarding a direct effect on vascular smooth muscle.

In conclusion, HGHH at low concentrations, but not nGHH or LGHH, appears to play a role in determining impaired endothelium-dependent responsiveness by NO blockade. It is noteworthy that some authors have found small amounts of free hemoglobin in plasma from normal subjects that are in the range of nanomolar concentrations. So, it could be hypothesized that if this free hemoglobin was abnormally glycosylated, our results would be of clinical relevance, adding new perspectives in the relationship among diabetes, hypertension, and other vascular diseases. Interestingly, a recent study showing increased mean apical capillary pressure in diabetic patients found a positive correlation between that increased capillary pressure and the glycosylated hemoglobin concentration. On the other hand, preliminary data from the Diabetes Control and Complication Trial show a relationship between levels of plasmatic glycosylated hemoglobin and the development of microangiopathic and macroangiopathic complications of diabetes. More studies are needed to confirm our results in other vascular beds and models of disease to subsequently establish the cutoff point of glycosylated hemoglobin concentration producing those effects as well as the mechanisms involved. These studies will help to improve our knowledge of the pathophysiologic links between diabetes and other vascular diseases and will raise new insights into therapeutic goals for diabetic patients.

Fig 5. Plot of effect of 10 nmol/L high- and low-glycosylated human oxyhemoglobin (HGHH and LGHH) on the responses elicited by norepinephrine (NE) in deendothelialized vessels. Data are expressed as mean±SEM of the percentage of contraction induced by cumulative concentrations of NE. 100% is the contraction induced by 75 nmol/L K+ (1993±203 mg, 2017±108 mg, and 1556±179 mg for control, LGHH, and HGHH, respectively). Three animals were used for the experiments. Number of segments used are in parentheses.

Fig 6. Plots of effect of 10 nmol/L high-glycosylated human oxyhemoglobin (HGHH) on the responses elicited by clonidine (A) and methoxamine (B). Data are expressed as mean±SEM of the percentage of contraction induced by cumulative concentrations of the agonists. 100% is the contraction induced by 75 nmol/L K+ (1779±182 mg and 1755±172 mg for control and HGHH, respectively). Three to five animals were used for each set of experiments. Number of segments used are in parentheses. *P<.05.
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