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ORIGINAL PAPER

Chronic oxidative stress as a mechanism for glucose toxicity of the beta cell in Type 2 diabetes

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Abstract Type 2 diabetes is characterized by a relentless decline in pancreatic islet beta cell function and worsening hyperglycemia despite optimal medical treatment. Our central hypothesis is that residual hyperglycemia, especially after meals, generates reactive oxygen species (ROS), which in turn causes chronic oxidative stress on the beta cell. This hypothesis is supported by several observations. Exposure of isolated islets to high glucose concentrations induces increases in intracellular peroxide levels. The beta cell has very low intrinsic levels of antioxidant proteins and activities and thus is very vulnerable to ROS. Treatment with antioxidants protects animal models of type 2 diabetes against complete development of phenotypic hyperglycemia. The molecular mechanisms responsible for the glucose toxic effect on beta cell function involves disappearance of two important regulators of insulin promoter activity, PDX-1 and MafA. Antioxidant treatment in vitro prevents disappearance of these two transcription factors and normalizes insulin gene expression. These observations suggest that the ancillary treatment with antioxidants may improve outcomes of standard therapy of type 2 diabetes in humans.

Keywords Oxidative stress · Glucose toxicity · Beta cell

Introduction

Glucose in chronic excess causes toxic effects on cells, tissues and organs: a phenomenon termed glucose toxicity. These structural and functional abnormalities are well documented in vascular, retinal, renal, and neural tissue. Considerably less is known about the fate of islet tissue.

Multiple biochemical pathways and mechanisms of action for glucose toxicity have been suggested. These include glucose autoxidation, protein kinase C activation, methylglyoxal formation and glycation, hexosamine metabolism, sorbitol formation, and oxidative phosphorylation [1]. Reactive oxygen species (ROS) are formed by all of these pathways (Fig. 1). In physiologic concentrations, ROS help maintain homeostasis. However, when ROS accumulate in excess levels for prolonged period of time, they cause chronic oxidative stress and adverse effects. This is particularly relevant and dangerous for the islet, which is ranked among those tissues with the lowest levels of intrinsic antioxidant defenses [2, 3].

Molecular mechanisms of glucose toxicity and defective insulin gene expression

We have reported that important aspects of the relentless decline in beta cell function found in chronic glucotoxic states include molecular abnormalities in insulin gene expression, decrease in insulin content, and defective glucose-induced insulin secretion [1, 4]. The molecular defects involve the loss of two critical proteins that activate the insulin promoter (Fig. 2). One is PDX-1 [5, 6] and the other is RIPE-3b1 activator [7, 8], recently identified as MafA [9, 10].

Both Western analysis and Northern analysis demonstrated absence of PDX-1 protein and mRNA, respectively, in HIT-T15 cells chronically cultured in supraphysiologic glucose concentrations [5, 6]. We found that disappearance of PDX-1 mRNA was preventable if a physiologic rather

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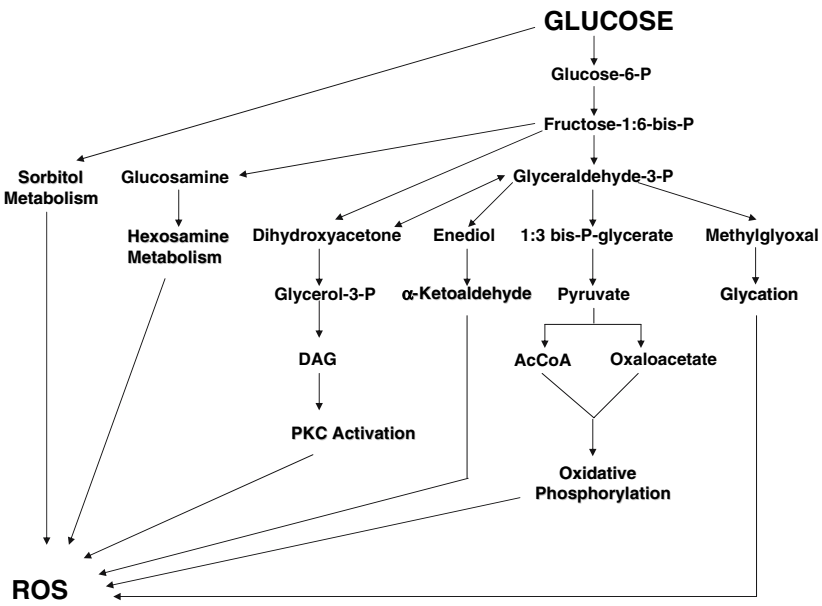

Fig. 1 Pathways through which glucose can form ROS. In normal concentrations glucose forms ROS via oxidative phosphorylation in physiologic concentrations which support normal functions, such as gene transcription and white blood cell function. However, in excess concentrations, glucose can form abnormally high ROS concentrations, especially if mitochondrial metabolism becomes overwhelmed and glucose is shunted to other pathways in excessive amounts, where it can also form ROS in pathological concentrations. Taken from Ref. [1]

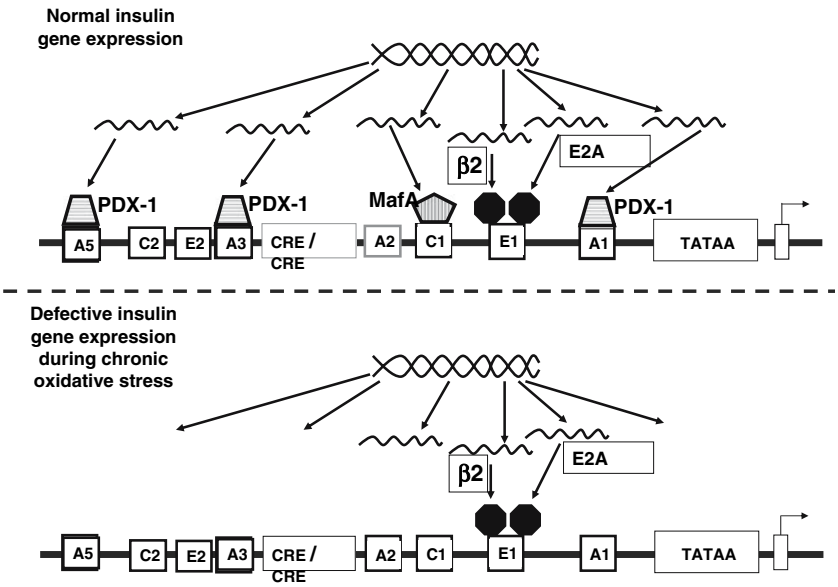

Fig. 2 Molecular mechanisms of defective insulin gene expression in glucotoxic beta cells. Two critical transcription factors, PDX-1 and MafA, are greatly diminished in beta cells chronically exposed to supraphysiologic glucose concentrations. PDX1 protein loss is a post-transcriptional whereas MafA protein loss is post-translational. Taken from Ref. [1]

than a supraphysiologic glucose concentration was used in the cell culture media [4], and that when the glucose concentration was switched from high to low, cells that no longer expressed insulin mRNA recovered this ability [9, 10]. This recovery, however, was limited, depending upon how soon after insulin mRNA disappearance in the glucose concentration in the media was decreased as well as the level of the original glucose concentration. PDX-1 transcription, measured by nuclear run-ons, was normal in cells chronically exposed to supraphysiologic glucose concentrations [6] and transient transfection of late passage glucotoxic HIT-T15 cells with PDX-1 cDNA demonstrated partially reconstituted insulin promoter activity [11]. We concluded that loss of PDX-1 protein in glucotoxic HIT-T15 cells was post-transcriptional.

We more recently published studies focusing on the mechanism of disappearance of MafA [12]. Northern analysis of HIT-T15 cells cultured chronically in a high glucose concentration revealed normal amounts of MafA mRNA, but Western analysis demonstrated a marked reduction in MafA protein (Fig. 3). We also observed that MafA is ubiquitinated and that exposure to lactacystin, an irreversible proteasome inhibitor, resulted in accumulation of cytosolic MafA protein. These findings suggest that the proteasome is a potential site for accelerated MafA degradation when cells are exposed to high glucose

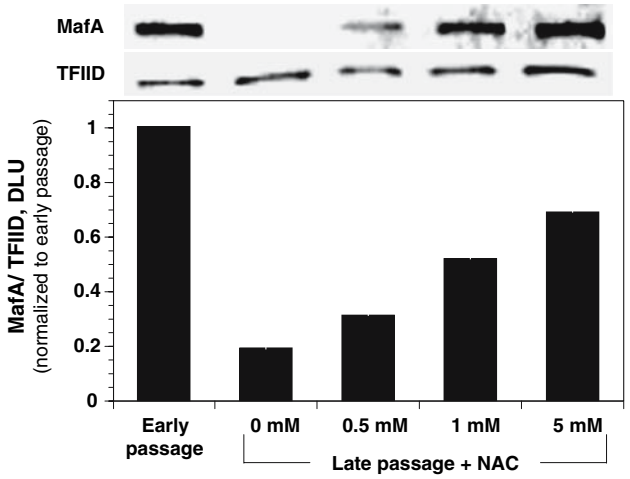

Fig. 3 Loss of MafA protein in HIT-T15 cells after prolonged culture in 11.1 mM glucose and prevention of this loss by including the antioxidant NAC in the culture media. Taken from Ref. [12]

concentrations. Combined transient transfection of PDX-1 and MafA cDNAs into late passage cells grown in media containing high glucose concentrations led to increased PDX-1 and MafA protein levels and fully restored insulin promoter activity [12] (Fig. 4). Since recovery of promoter activity with transient transfection was not accompanied by recovery of endogenous insulin mRNA, we turned to adenoviral infection of MafA and PDX-1 and observed 93% reconstitution of endogenous insulin mRNA in glucotoxic cells. We concluded that disappearance of MafA protein from beta cells via chronic oxidative stress contributes importantly to the progressive loss of endogenous insulin gene expression as glucose toxicity develops and that the mechanism of MafA loss is post-translational.

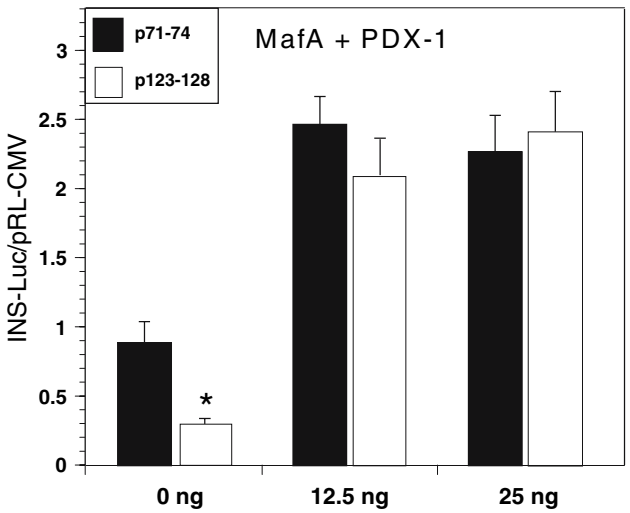

Fig. 4 Restoration of insulin promoter activity in glucotoxic HIT-T15 cells by transient transfection of PDX-1 and MafA cDNAs. Taken from Ref. [12]

Since excessive glucose can generate ROS, we examined the effects of n-acetylcysteine (NAC), a potent antioxidant, on protein expression of the two transcription factors. The disappearance of PDX-1 mRNA and PDX-1 binding in nuclear extracts of HIT-T15 cells chronically cultured in high glucose was completely prevented by including 5 mM NAC in the culture media [13, 14]. Similarly, NAC prevented loss of MafA protein and MafA binding to the insulin promoter in these cells [12] (Fig. 3). These findings reinforced our hypothesis that the generation of reactive oxygen species and attendant oxidative stress is a major mechanism for glucose toxicity of the beta cell.

To test this hypothesis in vivo, we evaluated the Zucker Diabetic Fatty (ZDF) rat, a model of type 2 diabetes. We found that the development of hyperglycemia in this animal is accompanied by loss of insulin gene expression and islet PDX-1 mRNA [13]. Others demonstrated a similar phenomenon in another model of type 2 diabetes, the *db/db* mouse [14]. Consequently, we treated ZDF animals with two antioxidants, NAC and aminoguanidine. Both drugs partially prevented the loss of insulin mRNA and lessened the degree of hyperglycemia found in the animals [13]. This result was consistent with the hypothesis that the onset of type 2 diabetes is genetically determined, but the worsening of diabetes was in large part secondary to hyperglycemia-induced generation of ROS, which subsequently further damages beta cells.

Prolonged exposure to high glucose concentrations has also been reported to upregulate the levels of transcription factor C/EBPβ, a repressor of insulin promoter activity [15]. Decreases in PDX-1 binding to the insulin promoter caused by oxidative stress were reported to be preceded by activation of the mitogen activated protein kinase JNK, and DN-JNK overexpression preserved insulin gene expression under hyperglycemic conditions [16].

Potential glucose- and lipid-related mediators of oxidative stress

D-glyceraldehyde (D-Glyc), a metabolite of glucose, is traditionally considered to be a stimulator of insulin secretion. However, we observed time- and concentration-related biphasic effects [17]. We considered that D-Glyc might also form ROS (Fig. 1) and thereby inhibit beta cell function. Examination of time- and concentration-dependent curves revealed that a 24-h exposure to 2 mM D-Glyc increased intracellular peroxide levels, decreased insulin content, and inhibited glucose-stimulated insulin secretion. NAC prevented these changes. Koningic acid, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase and D-Glyc metabolism, increased intracellular peroxide levels in the

presence of a high glucose concentration (but not in the presence of a low glucose concentration), and also inhibited glucose-induced insulin secretion. To determine whether oxidative phosphorylation was the source of reactive oxygen species formation, we cultured rat islets with mitochondrial inhibitors. We determined that neither mitochondrial inhibitors (rotenone, myxothiazol) nor adenoviral overexpression of manganese superoxide dismutase prevented the increase in reactive oxygen species caused by glyceraldeyde. These observations [17] indicate that exposure to excess D-Glyc concentrations, which are caused by a superabundance of glucose in the beta cell environment [18], can be a mechanism for glucose toxicity via oxidative stress, and that a non-mitochondrial pathway for formation of reactive oxygen species is involved.

Since the ZDF rat is both hyperglycemic and hyperlipemic, lipotoxicity is another potential cause of progressively deteriorating beta cell function. Consequently, we performed experiments to differentiate the adverse effects of hyperglycemia versus hyperlipemia. ZDF rats treated with either bezafibrate, which lowers lipid levels but not glucose levels, or with phlorizin, which lowers plasma glucose levels but not lipid levels. We observed specifically that lowering blood glucose levels while not altering blood lipid levels, with phlorizin protected the ZDF animals from developing elevated islet triglyceride levels and preserved insulin gene expression [19]. On the other hand, lowering blood lipid levels but not glucose levels with bezafibrate failed to prevent increased islet triglyceride and defective insulin gene expression. We concluded that hyperglycemia has toxic effects on beta cell function that do not depend upon high lipid levels, whereas lipotoxic effects on beta cell function depend on coexistant hyperglycemia. Similar observations have been reported from studies in vitro comparing the consequences of culturing islets in palmitate and glucose alone and in combination.

Interleukin-1β is another candidate molecule for causing oxidative stress during hyperglycemia. This cytokine has long been considered to be operative in the pathogenesis of type 1 diabetes. More recently, studies have indicated that pancreatic islets produce interleukin-1β in response to glucose; thereby raising the possibility that chronic exposure to high glucose concentrations may lead to chronic overproduction of interleukin-1β as part of an inflammatory response [20]. Our previous work using specific cyclooxygenase-2 (COX-2) inhibitors suggested functional interrelationships between inhibition of insulin secretion by interleukin-1β and endogenous synthesis of prostaglandin E2 [21]. We also showed that sodium salicylate, an inhibitor of COX, prevented interleukin-1β from inducing EP3 receptor mRNA and also prevented IL-1β-induced COX-2 gene expression. Sodium salicylate also inhibited NF-κB activation and prevented interleukin-1β from inhibiting glucose-induced insulin secretion. These findings suggest that mechanisms of action by which sodium salycilate inhibits the negative effects of interleukin-1β on beta cell function include inhibition of EP3 gene expression, NFκB activation, and COX-2 activation [22].

Antioxidant protection of the beta cell from the adverse effects of glucose toxicity

Over time high glucose concentrations increase intracellular peroxide levels in islets raises the issue of intrinsic antioxidant host defenses within the islet and whether augmentation of these defenses might be an appropriate therapeutic strategy to lessen the impact of diabetes and hyperglycemia on the beta cell. This possibility is supported by the observation that antioxidant drugs protect ZDF rats [13] and *db/db* mice [14] from deterioration of beta cell function.

Clinical reports of elevated levels of oxidative stress markers in patients with type 2 diabetes are numerous [23–28]. It has also been reported that the islet is among the least well-endowed tissues in terms of intrinsic antioxidant enzyme expression and activities, including superoxide dismutases (SOD-1, SOD-2), catalase, and glutathione peroxidase (GPx) [2, 3]. In contrast, we reported that gene expression of the catalytic subunit of γ-glutamylcysteine ligase (GCLC), the rate-limiting enzyme for glutathione (GSH) synthesis, is well expressed in islets [29]. In our laboratory the levels of GCLC mRNA are comparable to those found in liver and greater than those found in muscle, lung and fat. Importantly, however, long-term exposure to high glucose conditions decreases GCLC expression in mesangial as well as retinal cells and this is associated with a decrease in GSH levels [30, 31]. Thus, type 2 diabetes mellitus and hyperglycemia are associated with elevated markers of chronic oxidative stress; pancreatic islets contain relatively low levels of antioxidant gene expression; and elevated glucose levels down-regulate the rate-limiting enzyme for GSH synthesis. Injections of superoxide dismutase have been reported to act prophylactically against alloxan-induced diabetes [32].

Transgenic animals overexpressing superoxide dismutase have enhanced beta cell tolerance to oxidative stress-induced diabetes [33] and transgenic mice overexpressing GPx have increased protection against oxidants as well [34]. Overexpression of antioxidant enzymes in beta cell lines provides protection against oxidants and combinatorial rather than single overexpression of antioxidant enzymes is more efficacious [35]. Transgenic overexpression of catalase provided protection for the beta cell against streptozotocin and hydrogen peroxide [36]. Adenoviral

overexpression of catalase and superoxide dismutase have been shown to protect human islets [37, 38] and a beta cell line [39] against oxidative stress.

We examined adenoviral overexpression of glutathione peroxidase (GPx) and gamma-glutamylcysteine ligase (GCLC) as strategies to increase islet resistance to oxidant stress [29, 40]. We observed that high glucose concentrations increased intracellular peroxide levels in human islets and in a beta cell line [40]. GCLC primarily regulates the synthesis of glutathione (GSH) and is central to the antioxidant capacity of the cell. We observed that inhibition of GCLC activity and GSH production by buthionine sulfoximine (BSO) abrogated the protective effect of NAC against the augmented increases in islet peroxide and decreases in insulin mRNA levels, content, and secretion induced by ribose in islets [40]. Adenoviral overexpression of GPx increased GPx activity 6-fold in islets and protected against the adverse effects of ribose on insulin gene expression, insulin content, and glucose-induced insulin secretion [40] (Fig. 5). These results demonstrated that glucose and ribose increase human and rat islet peroxide accumulation, respectively; that protection by NAC against the adverse consequences of ribose-induced oxidative stress on insulin gene expression and insulin secretion can be worsened by a GSH synthesis inhibitor; and that the adverse effects of ribose can be prevented by increasing islet GPx activity.

In other studies we examined whether overexpression of GCLC would protect pancreatic islets against oxidative stress. Our experiments ascertained whether GCLC is upregulated by interleukin-1β. Interleukin-1β upregulated GCLC expression via the p38 form of MAP kinase and NFκB and also increased reactive oxygen species levels [29]. This was accompanied by an increase in islet GSH/GSSG ratio. Adenoviral overexpression of GCLC prevented the adverse effects of IL-1β on glucose-induced insulin secretion and this was accompanied by increased intraislet GSH levels.

It is notable that the degree of protection of the islet we observed was more complete in the GPx adenoviral overexpression studies when compared with our GCLC overexpression studies. This makes an intuitive sense because, although the islet has a very low concentration of antioxidant enzymes, islet GCLC and GSH levels are closer to that of other tissues. Because of these observations we concluded that in vivo overexpression of GPx is the more attractive approach to transgenic mice studies. In this context it also is notable that GPx catabolizes both hydrogen peroxide and lipid peroxide whereas catalase overexpression would only provide protection effects against hydrogen peroxide.

In our earlier studies of NAC in ZDF rats, we began the drug at 6 weeks of age. The degree of hyperglycemia developed in the NAC-treated animals was only 26% of that developed in placebo-treated rats [13]. Recently, we have started treatment beginning at 7, 9, and 11 weeks of age in the same animal model to address the issue of intervention as opposed to prevention. The animals are normoglycemic at 6 weeks of age. We observed that even when therapy was begun as early as 7 weeks of age, a dramatic fall off of the beneficial effects of NAC occurred (Fig. 6). This suggests a very early window of time in which antioxidant treatment must be started to have a substantial preventive effect on the development of

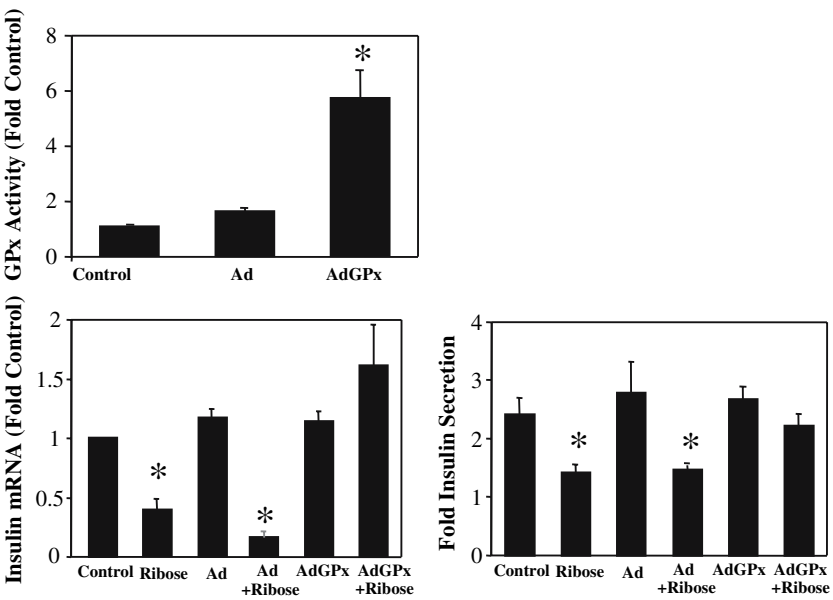

Fig. 5 Six-fold adenoviral overexpression of glutathione peroxidase in isolated pancreatic islets with resultant protection against ribose-induced oxidative stress and prevention of decreased insulin gene expression and defective beta cell function. Taken from Ref. [27]

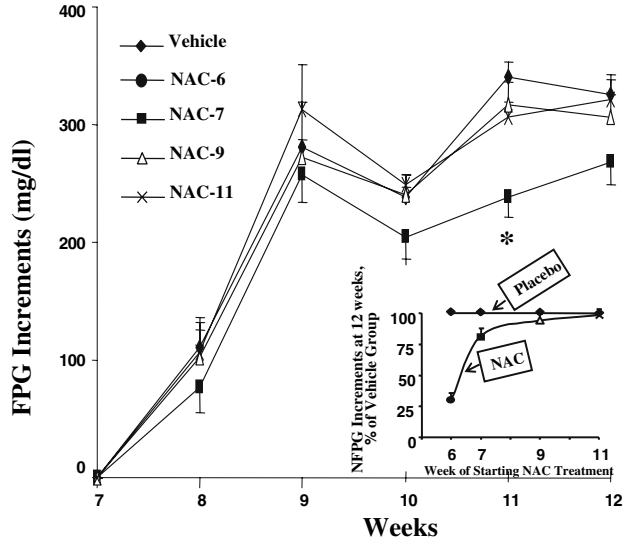

Fig. 6 Protection against full development of hyperglycemia by in vivo NAC treatment of ZDF rats. Starting treatment at 6 weeks of age diminished the level of hyperglycemia by 74%, whereas delaying treating by as little as 1 week markedly compromised this beneficial effect

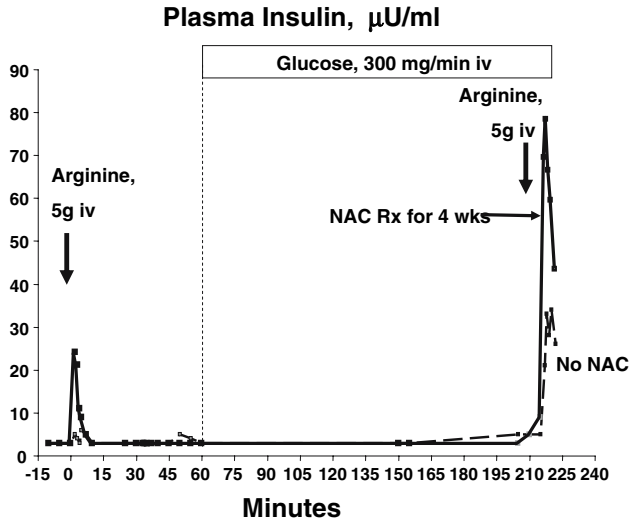

Fig. 7 Augmentation of glucose potentiation of arginine-induced insulin secretion in a type 2 hyperglycemic diabetic subject. This measure of insulin secretory reserve was increased after 28 days of NAC treatment compared to the control period in which no antioxidants were given. No changes in the subject's usual anti-diabetic medications were made

hyperglycemia in a ZDF rat. This finding is consistent with our work with HIT-T15 cells in which a similar phenomenon was demonstrated. Cells were cultured in progressively greater glucose concentrations for progressively greater periods of time [10]. Intervention by decreasing the high glucose concentrations to a low glucose concentration at earlier time points resulted in a return of normal beta cell function (insulin mRNA levels, insulin content, and glucose-induced insulin secretion). However, the success in recovering function was inversely related both to the height of the initial glucose concentration and to the amount of time that had elapsed prior to intervention with the lowest glucose concentration. Thus, in vivo and in vitro, the likelihood that glucotoxic effects on beta cells can be reversed seems to be increased if the interventional step is taken early in the course of deterioration in beta cell function.

We have completed preliminary studies in four subjects with type 2 diabetes mellitus for >5 years. These subjects had hemoglobin A1c levels of 6.5–8% and were being treated with oral hypoglycemic agents and insulin sensitizers. A double blinded, crossover design was used in which NAC (450 mg) or placebo was given twice a day for 28 consecutive days. A 2 week washout period was used between the two treatment periods. After each treatment, insulin secretory reserve was determined using the method of glucose potentiation of arginine-induced insulin secretion. Four of four subjects studied had improved acute arginine-induced insulin responses (AIRa) after treatment with NAC compared to no treatment (increase after NAC over control = 15, 23, 9, and 13 μU/ml; an example is shown in Fig. 7). Insulin secretory reserve (AIRaMAX) was improved in three of three patients studied (increase after NAC over control = 35, 64, and 45 μU/ml). The fourth patient did not complete the AIRaMAX study. These data are supportive of our overall hypothesis that interference with oxidative stress will result in improved beta cell function in patients with type 2 diabetes mellitus. Currently, we are studying patients over a greater period of time with emphasis on stratifying them with regards to duration of diabetes and glycemic control.

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