Exogenous nitric oxide inhibits IRS-1 expression in rat hepatocytes and skeletal myocytes

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Summary

Accumulative evidence has supported the role of nitric oxide (NO) in a variety of normal physiological functions as well as many pathological conditions. In this study, we examined the possible diabetogenicity of NO by measuring the expression of the insulin receptor substrate (IRS)-1 in rat hepatocytes and skeletal myocytes. IRS-1 is important in the insulin-mediated signal transduction pathway in both liver and skeletal muscle. Exogenous NO donated by S-nitroso-N-acetylpenicillamine (SNAP) and S-nitrosoglutathione (GSNO) resulted in significant reduction in levels of IRS-1 in both cells, when compared to the insulin-stimulated control ($p < 0.001$). Reversal to near normal levels was achieved using the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO). SNAP was the more potent drug, and the skeletal myocytes were the more sensitive cells to the inhibitory effects of NO released from the drugs. These results provide further evidence that exogenous NO is a potent modulator of insulin-mediated signal transduction and may play a significant role in the pathogenesis of type 2 diabetes mellitus.

Introduction

Type 2 diabetes is a heterogeneous disorder characterized by at least two pathological defects, namely β cell dysfunction and insulin resistance [1, 2]. Both skeletal muscle and liver are important targets for insulin action, and insulin resistance in skeletal muscle is a characteristic feature of type 2 diabetes [3].

Insulin is the principal hormone controlling blood glucose and acts by stimulating glucose influx and metabolism in muscle and adipocytes and inhibiting gluconeogenesis by the liver [4]. Insulin action is mediated through the insulin receptor, a

transmembrane glycoprotein with intrinsic protein tyrosine kinase activity [4]. The level of tyrosine kinase reflects the serum concentration of insulin and appears to mediate the insulin response through tyrosine phosphorylation of the receptor itself and substrates like insulin receptor substrate (IRS)-1 [5–7]. IRS-1 contains 21 potential tyrosine phosphorylation sites, of which at least eight undergo phosphorylation by the activated receptor [4].

Nitric oxide is a short lived free radical gas and biological signalling molecule produced by the intracellular enzyme NO synthase [8]. The reactivity of NO towards molecular oxygen, thiols, transition metal centres, and other biological targets enables NO to act as a ubiquitous cell-signalling molecule with diverse physiological and pathological roles [8, 9]. In this regard, NO can

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react with thiols to form S-nitrosothiols *in vivo*, such as S-nitrocysteine and S-nitrosoglutathione (GSNO), although the mechanism of this reaction remains unclear [10].

Nitric oxide drugs are useful in the treatment of several disorders, and are generally indicated in cases of NO insufficiency. Previously, we have established that exogenous NO (from NO-releasing drugs) inhibited *in vivo* insulin binding to its receptor on erythrocytes and mononuclear leukocytes [11], and *in vitro* glucose uptake in skeletal muscle cells [12] and adipocytes [13]. Our current objective was to characterize the *in vitro* effects of exogenous NO generated by S-nitroso-N-acetyl penicillamine (SNAP) and S-nitrosoglutathione (GSNO) on expression of IRS-1 in isolated rat hepatocytes and skeletal myocytes.

Materials and methods

Chemicals and reagents

Anti-insulin receptor substrate (IRS)-1 and associated alkaline phosphatase conjugate were obtained from Chemicon International Inc. (Temecula, CA, USA). All biochemicals and enzymes were of analytical grade and were purchased from commercial suppliers.

Preparation of dissociated cells

Female and male Sprague-Dawley rats (6–8-weeks old weighing 200–300 g) were housed at the University of the West Indies Preclinical Animal House, Mona, Jamaica, with free access to water and food. Food was withdrawn on the evening prior to experimentation and rats were euthanized using diethyl ether. The ethics committee of the University of the West Indies/University Hospital of the West Indies approved the experiments involving animals and method of euthanasia.

Approximately 5–10 g of liver and skeletal muscle tissue were aseptically removed, washed 2–3 times with sterile phosphate buffered saline (PBS, pH 7.2) and minced using a sterile scalpel. Hepatocytes and skeletal myocytes were isolated using a modification of the method by Freshney [14]. Briefly, minced tissues were incubated in Krebs' Ringer Bicarbonate (KRB) containing 200 U/ml collagenase, 10% bovine serum albumin

(BSA) and 1×penicillin/streptomycin/neomycin (PSN) (Sigma, St. Louis, MO, USA) at 37 °C in a humidified incubator containing 95% air and 5% CO₂. Dissociated cells were collected by centrifugation at 100 rpm for 5 min, washed twice in 5 ml KRB buffer containing 2.5 mM glucose and 2% BSA and resuspended in 20–30 ml KRB without any additives. Viability of dissociated cells was verified using Trypan blue.

Treatments of dissociated cells

After an incubation period of 30 min, cells (1 ml packed cells/1.5 ml Eppendorf tube) were treated with 25 and 50 ng/ml SNAP or GSNO (Sigma), in the absence or presence of 100 or 1000 nM insulin at 37 °C for 1 h. Nitric oxide released from the drugs was determined as nitrite using the Greiss reagent (BDH, Poole, UK). Experiments with 25 ng/ml GSNO and SNAP were repeated in the presence of 0.1 µM of the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO; Sigma).

Immunoblotting

Cells were washed and solubilized in 750 µl of 1% Triton X-100 lysis buffer in the presence of protease inhibitors for 30 min at 37 °C. The protein content of cell lysates was determined using the Bradford method [15]. Protein samples (30–50 µg) in 25 µl of lysis buffer were applied to PVDF membrane using a manifold apparatus (Scie-Plas, Warwickshire, UK) and a vacuum pressure pump (Barnant Co., USA). Blots were probed with anti-IRS-1 antibodies (1:1000) followed by the alkaline phosphatase conjugated secondary antibody and developed according to the manufacturer's instructions (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Membranes were scanned and densitometric analysis carried out using NIH Image J programme for PC [16].

Statistical analysis

Statistical analysis was performed using either unpaired Student's t test or ANOVA (Fisher, multiple comparisons), as applicable. Values are presented as means ± S.E.M. of 3–6 observations,

and differences among means were considered significant at $p < 0.05$.

Results

Nitric oxide released from drugs

Figure 1 shows the concentration-dependent increase in nitric oxide (measured as nitrite) released from SNAP and GSNO in aqueous solution. In all cases there was a gradual increase in NO released, with a greater amount of NO being released from drugs at the higher concentration. Carboxy-PTIO, when added either at the start of the experiment or after 30 min resulted in a sharp decline in the amount of NO released from either drug (Figure 2).

Effect of NO released from SNAP and GSNO on IRS-1 expression in hepatocytes and skeletal myocytes

The inhibitory effects of NO released from SNAP and GSNO on IRS-1 expression in isolated rat

hepatocytes and skeletal myocytes are shown in Figures 3 and 4. The mean basal (unstimulated) expression or content of IRS-1 in rat hepatocytes was $51.70 \pm 10.85\%$ when compared to the insulin-stimulated control (insulin at 100 nM), which was normalized to 100% (Figure 3). Incubation with SNAP at the concentrations studied significantly decreased expression of IRS-1 compared to the insulin-stimulated control by 60–100% ($p < 0.01$). Total inhibition of IRS-1 expression occurred when cells were incubated with 25 ng/ml SNAP in the presence of 100 nM insulin. By comparison, in treatments with 50 ng/ml SNAP, expression of IRS-1 was 19–43% of the insulin-stimulated control, indicating that there was a greater inhibition at the lower concentration, especially in the presence of 100 nM insulin. Further, SNAP at 50 ng/ml decreased basal expression of IRS-1 over that of 25 ng/ml. Generally, reduction of IRS-1 content by SNAP was to the extent of the basal cellular levels, or to that in the presence of high (1000 nM) insulin. In the absence of insulin, there was no significant difference in the effect of SNAP at either concentration ($p > 0.05$).

Generally, GSNO had a greater effect in reducing IRS-1 expression in these hepatocytes, although not significantly different from that with SNAP. Except for 25 ng/ml GSNO in the presence of 100 nM insulin, the reduction in IRS-1 expression was significantly lower than that of the basal levels. Further, in treatments with 25 or 50 ng/ml GSNO, reductions in the content of cellular IRS-1 was to 13–40% or 21–27% of the insulin-stimulated control, respectively ($p < 0.01$).

In the absence of insulin, there was also no significant difference in the effects of GSNO at either concentration ($p > 0.05$).

The mean basal expression of IRS-1 in rat skeletal myocytes was $54.34 \pm 7.58\%$ of the 100 nM insulin-stimulated control (Figure 4). Incubation with SNAP at either concentration significantly reduced IRS-1 expression in these cells ($p < 0.01$; Figure 4). However, 25 ng/ml SNAP had a slightly greater effect than 50 ng/ml SNAP in reducing IRS-1 expression in the absence ($10.74 \pm 1.98\%$ versus $12.70 \pm 1.23\%$) or presence of 100 nM insulin ($6.03 \pm 0.98\%$ versus $11.72 \pm 1.40\%$). These differences were significant only in the presence of 100 nM insulin ($p < 0.05$). Further, 50 ng/ml SNAP caused a marginally (though

Figure 1. Nitric oxide (NO) released from (a) SNAP and (b) GSNO at 25 and 50 ng/ml. Values are expressed as means $\pm$ SEM; * $p < 0.05$ vs the drug at 25 ng/ml.

Figure 2. Nitric oxide (NO) released from (a and b) SNAP and (c and d) GSNO at 25 and 50 ng/ml in the presence of the NO-scavenger, carboxy-PTIO, added at time 0 min (a and b) and after 30 min (c and d).

not significantly) greater reduction than 25 ng/ml SNAP in expression of IRS-1 in the presence of 1000 nM insulin ($7.23 \pm 0.75\%$ versus $8.20 \pm 1.06\%$; $p > 0.05$).

Both concentrations of GSNO (Figure 4) were effective in reducing IRS-1 expression ($p < 0.05$). However, 25 ng/ml GSNO compared to 50 ng/ml GSNO was more effective in reducing IRS-1 content in the absence ($14.79 \pm 3.12\%$ versus $34.22 \pm 8.90\%$) or presence of 100 nM insulin ($4.37 \pm 1.03\%$ versus $41.49 \pm 9.02\%$; $p < 0.05$). There was no significant difference between the effects of 25 or 50 ng/ml GSNO on IRS-1 content in the presence of 1000 nM insulin ($12.89 \pm 2.56\%$ versus $12.30 \pm 3.05\%$).

Generally, in the presence of 100 nM insulin, 25 ng/ml GSNO was more potent than 25 ng/ml SNAP in reducing IRS-1 expression ($p < 0.001$), and a greater reduction was observed for 50 ng/ml SNAP in the absence or presence of insulin when compared to 50 ng/ml GSNO ($p < 0.002$). However, the general observation was 25 ng/ml SNAP

effected a greater overall reduction of IRS-1 expression in skeletal myocytes.

We tested whether the NO scavenger, carboxy-PTIO was able to reverse the effect of NO-mediated reduction in expression of IRS-1 in the hepatocytes and skeletal myocytes. We found a near normal expression of IRS-1 in hepatocytes co-treated with carboxy-PTIO and 25 ng/ml SNAP or GSNO in the presence of 100 nM insulin ($87.5 \pm 1.6\%$ versus $93.0 \pm 0.0\%$) or 1000 nM insulin ($76.8 \pm 1.1\%$ versus $75.0 \pm 0.9\%$; Figure 5). These were not significantly different from the respective controls with 100 or 1000 nM insulin ($p > 0.8$).

A similar effect was seen in skeletal myocytes exposed to 25 ng/ml SNAP or GSNO when the NO scavenger carboxy-PTIO was added in the presence of 100 nM insulin ($97.80 \pm 0.60\%$ versus $85.96 \pm 0.18\%$) and 1000 nM insulin ($73.60 \pm 4.34\%$ versus $43.50 \pm 4.67\%$; Figure 5). These were not significantly different from the respective controls with 100 or 1000 nM insulin ($p > 0.2$).

Figure 3. The effects of NO donor on content of IRS-1 in rat hepatocytes. Rat hepatocytes were treated with the indicated concentrations of (a) SNAP, (b) GSNO, in the presence the indicated concentrations of insulin, with or without 25 mg/ml glucose (Glu). Values are expressed as means $\pm$ SEM; * p < 0.05 vs the insulin-stimulated control (100 mM insulin).

Discussion

The major conclusion to be drawn from this study is that the pathophysiologically relevant concentrations of NO are sufficient to reduce the expression, and hence availability, of IRS-1 in two

Figure 4. The effects of NO donor on content IRS-1 in rat skeletal myocytes. A, Rat skeletal myocytes were treated with the indicated concentrations of (a) SNAP, (b) GSNO, in the presence the indicated concentrations of insulin, with or without 25 mg/ml glucose (Glu). Values are expressed as means $\pm$ SEM; * p < 0.05 vs the insulin-stimulated control (100 mM insulin).

insulin-sensitive tissues. We found that rat hepatocytes and skeletal myocytes treated with SNAP or GSNO had significantly reduced IRS-1 content. Further, the fact that we were could completely abrogate the inhibitory effects of both drugs with the NO scavenger, carboxy-PTIO, where drugs had varying effects in the two tissues, suggests that the effects of SNAP and GSNO were related to the generation of NO and not to a non-specific effect

Figure 5. The effects of a NO scavenger, carboxy-PTIO and NO donors on content of IRS-1 in rat hepatocytes and skeletal myocytes. Rat hepatocytes (a) or skeletal myocytes (b) were treated with the NO-scavenger, carboxy-PTIO (0.1 μ M) with the indicated concentrations of GSNO or SNAP in the presence the indicated concentrations of insulin, with or without 25 mg/ml glucose (Glu). Values are expressed as means $\pm$ SEM; * p < 0.05 vs the insulin-stimulated control (100 nM insulin).

of the NO donors. Further, these results suggest that the effects of NO inhibition might be reversible if treated early, and before the pathologic sequelae associated with diabetes is evidenced.

Insulin action is initiated through its binding to the cell-surface receptor, initiating a series of signal transduction reactions, which stimulate various effectors to produce its physiological effects. Therefore, impairment of insulin signal transduction results in attenuation of insulin action and leads to insulin resistance resulting in type 2 diabetes mellitus. Because the molecular mechanisms of insulin resistance are still obscure, it is

indispensable to establish in vitro models of basal and insulin-mediated signal transduction to elucidate these mechanisms and suggest treatments where appropriate.

NO and NO-related compounds can function as both protective and cytotoxic, dependent on the cellular context and the nature of the NO group [17, 18]. The multifaceted actions of the NO group can be classified into true NO-mediated, cGMP-dependent, and reactive nitrogen species-mediated, cGMP-independent actions. The cGMP-dependent actions play critical roles in a variety of physiological processes, including NO-mediated vasodilation. In contrast, cGMP-independent, nitrosative protein post-translational modifications, including protein S-nitrosylation and tyrosine nitration are important mechanisms for regulating signal transduction. These reversible reactions are postulated to be involved in the pathological responses [17, 18]. SNAP and GSNO release NO in a quantitative manner, and in the absence of a quenching agent, will produce quantities of NO which will diffuse across the cell membranes and disrupt the normal cellular processes. Some of the differences observed in this study could be attributable to differences in concentration as well as in potency of the NO-releasing capacity of the drugs. However, it appears that there are some environmental influences which suggest 'tissue-specific' activities, possibly due to the different physiological milieus of the cells (tissues) used in this study.

Important recent findings highlight the involvement of S-nitrosylation (or S-nitrosation) of proteins in vesicle-mediated insulin release [19], or of IRS-1 [20] by NO donors. In the latter study [20], as well as another [21], the authors found that the treatment of cultured myocytes with NO donors induced IRS-1 degradation and consequently reduced protein content in a time- and concentration-dependent manner. However, S-nitrosation was implicated as a precursor to IRS-1 degradation only after chronic exposure to GSNO [20]. In the present study, in the absence of measuring S-nitrosation, we noted that acute treatment of skeletal myocytes and hepatocytes by either SNAP or GSNO resulted in a significantly reduced content of available IRS-1 for participating in insulin-mediated signal transduction. Prior to the publication by Carvalho-Filho et al. [20], the postulated mechanisms for insulin resistance involved either increased phosphotyrosine phosphatase activity or

increased serine phosphorylation of IRS-1 [22, 23], and only increased or preferential serine phosphorylation of IRS-1 had been linked to insulin resistance mediated by IRS-1 degradation [24, 25]. It is well established that changes in the level of phosphorylation at any of the possible sites on IRS-1 could potentially alter its ability of IRS-1 to bind and activate the various downstream effectors in the insulin-mediated signal transduction pathway [4]. It is our view that S-nitrosation is secondary to serine phosphorylation as the means whereby NO mediates insulin resistance in skeletal muscle. This is based on the fact that IRS-1 degradation potentiated by S-nitrosation occurs only after chronic *in vitro* exposure to the NO donor [20], unlike proteasome-mediated degradation subsequent to serine phosphorylation [26]. Whether these NO donors mediate insulin resistance and/or impaired insulin-mediated signal transduction via increased serine and/or decreased tyrosine phosphorylation, or via perturbation of other effectors are being investigated in our laboratory.

While some studies suggest that insulin down-regulates the expression of IRS-1 [27, 28], we found that this was true only for hepatocytes and skeletal myocytes exposed to high (1000 nM) insulin concentration. However, we noted that IRS-1 expression was significantly enhanced in the presence of 100 nM insulin concentrations in both tissues. This is not unexpected as the tissues and cells used in this study were expressing normal amounts of insulin receptors and would naturally have enhancement of IRS-1 expression when exposed to 100 nM insulin in the presence of glucose. In contrast, those authors [27, 28] reported a decrease in IRS-1 expression after chronic (12 h) exposure of CHO cells expressing insulin receptors and IRS-1 due to ubiquitination and degradation of IRS-1. This is also logical as chronic exposure to insulin would mimic insulin resistance, which would lead to internalization of the insulin receptors (a feature seen in acute exposure to high insulin concentrations) and a reduction of available IRS-1 molecules.

Carboxy-PTIO reacts rapidly with NO to yield NO₂ and has been increasingly used as a scavenger to test the importance of NO in various physiological conditions. Our data strongly support this thesis, and these findings have been corroborated with studies involving other NO donors, including diethylamine/NO [29], SIN-1 [30], streptozotocin [31], and NOC7 [32].

In conclusion, it is clear that NO released from the NO donors have a negative effect on IRS-1 expression in rat hepatocytes and skeletal myocytes. This reduction would have a direct effect on the number of IRS-1 molecules available for phosphorylation of tyrosine residues and transduction of the insulin signal. Increasing insulin concentrations in this context did not help but rather further reduced the content. Taken together, these results indicate a direct role of NO in the impairment of insulin-mediated signal transduction in insulin-sensitive tissues, and possibly in the pathogenesis of type 2 diabetes mellitus.

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