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Alteration of β-cell constitutive NO synthase activity is involved in the abnormal insulin response to arginine in a new rat model of type 2 diabetes

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Abstract

We have previously obtained a new type 2 diabetic syndrome in adult rats given streptozotocin and nicotinamide, characterized by reduced β-cell mass, partially preserved insulin response to glucose and tolbutamide and excessive responsiveness to arginine. We have also established that the neuronal isoform of constitutive NO synthase (nNOS) is expressed in β-cells and modulates insulin secretion. In this study, we explored the kinetics of glucose- and arginine-stimulated insulin release in perifused isolated islets as well as the effect of *N*-ω-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, to get insight into the possible mechanisms responsible for the arginine hypersensitivity observed in vitro in this and other models of type 2 diabetes. A reduced first phase and a blunted second phase of insulin secretion were observed upon glucose stimulation of diabetic islets, confirming previous data in the isolated perfused rat pancreas. Exposure of diabetic islets to 10 mM arginine, in the presence of 2.8 mM glucose, elicited a remarkable monophasic increment in insulin release, which peaked at 639 ± 31 pg/islet/min as compared to 49 ± 18 pg/islet/min in control islets ($P \ll 0.01$). The addition of L-NAME to control islets markedly enhanced the insulin response to arginine, as expected from the documented inhibitory effect exerted by nNOS activity in normal β-cells, whereas it did not further modify the insulin secretion in diabetic islets, thus implying the occurrence of a defective nNOS activity in these islets. A reduced expression of nNOS mRNA was found in the majority but not in all diabetic islet preparations and therefore cannot totally account for the absence of L-NAME effect, that might also be ascribed to post-transcriptional mechanisms impairing nNOS catalytic activity. In conclusion, our results provide for the first time evidence that functional abnormalities of type 2 experimental diabetes, such as the insulin hyper-responsiveness to arginine, could be due to an impairment of nNOS expression and/or activity in β-cells.

Keywords: Experimental diabetes; Constitutive nitric oxide synthase; Isolated islets; Insulin secretion; Arginine

1. Introduction

We have previously developed a new experimental model of type 2 diabetes in adult rats (Masiello et al., 1998), which is obtained by the combined injection of streptozotocin (STZ) and a partially protective dose of nicotinamide (NA). This model is characterized by a 40% reduction in β-cell mass (Novelli et al., 2001), which results into a moderate and stable hyperglycemia, glucose intolerance, altered but still present ability of β-cells to respond to glucose and preserved responsiveness to tolbutamide (Masiello et al., 1998), and thus shares a number of similarities with human type 2 diabetes. Actually, the insulin responsiveness to glucose and sulfonylureas, that is not present in other established models of type 2 diabetes, such as neonatally streptozotocin-induced diabetic rats (nSTZ), GK rats and partially pancreatectomized rats (Weir et al., 1981; Giroix et al., 1983; Portha et al., 1991), makes this novel diabetic syndrome particularly suitable for both biochemical and pharmacological studies aimed at assessing the effectiveness

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of new potential anti-diabetic compounds and their mechanisms of action. Indeed, streptozotocin-nicotinamide (STZ-NA) diabetic rats are being increasingly utilized in pharmacological research (e.g. Broca et al., 1999; Kuntz et al., 2002). It should be noted that, as in the other above mentioned animal models but unlike in human type 2 diabetes, STZ-NA-induced diabetic syndrome is associated with neither overweight (the weight gain of diabetic rats is normal), nor insulin resistance, at least in the first weeks. Insulin resistance appears to develop later: we have evidence of reduced insulin sensitivity in the adipose tissue 2 months after diabetes induction (Fierabracci et al., 2002), but this issue needs further investigation. With regard to circulating lipid values in STZ-NA rats, plasma triglyceride levels are stably elevated by 40%, cholesterol is unchanged and free fatty acids show a slight trend to increase (unpublished data).

Another interesting feature of the STZ-NA model is a striking monophasic insulin secretion elicited by arginine in the presence of non-stimulating glucose concentrations, as assessed in the isolated perfused pancreas (Masiello et al., 1998). A similar hyper-responsiveness to arginine has also been observed in several other diabetic models, including nSTZ rats (Giroix et al., 1983; Leahy et al., 1984), GK rats (Portha et al., 1991), partially pancreatectomized rats (Rossetti et al., 1987), glucose-infused rats (Leahy et al., 1987), and SHR/N-cp rats (Voyles et al., 1988). The reason for such an exaggerated arginine effect, shared by different experimental diabetic syndromes, remains unclear. In healthy β-cells, arginine is considered as a potentiator of glucose-stimulated insulin secretion, mainly acting through its electrogenic properties leading to cell membrane depolarization. However, in the past few years, evidences have accumulated suggesting that arginine could also influence insulin secretion in a more complex fashion, by exerting a metabolic action based on its monooxygenation by a constitutive nitric oxide synthase to yield NO and citrulline. Indeed, a constitutive neuronal isoform of NOS (nNOS) has been recently characterized in rat islets and in β-cell lines (Lajoix et al., 2001) and shown to be implicated in an inhibitory modulation of insulin secretion in experiments mainly based on the use of pharmacological NOS inhibitors, such as *N*-ω-nitro-L-arginine methyl ester (L-NAME) (Panagiotidis et al., 1995; Gross et al., 1997; Salehi et al., 1998; Lajoix et al., 2001). In particular, it was observed that L-NAME markedly enhanced arginine-induced insulin release at basal glucose concentrations (Panagiotidis et al., 1995; Gross et al., 1997). The analogy of this pattern of insulin secretion with that occurring in the perfused pancreas of STZ-NA rats in the presence of arginine alone, prompted us to hypothesize that an alteration of nNOS activity could be present in experimental diabetes and account for β-cell hypersensitivity to the amino acid.

On the basis of such considerations, the present study was designed (a) to investigate the kinetics of glucose-stimulated insulin release in perifused isolated islets of STZ-NA diabetic rats (not yet documented); (b) to address nNOS functional activity by testing the effect of a pharmacological blockade of the enzyme on arginine-induced insulin secretion; (c) to evaluate the possible alteration of nNOS expression in diabetic islets, to thereby get insight into the mechanisms responsible for the arginine hypersensitivity in experimental diabetes.

2. Materials and methods

2.1. Animals

Male Wistar rats of 2–3 months of age were administered intraperitoneally 270 mg/kg of nicotinamide (Sigma, St. Louis, MO, USA) dissolved in saline, 15 min before an intravenous injection of 60 mg/kg STZ (Sigma), dissolved in citrate buffer (pH 4.5) immediately before use. Each group of treated animals was paralleled by a group of controls receiving the vehicles of both substances. Animals were used for the experiments 5–8 weeks after diabetes was induced. Pancreatic islets were isolated by the collagenase method using the procedure of pancreatic duct cannulation and density gradient purification (Malaisse-Lagae and Malaisse, 1984). After appropriate washing, the islets were immediately used for perifusion experiments.

2.2. Perifusion experiments

The kinetics of insulin release in vitro was studied using a perifusion apparatus. Batches of 60 islets were housed in the bottom of small chambers (subsequently sealed with top adaptors) and perifused at 37 °C in Krebs–Ringer–bicarbonate (KRB)/HEPES buffer (pH 7.4) containing 0.5% bovine serum albumin, at a flow rate of 0.6 ml/min. Islets were exposed to test agents as indicated in the Figures. After a 30-min equilibration period, buffer fractions were collected at 1-min intervals and stored at −20 °C until assayed for insulin by radioimmunoassay.

2.3. RNA isolation and nNOS expression by reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA from islets isolated from either control or diabetic rats was extracted with TRIzol reagent (Life Technologies, Rockville, MD, USA). The integrity of RNA and the absence of contaminating genomic DNA were assessed after migration on agarose gel and ethidium bromide staining. First-strand cDNA was synthesized from approximately 5 μg of total RNA in the presence of both 3 μg of random hexanucleotide primers (Life Technologies) and 1 μg oligo(dT) (Life Technologies) using Superscript II RNase H-Reverse Transcriptase (Life Technologies). PCR was then performed using Taq polymerase (Life Technologies) and the following pairs of primers: 5′-ATGGAAGAGAACACGTTTGGGGTT-3′ and 5′-TTAGC-TTGGGAGACTGAGCCAGCT-3′ for nNOS, and 5′-ATC-

TTTCTGGTGCTTGTCTC-3′ and 5′-AGTGTGAGCCAG-GATGTAG-3′ for the internal control β_2-microglobulin.

2.4. Data presentation and statistical methods

The insulin secretion rate during the perifusion experiments was calculated by multiplying the insulin concentration in the samples by the flow rate, and was expressed as pg/islet/min. Statistical analysis of results was performed using Student's *t* test for unpaired data.

3. Results

3.1. Plasma glucose concentrations

The diabetic rats used in the present study exhibited non-fasting plasma glucose concentrations significantly higher than controls (151 ± 7.5 mg/dl versus 122 ± 3.0 mg/dl; $P < 0.01$), whereas body weights and plasma insulin levels were not different from controls.

3.2. Glucose-stimulated insulin secretion in perifused islets

The insulin secretory responses of perifused control and diabetic islets to 16.7 mM glucose are shown in Fig. 1. Basal insulin release was not significantly different between control and diabetic islets. High glucose elicited a biphasic insulin secretion in control islets with a first phase peaking at 4–5 min (five- to sixfold over basal), and a subsequent second phase (approximately twofold over basal) lasting the whole duration of the stimulus and promptly returning to basal values upon discontinuation of high glucose. The pattern in diabetic islets was considerably different: 16.7 mM glucose stimulated a rapid release of insulin which peaked at 4 min (threefold over basal), returned to basal values and remained constant thereafter, without generation of a significant second phase. When the insulin release was integrated as the area under the curve (AUC), diabetic islets released significantly less insulin than control islets ($P < 0.05$ at least) in both the first and the second phase (see insert to Fig. 1).

3.3. Insulin secretion in response to arginine and L-NAME in perifused islets

Fig. 2, panel A, shows the secretory response of islets perifused in the presence of 2.8 mM glucose and 10 mM arginine. In control islets, arginine provoked a rapid (4–5 min) monophasic insulin release (peak at 49 ± 18 pg/islet/min), smaller in magnitude than that produced by 16.7 mM glucose, which declined sharply to basal values. Conversely, in diabetic islets, arginine induced an abnormally high response, characterized by a striking peak at 4 min (639 ± 31 pg/islet/min) which promptly returned to baseline as in controls.

The effect of addition of L-NAME, a NO synthase inhibitor, to the perifusion buffer 10 min before and during arginine administration, is shown in Fig. 2, panel B. In both control and diabetic islets, L-NAME did not influence the insulin release in the presence of 2.8 mM glucose. When L-NAME was concomitantly present with arginine, it

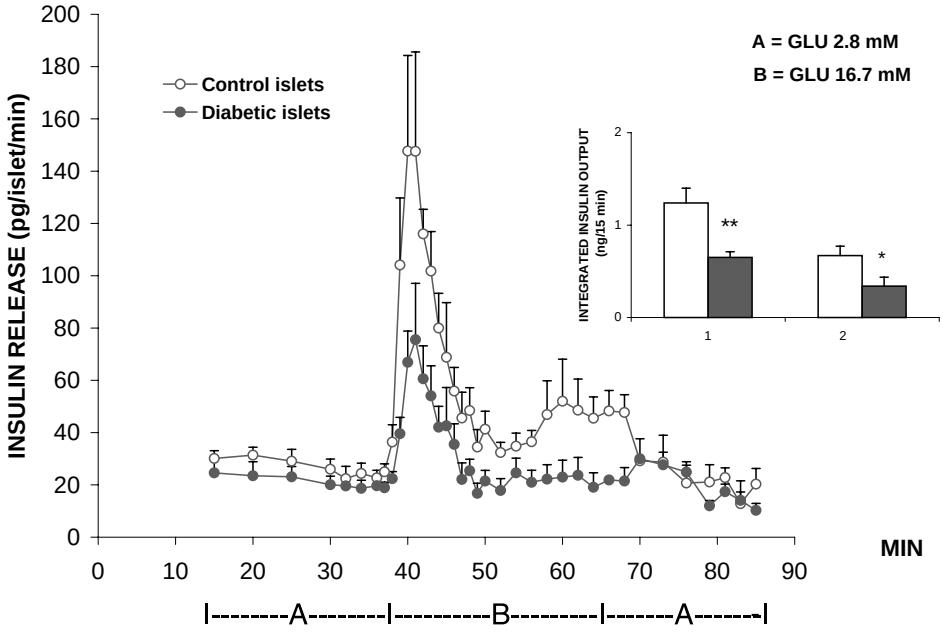

Fig. 1. Glucose-stimulated insulin secretion in perifused isolated islets from control (○) or diabetic (●) rats. Batches of 60 islets were perifused with KRB/HEPES buffer in the presence of 2.8 or 16.7 mM glucose (GLU), as indicated. Data are the means ± S.E.M. of eight perifusions from four separate experiments in each group. The insert shows the integrated insulin output in response to high glucose during two consecutive 15-min periods, in control (□) and diabetic (■) rats.

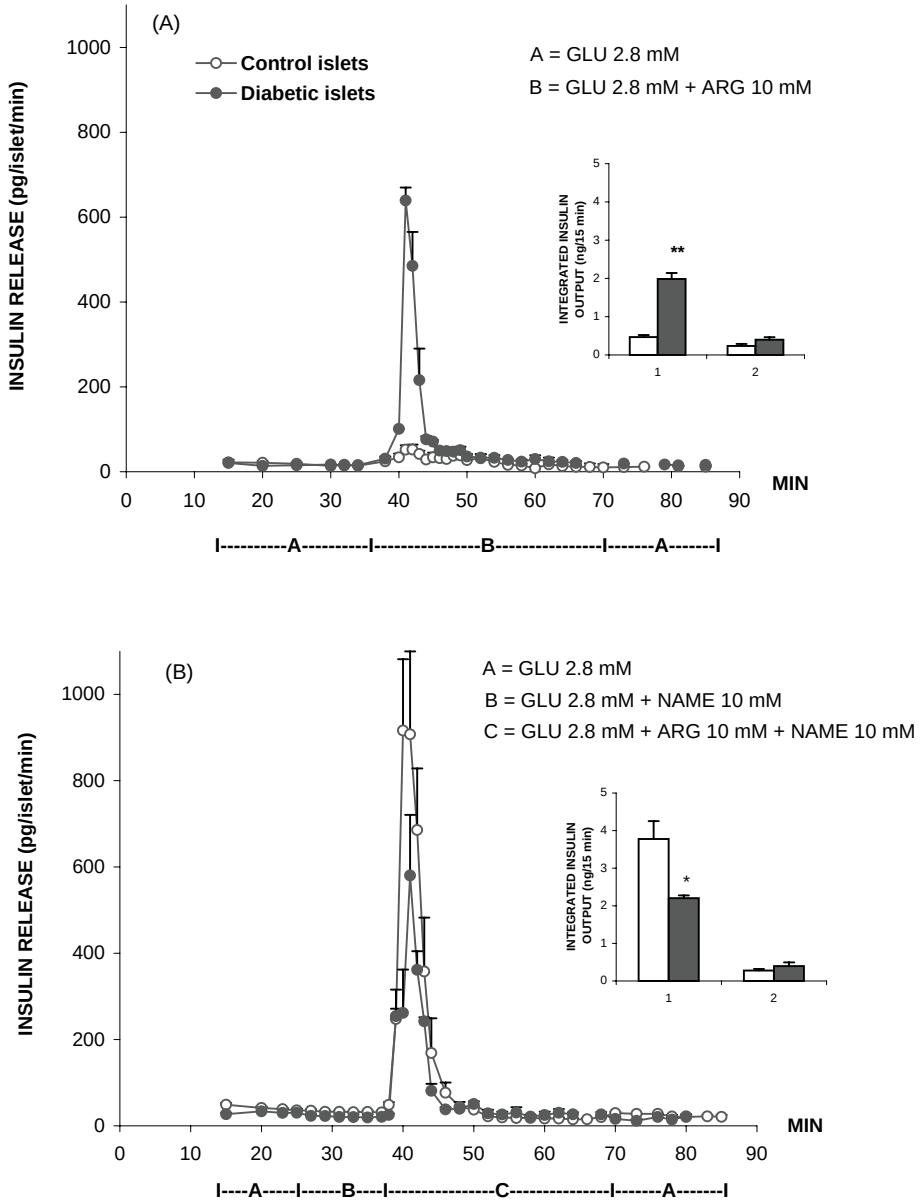

Fig. 2. Insulin secretion in response to arginine alone (panel A) or in combination with L-NAME (panel B) in perifused isolated islets from control (○) or diabetic (●) rats. Batches of 60 islets were perifused with KRB/HEPES buffer in the presence of 2.8 mM glucose (GLU), 10 mM arginine (ARG) and 10 mM L-NAME, as indicated. Data are the means ± S.E.M. of 6 and 8–10 perifusions (from three and four to five separate experiments) for control and STZ-NA rats, respectively. The inserts show the integrated insulin outputs in response to arginine (panel A) or arginine + L-NAME (panel B) during two consecutive 15-min periods, in control (□) and diabetic (■) rats.

increased markedly the monophasic insulin secretion in controls only (eightfold with respect to 10 mM arginine alone). In fact, in diabetic islets, L-NAME did not modify the kinetics of the exaggerated insulin release induced by arginine alone, as it can also be argued from the AUCs reported in the inserts to Fig. 2.

3.4. Expression of nNOS in pancreatic islets from control and diabetic islets

RT–PCR was performed with primers based on the sequence of rat nNOS. As shown in Fig. 3, a single band at the predicted size was obtained in both control and diabetic islet preparations. In control islets, nNOS expression was found fairly homogeneous. In most diabetic islet preparations, nNOS expression was reduced, but showed a certain variability with regard to the extent of reduction. Such variability was independent on plasma glucose levels of diabetic rats. Quantification of band intensities by a computerized program of image processing (SCION image) revealed that the ratio between nNOS and β_2-microglobulin expression was 51.6 ± 15.0 arbitrary units (AU) in diabetic islets versus 100.7 ± 8.3 AU in control islets, being the difference significant ($P < 0.02$, Student's t test).

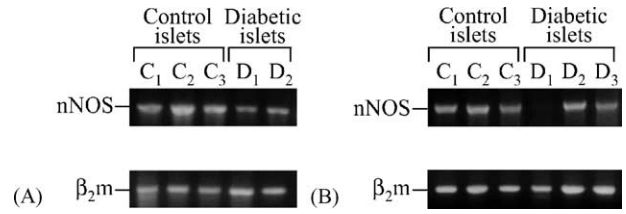

Fig. 3. RT–PCR analysis of nNOS expression from control and diabetic islets issued from two sets of experiments (panels A and B). Total RNA from pancreatic islets was isolated and cDNA was amplified with primers based on the sequence of nNOS and β_2-microglobulin (β_2m), this latter as an internal control. The ratios between nNOS and β_2-microglobulin band intensities, as quantified by SCION image processing, are 51.6 ± 15.0 arbitrary units (AU) in diabetic islets vs. 100.7 ± 8.3 AU in control islets, being the difference significant ($P < 0.02$, Student's t test).

4. Discussion

Our experiments show that the islets of STZ-NA diabetic rats, in contrast to those of nSTZ rats or GK rats which are blind to glucose in vitro (Giroix et al., 1983; Leahy et al., 1984; Portha et al., 1991), are still able to release insulin in response to glucose, even though both the first and especially the second phase of secretion are reduced with respect to control islets. The impairment in glucose-stimulated insulin secretion in perifused islets is quite similar to that observed in the perfused pancreas of STZ-NA diabetic rats (Masiello et al., 1998). It should be mentioned that in human type 2 diabetes the abnormality in insulin secretory kinetics is different, since the first phase is usually more impaired than the second phase (Hosken et al., 1989). The reason for this discrepancy remains unknown, although a possibility could be related to the occurrence of different alterations in the functional pools of secretory granules in β-cells and their regulatory mechanisms (Rorsman and Renstrom, 2003). With regard to the mechanisms of glucose-induced insulin release, there is a general consensus that glucose enters β-cells via facilitated transport involving GLUT-2 transporter, is phosphorylated by glucokinase and subsequently metabolized through glycolytic and mitochondrial oxidative pathways, leading to increased production of ATP at the expense of ADP. This in turn results in closure of ATP-regulated K^+-channels inducing membrane depolarization and opening of voltage-gated Ca^{2+}-channels and subsequent increase in intracellular Ca^{2+} concentrations which, in the presence of other amplification signals, finally trigger exocytotic events, releasing stored insulin from secretory granule pools.

Besides alteration in the kinetics of glucose-stimulated insulin release, another abnormality has been confirmed to occur in this diabetic model, i.e. the hypersecretion of insulin in response to arginine at low glucose concentrations, that is very striking all the more since the β-cell mass of STZ-NA rats is reduced by about 40% versus normal rats (Novelli et al., 2001). The hyper-responsiveness of β-cells to arginine has been documented in several other experimental models of type 2 diabetes, but the mechanisms involved remain elusive. In nSTZ rats, the abnormality was considered dependent on the failure of arginine-stimulated insulin secretion to be turned off by low glucose concentrations (Leahy et al., 1984). In GK rats, chronic exposure of β-cells to high glucose and consequent increased availability of endogenous substrates, such as glycogen, was supposed to be responsible (Portha et al., 1991).

The mechanism of action of arginine as an insulin secretagogue has not been fully elucidated. Besides its electrogenic effect leading to a direct membrane depolarization, arginine may also act through its metabolization by arginase into ornithine, which, as a precursor of glutamate and hence succinylCoA, can be considered an anaplerotic substrate refilling TCA cycle. Actually, arginine is not very effective as an initiator of insulin secretion, but is rather a potentiator of insulin release in the presence of stimulating glucose concentrations. Finally, arginine (at concentrations as low as 20–30 μM) is the substrate of nNOS activity leading to production of NO and citrulline. Although the role of endogenously produced NO (and of exogenous NO donors as well) on β-cell function remains controversial (Salehi et al., 1996; Smukler et al., 2002), consistent evidences coming from two independent groups converge at indicating that nNOS activity negatively regulates insulin secretion in the isolated perfused pancreas (Lajoix et al., 2001; Gross et al., 1995), isolated islets (Panagiotidis et al., 1995) and in cultured β-cells (Beffy et al., 2001).

The present results, based on the use of a pharmacological inhibitor of NO synthase, provide for the first time evidence that in the islets of the STZ-NA diabetic rats, the hyper-responsiveness to arginine could result from the loss of the inhibitory modulation exerted by nNOS activity in normal islets (Lajoix et al., 2001; Panagiotidis et al., 1995; Gross et al., 1997). Indeed, the arginine-induced insulin hypersecretion observed in diabetic islets could be reproduced in normal islets by treatment with the nNOS inhibitor L-NAME, as expected on the basis of the above-mentioned previous reports. Furthermore, exposure of diabetic islets to L-NAME completely failed to modify their response to arginine.

The basal maximal stimulation of arginine-induced insulin secretion in STZ-NA diabetic islets, which remained unchanged also in the presence of a nNOS inhibitor, could result from either a decreased nNOS expression or a reduced activity of the enzyme but also from both these defects. Although reduced in most cases, the discrete nNOS expression still present in diabetic islets and the absence of clear correlation between nNOS mRNA levels and the results obtained after a pharmacological blockade of the enzyme activity strongly suggest that the decreased catalytic activity responsible for β-cell hypersensitivity to arginine is likely to result from nNOS post-transcriptional defects. In this respect, it has already been reported that in STZ diabetic rats a reduced skeletal muscle nNOS activity was associated with unaltered nNOS mRNA expression, suggesting

that post-transcriptional mechanisms were responsible for the enzymatic defect (Perreault et al., 2000).

Taken together, our results, obtained through a pharmacological and molecular approach, support the conclusion that in STZ-NA diabetic animals, the abnormal insulin responsiveness to arginine, which might have a compensatory significance, is likely dependent on post-transcriptional defects in β-cells nNOS, leading to a reduced catalytic activity of the enzyme. Such an alteration might develop in this and other experimental models of type 2 diabetes as a consequence of the diabetic state, by unknown mechanisms which warrant further investigations.

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