THE EFFECT OF S-NITROSOGLUTATHIONE ON INSULIN RECEPTOR BEHAVIOR ON ERYTHROCYTES IN AN ANIMAL MODEL

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SUMMARY

Nitric oxide (NO) is a potent modulator of cellular function. This study was designed to examine the possible diabetogenicity of nitric oxide (NO) by investigating any differences in cellular binding of insulin between dogs treated with the NO donor S-nitrosoglutathione (GSNO) and controls treated with captopril. Glucose abnormality was attributed to decreased binding of insulin to its receptor on the cell membrane of erythrocytes, 27.53 ± 1.37% in GSNO treated dogs compared with 20.60 ± 3.36 in captopril controls at an unlabeled insulin concentration of 2.5 ng/ml (p<0.05). The decreased insulin binding was attributed primarily to a 22% decrease in the number of insulin receptor sites per cell (p<0.05) and secondarily to a decreased average receptor affinity. In conclusion, these findings suggest the evidence of NO as a modulator of insulin binding and the involvement of NO in the etiology of diabetes in dogs treated with this NO donor.

INTRODUCTION

The endogenous nitrovasodilator nitric oxide (NO), formed by endothelium nitric oxide synthase, plays a major physiological role in vascular biology (1). On the other hand, recent findings suggest that induced NO formation plays a role in the destruction of pancreatic β-cells during the development of type I diabetes (2). S-nitrosothiols are adducts of NO and free sulfhydryl (-SH) groups, and they are detectable in both blood and tissues (3). In addition, S-nitrosothiol formation may mediate the bioactivity of nitrovasodilator drugs (4). S-nitrosoglutathione (GSNO) is a common source of NO in the biomedical field, which has shown great biological diversity. GSNO, a physiological S-nitrosothiol compound, possesses potent antiplatelet aggregatory activity despite a slow rate of NO release and modest capacity to stimulate intraplatelet cyclic GMP accumulation. At low concentrations, exogenous GSNO has been shown to provide significant protection to the ischemic myocardium (5). It is also used therapeutically as an arterioselective vasodilator (6) and anti thrombotic agent (7).

A recent study conducted in our laboratory showed GSNO to induce a state of impaired glucose tolerance (8). Abnormalities at the level of insulin receptor have been implicated in a number of insulin-resistant states (9). In some disorders of carbohydrate metabolism, changes in insulin receptor affinity alone or in addition to changes in receptor number have been observed (10). The present study was so designed as to investigate the possible effects of GSNO on insulin binding to its receptor on erythrocyte cell membranes in dogs treated with GSNO and controls treated with captopril.
MATERIALS AND METHODS

The protocol was conducted in accordance with the guidelines of the University of the West Indies Animal Study Committee. Eighteen normal Mongrel dogs (9 male and 9 female) aged 2-3 years, mean weight 12.4 ± 0.4 kg, were obtained from the Preclinical Animal House of the Department of Basic Medical Sciences, University of the West Indies. The animals were kept in animal housing under the supervision of attendants. Dogs were fed a diet of Purina Laboratory Chow (Purina, St. Louis, MO, USA) and water ad libitum.

The dogs were divided into two groups of nine animals, test and controls. Oral glucose tolerance test was performed on each dog. Briefly, after an 18-h fast, dogs were anesthetized with sodium pentobarbital (30 mg/kg i.v.). Subsequently, fasting blood samples were obtained and control group animals were administered 20 mg/kg body weight of captopril (Sigma Aldrich, St. Louis, MO, USA) dissolved in water. GSNO was obtained by the method of Hart (11). GSNO was dissolved in water and administered to dogs at a dose of 35 mg/kg body weight. Blood samples were collected 1.5 h after glucose load of 1.75 g/kg body weight. This was the time point at which peak postprandial blood glucose had been observed in previous experiments (8). Each sample was collected in an EDTA tube and immediately placed on ice for subsequent biochemical analysis.

The erythrocyte receptor assay was performed according to a modification of the method of Ghambir et al. (12). Lyophilized unlabeled insulin (10 mg; Sigma, 23.5 U/mg) was reconstituted in 10 ml of 0.1 M HCl and 100 µl aliquots were stored at −70 °C until required. Serial dilutions were then performed in assay buffer for each of the following concentrations: 0.1, 0.5, 1.0, 2.5, 5.0, 10.0 and 100 ng/ml (physiological range 16.7 – 16700 pM). Monoiodinated A\textsuperscript{[125]I} insulin (Amersham, Arlington Heights, IL, USA; specific radioactivity 50 µCi/µg) was dissolved in deionized water (500 µl) and aliquots of 5 µl were stored at −70 °C until needed.

The erythrocytes were washed three times by centrifugation (4 °C, 4500 rpm) in 10 ml of Buffer G [Hepes (11.92 g), Tris (6.06 g), EDTA (0.74 g), glucose (1.60 g), NaCl (3.80 g), KCl (0.37 g), MgCl\textsubscript{2}·6H\textsubscript{2}O (2.03 g) and CaCl\textsubscript{2}·2H\textsubscript{2}O (1.47 g) dissolved in water (1 L), pH 7.8] and 1% human serum albumin for 10 minutes. The washed erythrocytes were resuspended in Hepes-Tris-phosphate buffer (pH 7.8) containing 1% of human serum albumin, and dispersed in triplicate for insulin binding studies. The erythrocyte suspension contained 5 x 10\textsuperscript{8} cells/ml.

One hundred and fifty-µl aliquots of the washed erythrocyte suspension were added to a series of Eppendorf tubes. To each sample tube 50 µl of unlabeled insulin and 50 µl of 125I-insulin (0.2 ng/ml) were added and tube contents were incubated for 3 hours. The erythrocyte reaction was stopped by washing with cold saline (1 ml) and centrifuged at 1100 rpm for 1 minute. The supernatant was discarded and 100 µl of 40% formalin were added to harden the red cell pellet. The radioactivity of the pellet was also determined using a gamma counter.

The data were analyzed by Scatchard analysis (13) and the average affinity profile was calculated according to the method of DeMeyts (14). The receptor affinity and receptor numbers were derived for the physiological range of insulin, i.e. between 0.1 and 100 ng/ml. Specific insulin binding (SB) was calculated as percentage of radioactive insulin bound by 5 x 10\textsuperscript{8} cells/ml of erythrocytes. Nonspecific binding was assessed by the amount of radioactive insulin bound in the presence of 100 ng/ml unlabeled insulin. Competitive binding curves were obtained for erythrocyte suspension. From these curves, the insulin receptor affinity and number of receptor sites were determined by Scatchard analysis.

Results of the binding studies are presented in three ways: (i) percentage binding of 125I-insulin as a function of total insulin concentration (competitive curve); (ii) bound-free 125I-insulin ratio plotted as a function of bound insulin (Scatchard plot); and (iii) average affinity profile calculated according to the method of De Meyts and Roth (13). The total binding capacity or concentration of the binding sites was derived from the point where the linear extrapolation of the curve intercepted horizontal axis.

The experimental data suggested the insulin receptor to consist of homologous binding sites undergoing negatively cooperative site-site interactions so that the affinity of the receptors for insulin was inversely related to the receptor occupancy. The average affinity profile expresses the relationship between the average affinity for insulin (K) and receptor occupancy (Y). The average affinity falls as a function of receptor occupancy.
(negative cooperativity) until the lowest observable affinity ($K_c$) is reached. The fractional occupancy necessary to produce $K_t$ is designated $y_f$, and $K_e$ represents the highest observable affinity of the receptors and is expressed in the native or 'empty site' state.

All results shown in the figures are expressed as means ± SEM. Integrated area under the curve (iAUC) was calculated by subtracting the rectangle corresponding to the basal value from the total area under the curve (15). Data analysis was done using the Sigma Plot and Sigma Statistics software packages (Jandel Scientific). To evaluate the effects of GSNO and captopril on the biochemical parameters, values for each group were compared by either paired Student’s test or two-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test (16). Values of $p$ less than 0.05 were considered to indicate significance in all cases. Linear regression analysis was used to determine correlation coefficients.

RESULTS

Figure 1 summarizes the ability of unlabeled insulin to competitively inhibit the binding of $^{125}$I-insulin to the insulin receptor on erythrocyte cell membranes in dogs treated with 35 mg/kg of GSNO and controls treated with 20 mg/kg of captopril. The percentage $^{125}$I-insulin bound to insulin receptor on erythrocyte cell membrane of captopril controls was 34.70 ± 3.58%, as compared with 30.38 ± 4.54 % for erythrocytes from dogs treated with 35 mg/kg of GSNO (p=0.158; Figure 1) in the absence of unlabeled insulin (0 ng/ml). Further comparison of the percentage of $^{125}$I-insulin bound to insulin receptor on the cell membrane of erythrocytes of GSNO-treated dogs and captopril-treated controls showed slopes which decreased steadily with increasing unlabeled insulin concentrations, with statistically significant differences observed at unlabeled insulin concentrations of 2.5 ng/ml (20.60 ± 3.36% in GSNO treated dogs compared with 27.53 ± 1.37% in captopril controls) and 10.0 ng/ml (p<0.05). The integrated area under the curve for erythrocytes from GSNO treated dogs was 186.36 ± 4.52% x 10 ng/ml compared with 212.40 ± 4.97% x 10 ng/ml in captopril controls (p<0.05).

In this study, the ratio of bound/free labeled hormone was expressed as a function of the bound hormone giving a Scatchard plot for erythrocytes (Figure 2). Curvilinear plots were obtained for both the controls and treated dogs. Statistical analysis showed significant differences in the bound/free $^{125}$I-insulin ratio of erythrocytes from dogs treated with GSNO (0.450 ± 0.05 and 0.394 ± 0.03) compared with those of captopril controls (0.531 ± 0.06 and 0.479 ± 0.02; p<0.05; Figure 2) at unlabeled insulin concentration of 0 and 0.1 ng/ml.

The data were plotted on an average receptor affinity graph as described by DeMeyts (14). Closer examination showed that erythrocytes from captopril controls had the highest 'empty site' affinity ($K_c$) of 38.36 ± 0.80 x 10⁻⁸ M⁻¹, which began to decrease when approximately 0.3% of total receptor sites were
occupied. With increasing occupancy of the receptor by insulin, the average receptor affinity progressively decreased to the 'filled site' affinity ($K_f$) of $13.40 \pm 2.73 \times 10^{-8} \text{M}^{-1}$, when 65.00% of available receptor sites were occupied (Figure 3). The comparable values of $K_e$ and $K_f$ for erythrocytes from dogs treated with GSNO were $28.30 \pm 2.55 \times 10^{-8} \text{M}^{-1}$ and $11.41 \pm 2.98 \times 10^{-8} \text{M}^{-1}$, respectively. Statistical analysis of the data showed significant differences in the average receptor affinity values between the groups at low unlabeled insulin concentration ($0.1 - 2.5 \text{ng/ml}$; $p<0.05$). Scatchard analysis of the data revealed that there was a 21% decrease in the number of insulin receptor sites per cell for erythrocytes from dogs treated with GSNO ($72 \pm 5$) as compared with those of captopril controls ($93 \pm 5$; $p<0.05$, Figure 4).

**DISCUSSION**

This study was a continuation of a previous study by McGrowder et al. (8), which found that GSNO-treated dogs displayed transient postprandial hyperglycemia. These results clearly demonstrated that erythrocytes isolated from dogs treated with 35 mg/kg of GSNO had a decreased ability to bind insulin. When erythrocytes were incubated with trace amount of $^{125}$I-insulin, the cells from dogs administered 35 mg/kg of GSNO showed a significant decrease in insulin binding at low unlabeled insulin concentrations. The decreased binding of insulin to its receptor on the erythrocyte cell membrane was attributed primarily to a significant reduction in the average affinity of the receptor for insulin, and secondarily to a decrease in the number of insulin receptor sites per cell.

Previous studies suggest that NO could control mitochondrial respiration and carbohydrate metabolism (17). The decreased insulin binding to its receptors in...
the high affinity regions could be attributed to the effects of high nonphysiological concentration of NO released from GSNO. Nitric oxide released from GSNO could possibly affect the intracellular processing of insulin by interfering with the formation of the insulin-receptor complex. Nitric oxide in nonphysiologically high concentrations can damage the cell membrane integrity and affect the formation of the insulin-receptor complex.

The curvilinear plot of bound/free against \([\text{bound}]\) erythrocytes yielded curvilinear plots with upward concavity. These results suggest that erythrocytes from the dogs had a similar binding kinetics as those reported in the literature for lymphocytes, mononuclear leukocytes, erythrocytes and insulin target tissues (18). The data in Figure 1 clearly demonstrate that the percentage \(^{125}\text{I}-\text{insulin} \) bound decreased at lower unlabeled insulin concentrations, but the curves converged at higher unlabeled insulin levels. This suggests a change in receptor affinity. The decrease in insulin binding attributed to a decrease in receptor affinity was confirmed by the decrease in the average receptor affinity \((K)\) for erythrocytes.

Nitric oxide is known to have cytotoxic effects on target cells. It is therefore likely that the insulin receptor on the cell membrane of erythrocytes could be affected by the cytotoxic effect of NO and become desensitized. Desensitization is associated with the total lack of insulin effect despite remaining insulin receptors. Several possibilities exist to explain the mechanism of changing receptor affinity and desensitization. First, membrane fluidity may be an important factor in modulating insulin binding and action. Second, insulin receptor may be covalently associated with another protein that modulates receptor affinity. It is therefore possible that NO released from GSNO alters the interaction of insulin with its receptor, thus affecting the ability of insulin to differentially regulate its receptor and this regulator protein (19). The third possibility is that the receptor undergoes some form of modification (conformational change) that alters the binding and signal transduction properties (18). The modification could involve a change in the redox state of the receptor. The insulin receptor is composed of major subunits linked by disulfide bonds to various oligomeric forms. Reduction of the oxidized forms of the receptor could modify the affinity of insulin (20).

The data were analyzed using Scatchard plot to investigate whether the decrease in insulin binding was attributable to a decrease in the number of insulin receptor sites per cell. The x-intercept represents the number of insulin receptor sites per cell. Calculations revealed that there was a 21% decrease in the number of receptor sites per erythrocyte in GSNO-treated dogs compared with captopril controls. The lower number of insulin receptor sites per cell in GSNO dogs could be the result of primary alteration in the receptor or may be secondary to some other alteration in the membrane (21). Nitric oxide released from 35 mg/kg of GSNO may damage erythrocytes in the short term with direct effects on membrane structure, membrane fluidity, cross-linking and function. These changes in membrane integrity could be responsible for the decreased number of insulin receptor sites per cell. The resulting membrane dysfunction in addition to the
decreased insulin production in these dogs could impair transport of glucose and other metabolites across the cell membrane resulting in the observed hyperglycemia (8).

Circulating erythrocytes express insulin receptor (22), and the main advantage of using erythrocytes for investigating the receptor status in humans and animals is that they are more easily accessible than cells of primary insulin target organs, such as adipocytes and muscle cells. It has been found that the characteristics of insulin binding to human adipocytes (including affinity constants for the binding reaction) were similar to the characteristics of insulin binding to erythrocytes and mononuclear leukocytes in obese subjects (23). It is our strong opinion that any changes in membrane properties resulting in changes of receptor behavior in response to extracellular environment will be evident in some measure in blood cells as well as in target tissues.

In conclusion, NO released from GSNO caused a decreased ability of insulin to bind to its receptor on the cell membrane of erythrocytes. This decreased binding is attributed primarily to a decreased average receptor affinity and reduction in the number of available insulin receptor sites per cell. The data thus support the modulatory role of exogenous NO in insulin binding. Whether NO released from GSNO plays a role in the inhibition of insulin action is presently under investigation in our laboratory.

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REFERENCES


