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Enhancement of S-Nitrosylation in Glycosylated Hemoglobin

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In this study, we report a novel differential nitric oxide interaction with nonglycosylated and glycosylated hemoglobin. After *in vitro* incubation of hemoglobin with *S*-nitroso *N*-acetyl penicillamine (SNAP), *S*-nitrosoglutathione, or *S*-nitrosocysteine, S-nitrosylation was significantly higher in human glycosylated hemoglobin purified from diabetic subjects compared to nondiabetic controls. Inversely, spontaneous decomposition was significantly lower for *S*-nitrosohemoglobin obtained from glycosylated hemoglobin. Bidimensional isoelectric focusing of hemoglobins incubated *in vitro* with SNAP also revealed a greater interaction of nitric oxide with glycosylated hemoglobin. In addition, a significantly higher level of *S*-nitrosohemoglobin was found in erythrocyte lysates from streptozotocin-induced diabetic rats compared to control rats. We suggest that highly glycosylated hemoglobin in diabetic subjects may favor S-nitrosylation, which may in turn impair vascular function, and participate in diabetic microangiopathy. © 2000 Academic Press

***Key Words:* S-nitrosohemoglobin; diabetes mellitus; nitric oxide; glycosylated hemoglobin.**

Hemoglobin is a complex protein, formed by a tetramer of 64.5 kDa composed of two α- and two β-polypeptide chains, each bound to an heme group with very high affinity for oxygen (1). In the capillaries, oxygen release from heme groups is favored by low oxygen gradient and by three known allosteric factors: carbon dioxide, hydrogen ions, and 2,3-diphosphoglycerate (DPG) (2). Alterations of these factors have been involved on several physiopathological conditions associated to tissue hypoxia, including diabetes mellitus (3).

The heme group of hemoglobin has also a very high affinity for nitric oxide (NO), and it has been described as a specific antagonist of NO in the vasculature (4). Depending on the presence of oxygen, the interaction of NO with the heme groups of hemoglobin may lead to the formation of methemoglobin plus nitrate (5), or to nitrosylhemoglobin (6), resulting on impaired vascular relaxing activity. In addition to such well-known reactions, a novel interaction of NO with hemoglobin has been recently reported, which preserves, rather than destroy, NO activity (7). This interaction results in the formation of *S*-nitrosohemoglobin and involves binding and release of NO from β 93 cysteine residues of hemoglobin, through a mechanism depending on the relaxed (R; oxygenated) or tense (T; deoxygenated) conformational status of the molecule (8). Indeed, it has been postulated that hemoglobin carries NO from the lungs to tissues as an *S*-nitrosothiol, playing an active role in vascular control and blood flow regulation (7–10).

Atherosclerosis, hypertension, or microangiopathy are the more frequent and threatening consequences of long-term diabetes mellitus (11). The early stages of diabetic vascular disease are linked to endothelial dysfunction and to a deficit in NO bioavailability (12, 13). Previous work from our laboratory indicates that such endothelial dysfunction may be induced by highly glycosylated hemoglobin, through the release of superoxide anions that inactivate NO (12, 13). In fact, poorly controlled diabetic patients exhibit a high percentage of non-enzymatically glycosylated hemoglobin (14). This spontaneous glycosylation of hemoglobin principally occurs at the amino terminus of β chains, interfering with the binding site for 2,3-DPG, thus increasing oxygen retention by the heme group (15), which might be involved on the reduced tissue oxygenation observed in diabetes (16). However, to our knowledge, there are no studies analyzing the influence of hemoglobin glycosylation on the formation of

Abbreviations used: NO, nitric oxide; G-Hb, highly glycosylated hemoglobin; C-Hb, control hemoglobin; SNO-Hb, *S*-nitrosohemoglobin; SNAP, *S*-nitroso-*N*-acetyl penicillamine; GSNO, *S*-nitrosoglutathione; CYSNO *S*-nitrosocysteine; CYS, L-cysteine; GSH, reduced glutathione; 2,3-DPG, 2,3-diphosphoglycerate.

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S-nitrosohemoglobin. Therefore, in this paper we explored the existence of alterations in the interaction of NO with human nonglycosylated and highly glycosylated hemoglobin *in vitro*. Further comparisons were carried out using fresh blood from control and streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Preparation of hemoglobins. For *in vitro* studies we used commercially available human control (C-Hb) and highly glycosylated (G-Hb) hemoglobins obtained from Sigma. As indicated by the manufacturer in the product specifications, control hemoglobin and highly glycosylated hemoglobin present a degree of glycation around 7 and 14%, respectively.

Stock solutions of C-Hb and G-Hb (1 mM) were prepared in deionized water, and reduced in presence of sodium dithionite (1 mM). Solutions were dialyzed three times for 60 min versus 0.5 mM EDTA in deionized water degassed with nitrogen. The reduction of hemoglobin and the resulting concentration were verified by spectrophotometry.

Nitric oxide donors. *S*-Nitroso *N*-acetyl penicillamine (SNAP), L-cysteine (CYS), reduced glutathione (GSH), and most of biochemical reagents were also purchased from Sigma (St. Louis, MO).

S-Nitrosocysteine (CYSNO) and *S*-nitrosoglutathione (GSNO) were synthesized for each experiment as previously described (17), by equimolar reaction between cysteine (CYS) or glutathione (GSH) (both at 1 M) with sodium nitrite in 1 M HCl. Synthesized CYSNO and GSNO were maintained on ice protected from light before developing each experience.

In vitro S-nitrosylation of hemoglobins. *In vitro* incubations of hemoglobins (50 μM) in the presence of different NO donors (SNAP, GSNO, or CYSNO) were always performed protected from light in PBS-EDTA (0.5 mM). Different concentrations and incubation times were scheduled, as indicated in every case.

Immediately after incubation, the reaction mixtures were desalted in 10-ml Sephadex G25 columns preequilibrated with PBS-EDTA (0.5 mM). *S*-Nitrosohemoglobin determinations in the eluted fractions were based on Saville's method (18). Briefly, this method consists on a Griess-based nitrite measurement (19) in the presence and in the absence of HgCl (1 mM).

S-Nitrosohemoglobin stability. *S*-Nitrosohemoglobin (SNO-Hb was first synthesized by incubating C-Hb and G-Hb (both at 50 μM) with CYSNO (5 mM) for 45 min, at 37°C protected from light, to be then desalted in 10 ml Sephadex G-25 columns. Comparison of stability was then carried out by serial determinations of SNO-Hb on each sample at several hours of interval at room temperature.

Bidimensional isoelectric focusing. Based on a protocol previously described by Moriguchi (20), human C-Hb and G-Hb (both at 50 μM), were analyzed by bidimensional isoelectric focussing after 2 h of incubation at 37°C with SNAP (5 mM). During 40 min, pH was range from 6 to 8 at 15°C in presence of constant power (10 W). Gels were soaked in 10% TCA for 20 min, washed several times with distilled water before drying and then staining with Coomassie Blue. Finally, the NIH Image software for Macintosh was used for densitometric comparison of major product spots observed in the scanned gels. Densitometric values for each spot are given as percentage of the total arbitrary units from each sample.

Induction of diabetes in rats. Diabetes was induced in 10-week old male Sprague–Dawley rats (250–300 g) by intraperitoneal inoculation of a single dose of streptozotocin (60 mg/kg) (21). Eight weeks after inoculation, blood samples were obtained by cardiac puncture to assess the levels of SNO-Hb in lysated erythrocytes. Briefly, packed erythrocytes were washed three times in PBS-EDTA (0.5 mM) and then lysed in a known volume of distilled water as previously reported (22).

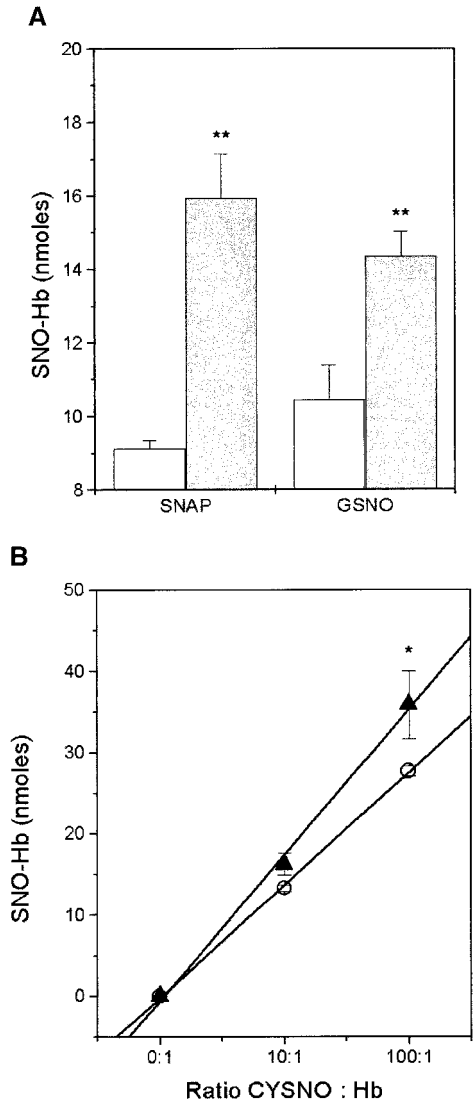

FIG. 1. Generation of SNO-Hb after 45 min of *in vitro* incubation of human control (open bars/open circles) and glycosylated hemoglobin (light gray bars/closed triangles), both at 50 μM, in the absence or in the presence of a 10-fold molar excess of SNAP or GSNO (A), and CYSNO in a 10- and 100-fold molar excess over protein (B). Significant difference between groups, evaluated by unpaired Student's t test, on A was accepted for ** $P < 0.01$ or * $P < 0.05$. Analysis of ANOVA on B indicates significant difference for * $P < 0.05$. Three to five independent experiments were performed.

Statistical analysis. Means ± SEM from replicated experiments were calculated by using the StatView software for Macintosh. Unpaired Student's t test or ANOVA analysis was carried out as indicated, with the level of significance chosen at $P < 0.05$.

RESULTS

The results on Fig. 1A show that, after 45 min of *in vitro* incubation at 37°C with SNAP or GSNO, at a 10-fold molar excess over protein, production of

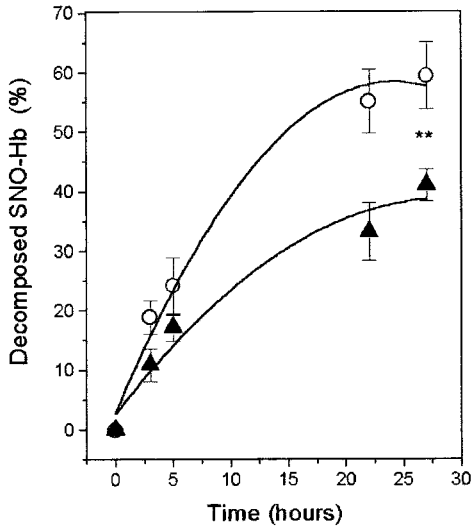

FIG. 2. Percentage of SNO-Hb synthesized from control (open circles) and glycosylated hemoglobin (closed triangles) that undergoes decomposition after several hours of incubation protected from light at room temperature. Analysis of ANOVA indicates significant difference for ** $P < 0.01$. Three to five independent experiments were performed.

SNO-Hb was significantly higher for human G-Hb compared with C-Hb. Furthermore, comparison of slopes by ANOVA between linear regression curves for CYSNO used at different proportions, corroborate a higher rate of SNO-Hb production in human G-Hb (Fig. 1B).

On the other hand, significant differences were also observed on the stability of the produced SNO-Hb (Fig. 2). The percentage of decomposed SNO-Hb after several hours of storage protected from light at room temperature was significantly lower for SNO-Hb obtained from human G-Hb compared to SNO-Hb obtained from C-Hb.

Supporting the idea of a higher interaction of NO with G-Hb (Fig. 3), after 2 h of incubation of C-Hb and G-Hb (both at 50 μM) with SNAP (5 mM), densitometry of the Coomassie blue stained gels from bidimensional isoelectric focusing, indicate a significantly higher magnitude of the reaction product spots from G-Hb compared to C-Hb. Two major spots from both hemoglobins were observed to be increased after incubation with SNAP, however in the case of G-Hb such effect was significantly higher.

Finally (Fig. 4), experiments *in vivo* showed significantly higher levels of SNO-Hb in erythrocyte lysates from streptozotocin-induced diabetic rats compared to controls. Furthermore, higher level of SNO-Hb in diabetic rats corresponded with higher levels of G-Hb in blood (11.7 $\pm$ 1.7% in diabetics compared to 4.07 $\pm$ 0.07% in controls).

DISCUSSION

As mentioned above, a novel mechanism of S-nitrosylation of hemoglobin suggested the idea of a new system for transporting NO, which may control vascular tone, blood flow and tissue oxygenation (7–10). The biochemical characterization of *S*-nitrosohemoglobin argues in favor of such hypothesis (23), while parallel studies have already explored the implication of the impairment of such mechanisms in hemoglobin from sicklemic cells (24).

On the other hand, some interesting possibilities arise in relation to the pathophysiological implications

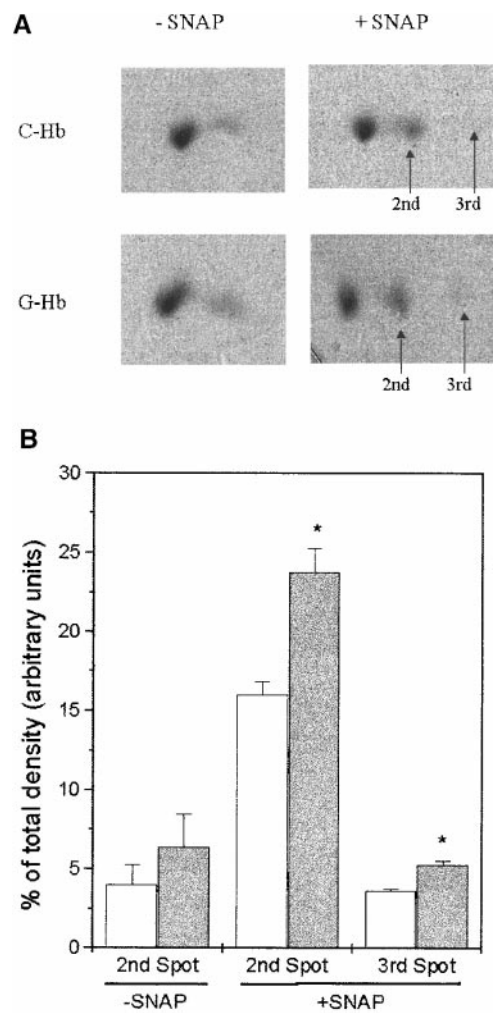

FIG. 3. Bidimensional isoelectric focusing of human control (C-Hb) and glycosylated (G-Hb) (both at 50 μM) after 2 h of incubation at 37°C with SNAP (5 mM). Arrows, in a representative scanning of the Coomassie blue staining gels, indicate the 2nd and 3rd spots considered as the two major distinguishable reaction products (A). Densitometric comparison of the 2nd and 3rd spot from control (open bars) and glycosylated hemoglobin (light gray bars) incubated or not with SNAP (5 mM) (B). Significant difference between groups, evaluated by unpaired Student's *t* test, was accepted for * $P < 0.05$. This experiment was reproduced two times.

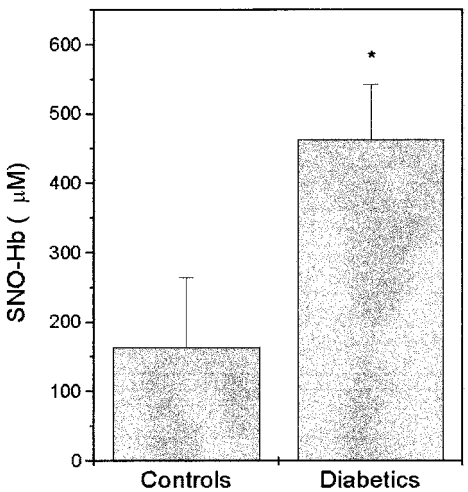

FIG. 4. Levels of SNO-Hb in erythrocyte lysates from control and 8 weeks of streptozotocin-induced diabetes in Sprague–Dawley rats ($n = 5$). Significant difference between groups, evaluated by unpaired Student's t test, was accepted for * $P < 0.05$. This experiment was reproduced two times.

of the interaction between NO and hemoglobin in several cardiovascular diseases. Indeed, endothelial dysfunction, as well as NO deficiency, precedes diabetic vasculopathy (12, 13), meanwhile hemoglobin glycosylation is a typical characteristic of poorly controlled diabetes (14). To our knowledge, there are no studies investigating the influence of glycosylation on the production of *S*-nitrosohemoglobin. It is well known, however, that hemoglobin glycosylation interferes in the binding site for 2,3-DPG (15). In fact, it has been suggested that this mechanism may be on the basis of the lower tissue oxygenation observed in diabetes (6), because of the lower oxygen release from the heme group in such conditions (16).

In the present work, we have reproduced experimental conditions analogous to those employed in the original description by Stamler and co-workers (7), and we have obtained evidence suggesting that human oxyhemoglobin containing high levels of glycosylation (14%) showed a higher *in vitro* S-nitrosylation when compared to non-glycosylated oxyhemoglobin. This effect occurred in a similar way when incubating hemoglobins with different NO donors, such as SNAP, GSNO or CYSNO.

On the other hand, we have also analyzed the rate of spontaneous decomposition of both types of *S*-nitrosothiols, formed with glycosylated and nonglycosylated oxyhemoglobin. We observed that the spontaneous S-denitrosylation occurred more slowly for *S*-nitrosohemoglobin formed with glycosylated hemoglobin.

As mentioned above, the so-called glycosylated oxyhemoglobin means that around 14% of the protein is glycosylated in the terminal amino group of β chain. Likely, the S-nitrosylation process observed in this type of hemoglobin occurred both in the glycosylated and non-glycosylated fractions. However, it seems reasonable to suggest that the differences obtained when compared to non-glycosylated hemoglobin are mainly due to the glycosylated percentage. We hypothesize that in glycosylated oxyhemoglobin the binding of NO to β93 cysteine residues may be increased while its release is impaired.

A previous report indicates that interaction of a NO donor with oxyhemoglobin can modify the N terminal groups of the β-chains, likely by deamination (20). These authors found that the products of such interaction were more electronegative than unmodified oxyhemoglobin, as determined by isoelectric focusing (20). Indeed, using this technique we observed that incubation of C-Hb with SNAP produced a change in protein migration in agreement to previously described (20). In our case, we have also observed that, in similar conditions, interaction of NO with G-Hb resulted in a significantly higher magnitude of product spots. These facts are consistent with our hypothesis of a higher interaction of NO with G-Hb.

To provide a possible explanation concerning the mechanism by which glycosylation of hemoglobin may influence the NO interaction, it is worth to consider the relevance of the R/T hemoglobin conformational status for processes of S-nitrosylation in β93 cysteines residues (7–10). Glycosylation of amino terminus in β chains of hemoglobin is known to diminish the binding of 2,3-DPG (15), increasing the stability of the R conformation, in which S-nitrosylation is favored (7–10).

Finally, we tested an easily demonstrable consequence of our hypothesis in glycosylated hemoglobin. If the formation of S-nitrosohemoglobin is enhanced while its S-denitrosylation process is reduced, this should lead to an increase in the total amount of *S*-nitrosohemoglobin when diabetes is present and blood levels of HbA_{1c} are increased. Indeed, we found higher levels of *S*-nitrohemoglobin using erythrocyte lysates from streptozotocin-induced diabetic rats, in which values of G-Hb were also significantly higher.

The pathophysiological relevance of the present results remains highly speculative, but we provide two interesting and apparently counteracting mechanisms. The first one leads to a higher formation of *S*-nitrosohemoglobin, therefore enhancing the transport of NO through peripheral circulation. However, the effective release of NO may be reduced because the lower rate of S-denitrosylation. At present, we have no evidence indicating the predominance of any of such mechanisms. Nevertheless, we have previous data indicating that glycosylated oxyhemoglobin can inactivate endothelium-derived NO by releasing superoxide anions, which may be an important mech-

anism for diabetic endothelial dysfunction (13). Therefore, it seems reasonable to propose that the combined final effect of hemoglobin glycosylation may be the reduction in the bioavailability of NO in diabetic subjects.

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