

Calmodulin Overexpression Causes Ca^{2+} -Dependent Apoptosis of Pancreatic β Cells, Which Can Be Prevented by Inhibition of Nitric Oxide Synthase

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SUMMARY: We investigated the mechanism of β -cell loss in transgenic mice with elevated levels of β cell calmodulin. The transgenic mice experienced a sudden rise in blood glucose levels between 21 and 28 days of age. This change was associated with development of severe hypoinsulinemia and loss of β cells from the islets. Ultrastructural analysis revealed that compromised granule formation and apoptotic changes in the transgenic β cells preceded the onset of hyperglycemia. Intraperitoneal injection of tolbutamide, an antidiabetic sulfonylurea, decreased blood glucose levels but increased the number of apoptotic β cells. Finally, injection of transgenic mice with N^{ω} -nitro-L-arginine methyl ester, which inhibits nitric oxide synthase activity, prevented hyperglycemia and lessened the changes in number and size of β cells. Because immunofluorescent staining revealed preferential distribution of neural nitric oxide synthase in pancreatic β cells, we speculate that overexpression of calmodulin sensitizes the β cells to Ca^{2+} -dependent activation of neural nitric oxide synthase, which mediates apoptosis. (*Lab Invest* 2002, 82:1229–1239).

Selective overexpression of calmodulin in pancreatic β cells of transgenic mice leads to early-onset nonimmune diabetes (Epstein et al, 1989). The onset of hyperglycemia is accompanied by decreased glucose-mediated insulin secretion caused by a metabolic defect in the β cells (Epstein et al, 1992; Ribar et al, 1995b). This metabolic defect results in compromised ability of glucose to lead to depolarization of the β -cell membrane and, thus, increase the activity of the voltage-dependent Ca^{2+} channels. In perfused islets isolated from neonatal mice, this secretory defect could be overcome by sulfonylureas, which bypass the metabolism of glucose and act directly on the plasma membrane to close ATP-dependent K^{+} channels (Aguilar-Bryan and Bryan, 1999). Closure of these K^{+} channels results in membrane depolarization, opening of voltage-dependent Ca^{2+} channels, and an influx in Ca^{2+} that is required for insulin secretion.

However, the transgenic mice experienced a decrease in the number of β cells as a function of age, which in the most severely affected line of mice reached 90% by 37 days of age (Epstein et al, 1989). The time course and profound nature of the decreased β -cell number suggested that this might be a major factor in the development of diabetes.

Calcium has long been implicated as a mediator of cell death by apoptosis (Nicotera and Orrenius, 1998), and studies have suggested that an increase in Ca^{2+} influx could trigger apoptosis of β cells in culture (Efanova et al, 1998; Wang et al, 1999). Such an increase in calmodulin might be expected to sensitize calmodulin-dependent enzymes to a rise in intracellular Ca^{2+} concentrations. Therefore, we have examined whether the β cells are subject to Ca^{2+} -mediated apoptosis in a line of mice that overexpress calmodulin to a lesser degree than the originally characterized line and are slower to develop the manifestations of diabetes. Indeed, we found that β -cell loss occurs in the prediabetic stage and is exacerbated by administration of the antidiabetic sulfonylurea, tolbutamide. The observation that an inhibitor of nitric oxide synthase (NOS), N^{ω} -nitro-L-arginine methyl ester (L-NAME), prevents β -cell apoptosis implicates this enzyme as a component of a Ca^{2+} /calmodulin-dependent pathway that signals cell death.

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Results

Blood Glucose and Insulin Measurements

As shown in Figure 1a, the transgenic mice were normoglycemic at birth, and no significant differences in the blood glucose levels were found during the first 20 days. However, glucose levels suddenly rose between 21 and 28 days of age and, by the end of 4 weeks after birth, had reached a maximum value of ~ 400 mg/dl. It is interesting to note that after 4 weeks of age, glucose levels of the male transgenic mice were consistently higher than those of the females (~ 350 mg/dl). Hyperglycemia in these transgenic animals continued to be manifest for at least 1 year (data not shown). Figure 1b is a plot of serum insulin levels of transgenic and nontransgenic mice as a function of blood glucose levels. This analysis reveals that hyperglycemia was positively associated with hypoinsulinemia in both male and female transgenic mice.

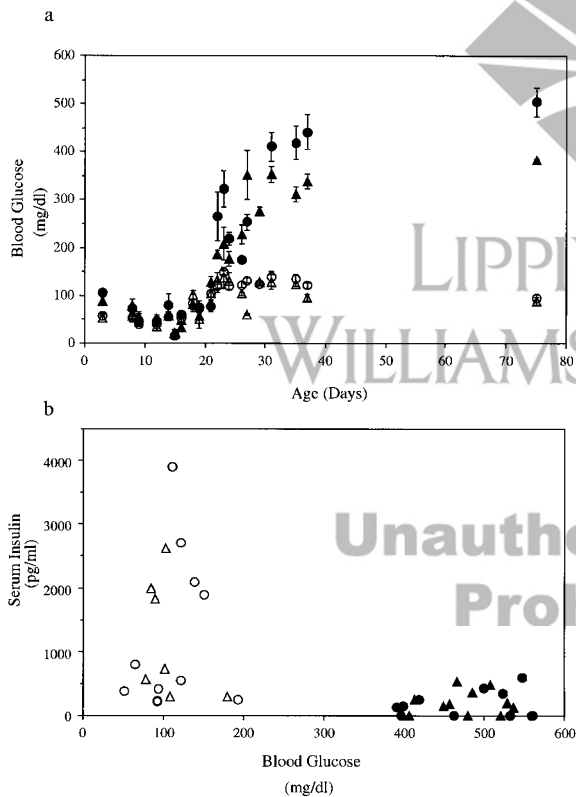


Figure 1.

Blood glucose and serum insulin levels of calmodulin-transgenic and nontransgenic mice. Blood samples were taken from calmodulin-transgenic and nontransgenic mice (3–75 days of age) fed ad libitum by tail cut. Blood glucose and serum insulin levels were measured using a compact glucose analyser and a radioimmunoassay kit, respectively. a, Profiles of blood glucose levels of male transgenic (closed circles), female transgenic (closed triangles), male nontransgenic (open circles), and female nontransgenic (open triangles) mice. Each symbol represents the mean \pm SE values ($n = 3-6$). b, Serum insulin levels plotted against blood glucose. Blood samples were taken from nontransgenic (male, open circles, $n = 11$; and female, open triangles, $n = 7$) and transgenic (male, closed circles, $n = 11$; and female, closed triangles, $n = 11$) mice at 5 to 6 months of age.

Quantification of Calmodulin by Radioimmunoassay

Calmodulin was quantified by radioimmunoassay in extracts of islets isolated from 21-day-old nontransgenic and transgenic mice and found to be ~ 1.3 ng/islet and 2.1 ng/islet, respectively. Thus, the ratio of calmodulin between normal and transgenic mice was 1:1.6 or only a 60% increase in the calmodulin content in transgenic islets. In the original FVBM line of mice, the ratio was $\sim 4:1$ between transgenic and normal islets (Epstein et al, 1992). It seems likely that the low expression of calmodulin in the present line of mice (maintained in the ICR genetic background) is responsible for the delay in development of hyperglycemia relative to the original line of mice (maintained in the CD1 genetic background).

Morphologic Development of Islets of Langerhans in Calmodulin Transgenic and Nontransgenic Mice

Figure 2 demonstrates stereoscopic images of pancreatic tails derived from 6-month-old calmodulin-transgenic and nontransgenic mice. Pancreatic islets can be seen as scattered white round spots in the nontransgenic tails (Fig. 2a), but most of the islets have disappeared from the transgenic mice (Fig. 2b). Figure 3 shows time-dependent changes in the morphologic properties of islets in pancreata from calmodulin-transgenic and nontransgenic mice by hematoxylin-eosin staining. Pancreatic islets in nontransgenic mice increased in size between 1, 3, and 5 weeks after birth (Fig. 3, a to c, and Table 1). The size of individual islet cells also increased in nontransgenic mice. In particular, the cytoplasmic area was considerably enlarged. In contrast, islets in calmodulin-transgenic mice were even bigger than those from

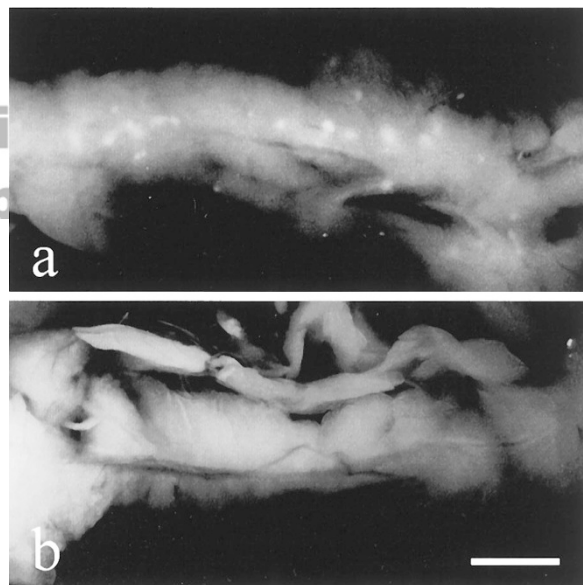


Figure 2.

Stereoscopic images of nontransgenic (a) and calmodulin-transgenic (b) pancreatic tails. Pancreata were removed from 6-month-old nontransgenic and calmodulin-transgenic mice and fixed with 2.5% glutaraldehyde; pancreatic tails were observed under a stereoscopic microscope. Bar = 1 mm.

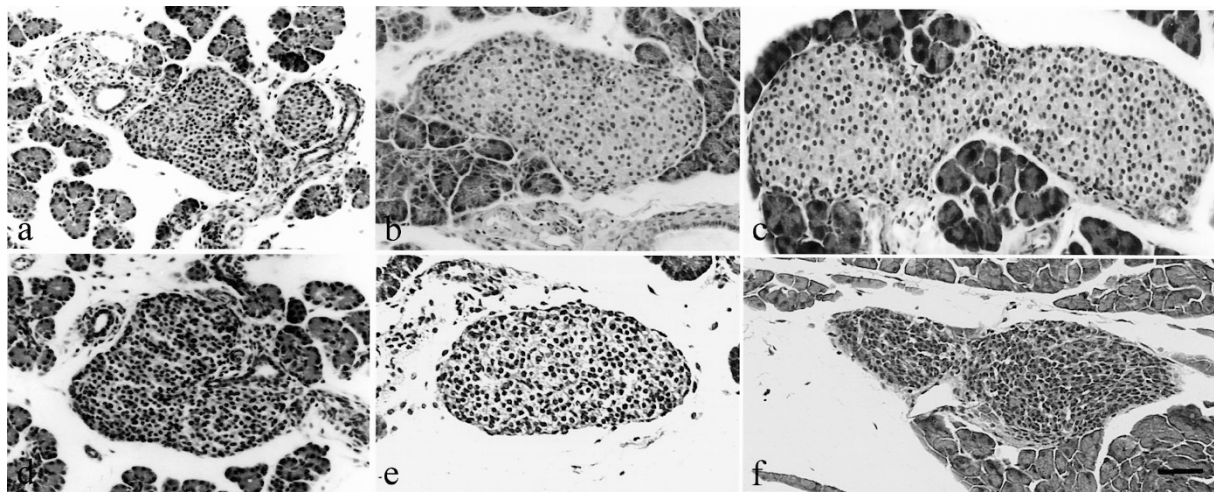


Figure 3.

Hematoxylin and eosin staining of pancreatic islets from nontransgenic (a to c) and transgenic (d to f) mice. Mouse pancreata were fixed with paraformaldehyde (4%) and embedded in paraffin, followed by staining with hematoxylin and eosin. Samples were taken from 1-week-old (a and d), 3-week-old (b and e), and 5-week-old (c and f) mice. Bar = 50 μm .

Table 1. Quantitative Analysis of the Islet Size of Pancreatic Islets from Tg and nT Islets

Age	Islet size (μm^2)	
	nT	Tg
1 Week	7036 \pm 510 (100)	8659 \pm 503 ^a (100)
3 Weeks	9233 \pm 774 (100)	8123 \pm 447 (100)
5 Weeks	19959 \pm 1319 (100)	11905 \pm 716 ^b (100)

Tg, calmodulin-transgenic; nT, nontransgenic. Pancreata from Tg and nT mice were stained by hematoxylin and eosin, and their islet sizes at 1, 3, and 5 weeks of age were quantified using an image analyzer. Data represent the mean \pm SEM values from 100 islets.

^a $p < 0.05$ and ^b $p < 0.001$ as compared with the values from nontransgenic islets at the same age.

nontransgenic mice at 1 week of age. The size of transgenic islets was unchanged between 1 and 3 weeks and was only moderately increased at 5 weeks of age (Fig. 3, d to f, and Table 1). The number of nuclei contained in a given area of a nontransgenic islet was 1.6 times more in 1-week-old mice than in 3- or 5-week-old ones (50 nuclei/cm² in 1-week-old nontransgenic mice and 32 nuclei/cm² in 3- and 5-week-old ones). In contrast, the number of nuclei/cm² remained unchanged in calmodulin transgenic islets from 1 to 5 weeks of age. These findings suggest that hyperglycemia may result from impairment of neonatal islet growth in calmodulin-transgenic mice and/or accelerated loss of islet cells. We have been unable to detect β -cell necrosis or infiltration of lymphocytes (insulinitis) at any age examined (not shown).

Immunofluorescent Microscopy

To examine islet cell distribution in calmodulin-transgenic islets, we performed immunostaining with

anti-islet hormone antibodies (Fig. 4). In nontransgenic mice, the typical arrangement of rodent islet endocrine cells was consistently observed: β cells were situated in the middle, and non- β cells were around the periphery of an islet (Fig. 4, a to c). Calmodulin-transgenic islets looked slightly bigger than nontransgenic ones in 1-week-old mice (Fig. 4d). However, a marked decrease in insulin-positive cells appeared 3 weeks after birth (Fig. 4e). Finally, insulin-positive areas became very sparse by 5 weeks after birth (Fig. 4f).

Electron Microscopic Observations of Pancreatic β Cells and Ultrastructural Apoptotic Changes

Figure 5 depicts ultrastructural images of β cells by transmission electron microscopy. Insulin granules were identified as spherical granules with a high electron-dense core and a surrounding wide halo, 200 to 300 nm in diameter, which was verified by immunoelectron microscopy (not shown). Nontransgenic islets contained numerous insulin granules, some of which were characterized by the presence of crystalline electron-dense material (Fig. 5, a to c). We quantified the number of β cells, measured the size of their nuclei, and counted the insulin granules present in representative β cells (Table 2). The size of nontransgenic β cells in 3- and 5-week-old mice became larger than in 1-week-old ones. In pancreata from 1-week-old mice, we could not detect many ultrastructural differences between nontransgenic (Fig. 5a) and calmodulin-transgenic (Fig. 5d) islets. In contrast, insulin granules dramatically decreased in number in 3-week-old transgenic mice (Fig. 5e). The size of nuclei in nontransgenic β cells remained unchanged throughout this period (Fig. 5b), but significant decreases in nuclear size occurred in calmodulin-transgenic cells (Fig. 5e). Mitochondria, Golgi complexes, and well-developed endoplasmic reticulum were distributed throughout the cytoplasm both in nontransgenic and transgenic β cells. In 1- and

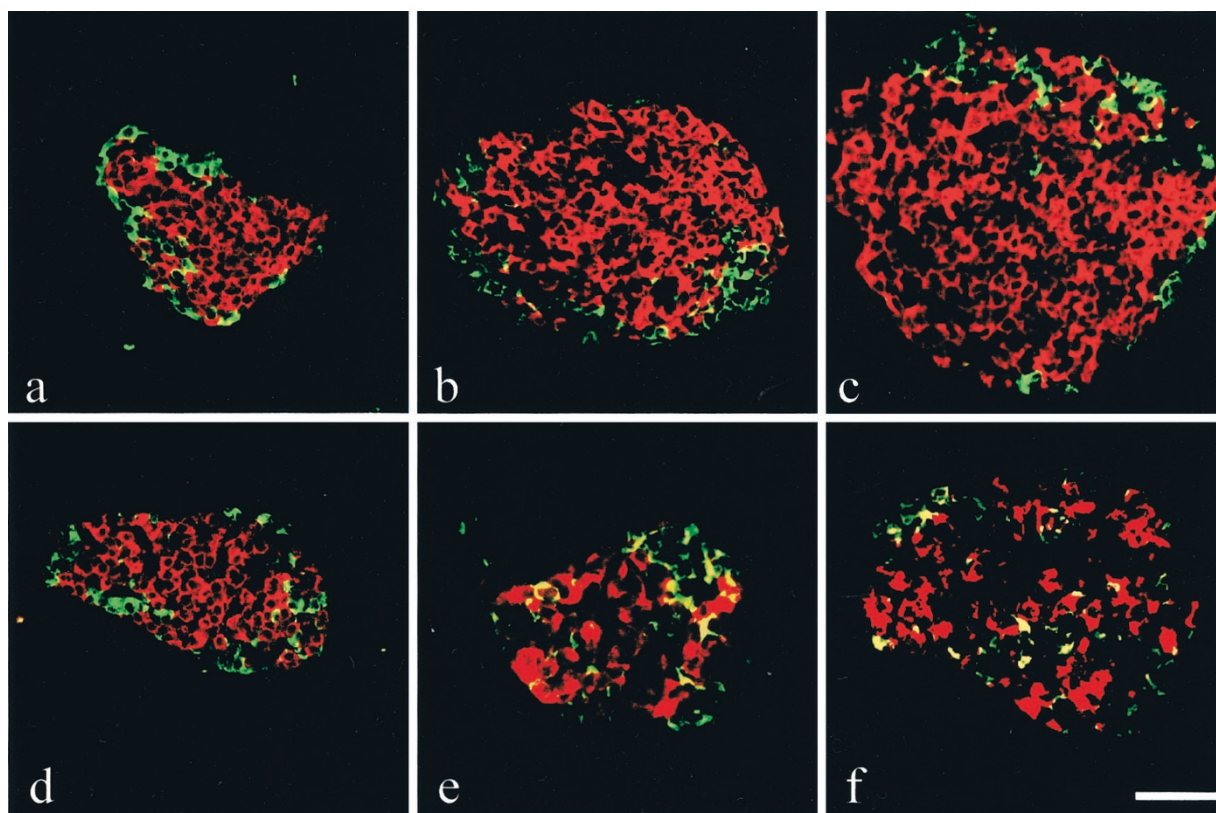


Figure 4.

β -cell distribution in nontransgenic (a to c) and transgenic (d to f) pancreatic islets. Frozen sections of the fixed pancreas were incubated in a mixture of anti-insulin guinea pig antibody and rabbit antibodies against glucagon and somatostatin, followed by incubation in a mixture of rhodamine-conjugated anti-guinea pig IgG antibody and FITC-conjugated anti-rabbit IgG antibody. All sections were analyzed with a confocal laser scanning microscope. Red, insulin; green, glucagon and somatostatin. Samples were taken at 1 week (a and d), 3 weeks (b and e), and 5 weeks (c and f) after birth. Bar = 50 μ m.

3-week-old calmodulin transgenic mice, we also found β cells that exhibited typical apoptotic changes (cell shrinkage, condensed chromatin, and abnormally convoluted nuclear outline), scattered throughout the islet (Fig. 6). β cells with apoptotic changes in the transgenic islet were relatively few in number, possibly because of the swift clearance of the apoptotic cells (Mauricio and Mandrup-Poulsen, 1998). In contrast, α and δ cells in calmodulin transgenic islets remained unaffected (not shown). These findings indicate that hyperglycemia in the calmodulin transgenic mice may be a result of preferential destruction of β cells in pancreatic islets.

Administration of Tolbutamide to Transgenic Mice

As shown in Figure 7a, intraperitoneal injection of an antidiabetic sulfonylurea, tolbutamide (75 μ g/mice daily from 21–26 days of age), did not cause changes in blood glucose levels of nontransgenic mice. On the other hand, in transgenic mice, hypoglycemic effects of tolbutamide were evident at 25 and 26 days of age. The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method was used to determine whether apoptosis of β cells occurred in the transgenic mice as a consequence of tolbutamide treatment. As shown in Figure 7b, a high incidence of apoptosis was evident in tolbutamide-treated transgenic

islets, whereas no apoptotic cell death occurred in the nontransgenic islets (Fig. 7c). Most of the TUNEL-positive cells in the transgenic islets were also positive for anti-insulin antibody (Fig. 7b), suggesting the tolbutamide-induced apoptotic changes take place specifically in pancreatic β cells.

L-NAME Treatment

Inducible NOS (iNOS) has been implicated in mediating the apoptosis of β cells that occurs in type 1 diabetes (Kaneto et al, 1995; Mauricio and Mandrup-Poulsen, 1998). On the other hand, although Ca^{2+} /calmodulin-dependent NOS isoforms such as neural NOS (nNOS) or endothelial NOS have been described, they have not been examined as participants in β -cell apoptosis. To determine whether the apoptotic cell death we observed in the calmodulin-transgenic islets might also involve an NOS isoform, we injected these mice with the NOS inhibitor, L-NAME (0.5 mg/gm body weight/day ip) from 4 to 27 days of age. In 13 of 22 mice, the onset of hyperglycemia between 20 and 27 days of age was completely prevented (Fig. 8a). These differences in blood sugar between the treated mice that responded to L-NAME and those that failed to respond to L-NAME are not a result of toxic effects of the drug that lead to malnutrition because the body weights of all 22 mice were similar both before and after

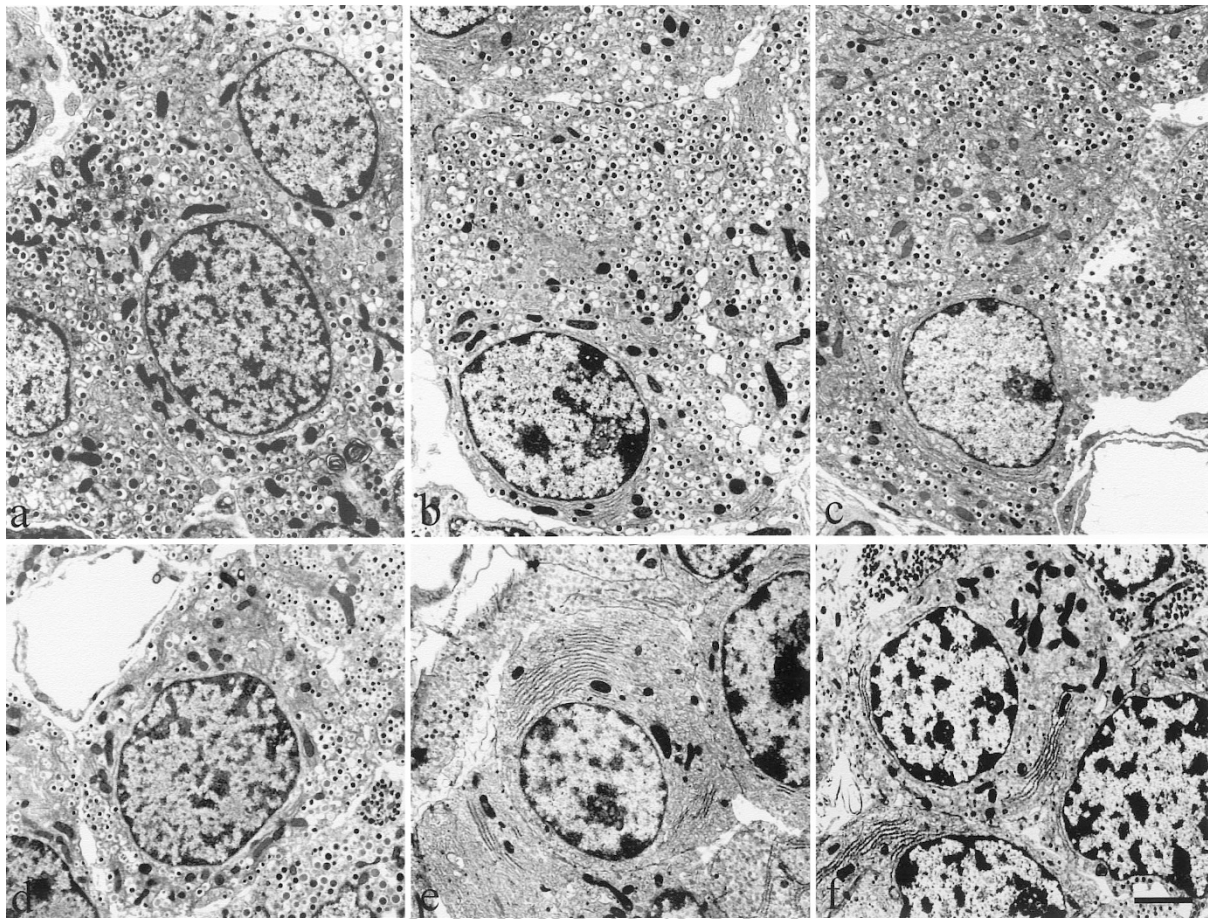


Figure 5.

Electron micrographs of β cells in nontransgenic (a to c) and calmodulin transgenic (d to f) islets. Pancreata of calmodulin transgenic and nontransgenic mice were fixed with 2.5% glutaraldehyde and processed for electron microscopy. Samples were taken at 1 week (a and d), 3 weeks (b and e), and 5 weeks (c and f) after birth. Bar = 2 μ m.

Table 2. Cell Size, Nuclear Size, and Granule Numbers in a β Cell in Tg and nT Islets

Age	Cell size (μm^2)		Nuclear size (μm^2)		Granule number (per β cell)	
	nT	Tg	nT	Tg	nT	Tg
1 Week	113.7 \pm 6.6 (6)	114.4 \pm 6.1 (6)	37.4 \pm 3.0 (6)	38.2 \pm 1.5 (6)	119 \pm 6 (6)	127 \pm 7 (6)
3 Weeks	180.4 \pm 4.8 (6)	102.7 \pm 2.6 (6)	37.0 \pm 1.9 (6)	32.6 \pm 1.6 (6)	229 \pm 22 (6)	42 \pm 8 (6)
5 Weeks	181.8 \pm 9.6 (6)	92.4 \pm 4.4 (6)	35.5 \pm 1.3 (6)	29.9 \pm 2.0 (6)	238 \pm 20 (8)	42 \pm 8 (8)

Pancreatic β cells from Tg and nT mice were observed by electron microscopy, and their size, nuclear size, and numbers of the β granules per cell were assessed as described in "Materials and Methods." Data represent the mean \pm SEM values from *n* individual β cells shown in the parentheses.

L-NAME administration. The reason for the mouse to mouse variability of L-NAME might be because of variability of L-NAME metabolism and/or endogenous NOS inhibitor. The efficacy could not be increased by administering more drug (not shown). The protective effects of L-NAME in responsive mice were profound. Immunostaining with anti-islet hormone antibodies showed maintenance of insulin-positive cells (Fig. 8b). In addition, there was considerable retention of insulin granules in β cells from drug-treated mice as shown by electron microscopy (compare Fig. 8d with Fig. 5c). Finally, as

might be expected from these results, the size of the islets was considerably bigger in L-NAME-treated than in saline-treated transgenic mice. The effects of L-NAME on preventing the hyperglycemia-induced changes in individual β -cell size and the number of the insulin granules are quantified in Table 3.

nNOS Distribution in Islet Cells

We used immunostaining to examine the isoform of NOS present in the pancreatic β cells. Figure 9 shows

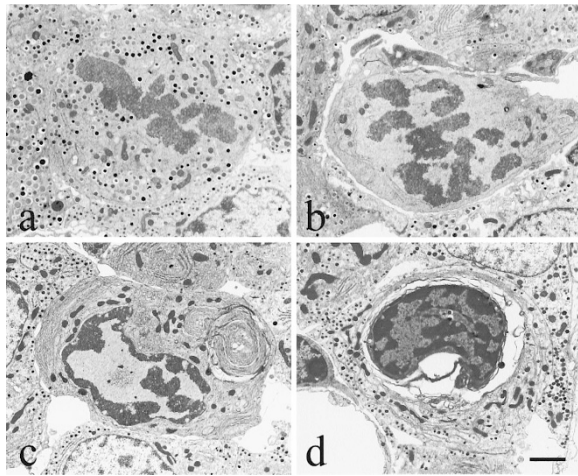


Figure 6.

Apoptotic changes in calmodulin transgenic β cells. Pancreata from 1-week-old (a and b) and 3-week-old (c and d) calmodulin transgenic mice were fixed and processed for electron microscopy. Typical ultrastructural changes for apoptosis are evident in c and d. Bar = 2 μ m.

immunofluorescence staining of nNOS and insulin in pancreatic islets from nontransgenic (Fig. 9, a to c) and calmodulin-transgenic (Fig. 9, d to f) mice. Double-staining of the nontransgenic pancreas demonstrated preferential distribution of nNOS in the insulin-positive cells. Because these samples were taken from 4-month-old mice, insulin-positive cells were rarely observed in the transgenic pancreas. However, a convergence of insulin and nNOS remained evident in these few β cells (Fig. 9, d to f). We failed to detect either endothelial NOS or iNOS in pancreatic islets from prediabetic or diabetic calmodulin-transgenic mice and nontransgenic ones (not shown). We conclude that nNOS is the likely target of L-NAME in pancreatic β cells.

Discussion

The present findings clearly demonstrate that NO-mediated postneonatal β -cell apoptosis occurs *in vivo* in calmodulin-transgenic mice. Acceleration of β -cell death by administration of tolbutamide, which causes Ca^{2+} influx selectively in the pancreatic β cell (Aguilar-Bryan and Bryan, 1999), suggests that the apoptotic changes are Ca^{2+} /calmodulin-dependent. Several calmodulin-dependent proteins, including calcineurin, the death-associated protein kinases, and NOS, have been suggested to be participants in Ca^{2+} -initiated apoptosis. It has been recently reported that the Ca^{2+} /calmodulin-dependent protein phosphatase, calcineurin, may play an important role in Ca^{2+} -dependent apoptotic cell death in cells deprived of growth factors (Shibazaki and McKeon, 1995). Moreover, overexpression of calcineurin increased vulnerability of neuronal cells to serum restriction or exposure to Ca^{2+} ionophore, both of which cause apoptotic cell death (Asai et al, 1999). However, injection of our transgenic mice with the calcineurin inhibitor cyclosporine (up to 2 μ g/gm body weight ip, twice

per week, from 7–28 days of age) failed to prevent or retard the onset of postnatal hyperglycemia (not shown). Thus, altered calcineurin activity does not seem to be responsible for the β -cell loss in our calmodulin-overexpressing mice.

Our results are compatible with the postulate that NO production by nNOS, which is activated in a Ca^{2+} /calmodulin-dependent manner, may be involved in the generation of β -cell apoptosis in calmodulin-transgenic mice. Previously, NO-dependent apoptosis was found to be more pronounced when pancreatic β cells were exposed to cytokines as a consequence of autoimmune diseases (Kaneto et al, 1995; Mauricio and Mandrup-Poulsen, 1998). For example, this seems to be true in type 1A diabetes mellitus (sudden onset and autoimmune-related) in which NO is produced from iNOS present in β cells attacking the pancreas in response to cytokines. The ability of NO production by iNOS to accelerate β -cell loss was also verified using transgenic mice that overexpressed iNOS in β cells. The transgene product caused insulin-deficient diabetes mellitus, which was accompanied by β -cell apoptosis (Takamura et al, 1998). In our study, induction of β -cell apoptosis by tolbutamide, prevention by L-NAME, and the absence of insulinitis suggest that the NO involved in apoptosis may be produced by the Ca^{2+} /calmodulin-dependent isoforms of the synthase, such as nNOS. Indeed, preferential distribution of nNOS in pancreatic β cells supports the idea that activation of nNOS is responsible for triggering β -cell apoptosis in this mouse model. Sudden onset of hyperglycemia and lack of insulinitis suggest Ca^{2+} -dependent and NO-mediated apoptosis of the β cell may be a cause of type 1B (nonimmune) diabetes mellitus (Imagawa et al, 2000). On the other hand, the reason why administration of L-NAME failed to completely prevent postnatal hyperglycemia is unclear. One possibility is that L-NAME may inhibit insulin release from the islets, because NO has been reported to potentiate the release (Ding and Rana, 1998). Another possibility is suggested by observations that NO-independent pathways can also result in β -cell apoptosis in response to immune attack (Rabinovitch et al, 1994). Perhaps the mechanism in this case involves other Ca^{2+} /calmodulin-dependent pathways that lead to apoptosis such as those involving the death-associated protein kinases (Cohen et al, 1997).

Our observations that the sulfonylurea, tolbutamide, induces apoptosis in β cells that overproduce calmodulin suggest that clinical use of these drugs could theoretically produce undesirable side effects. Although sulfonylureas have been widely used as antidiabetic agents for over 40 years, the molecular mechanism by which they elicit insulin release has been elucidated only recently (Aguilar-Bryan and Bryan, 1999; Miki et al 1999). Sulfonylureas bind to plasma membrane receptors in close proximity to pore-forming subunits of ATP-sensitive K^+ channels. Resultant closure of these K^+ channels causes membrane depolarization, which opens voltage-dependent Ca^{2+} channels, promoting Ca^{2+} influx. A rise in intra-

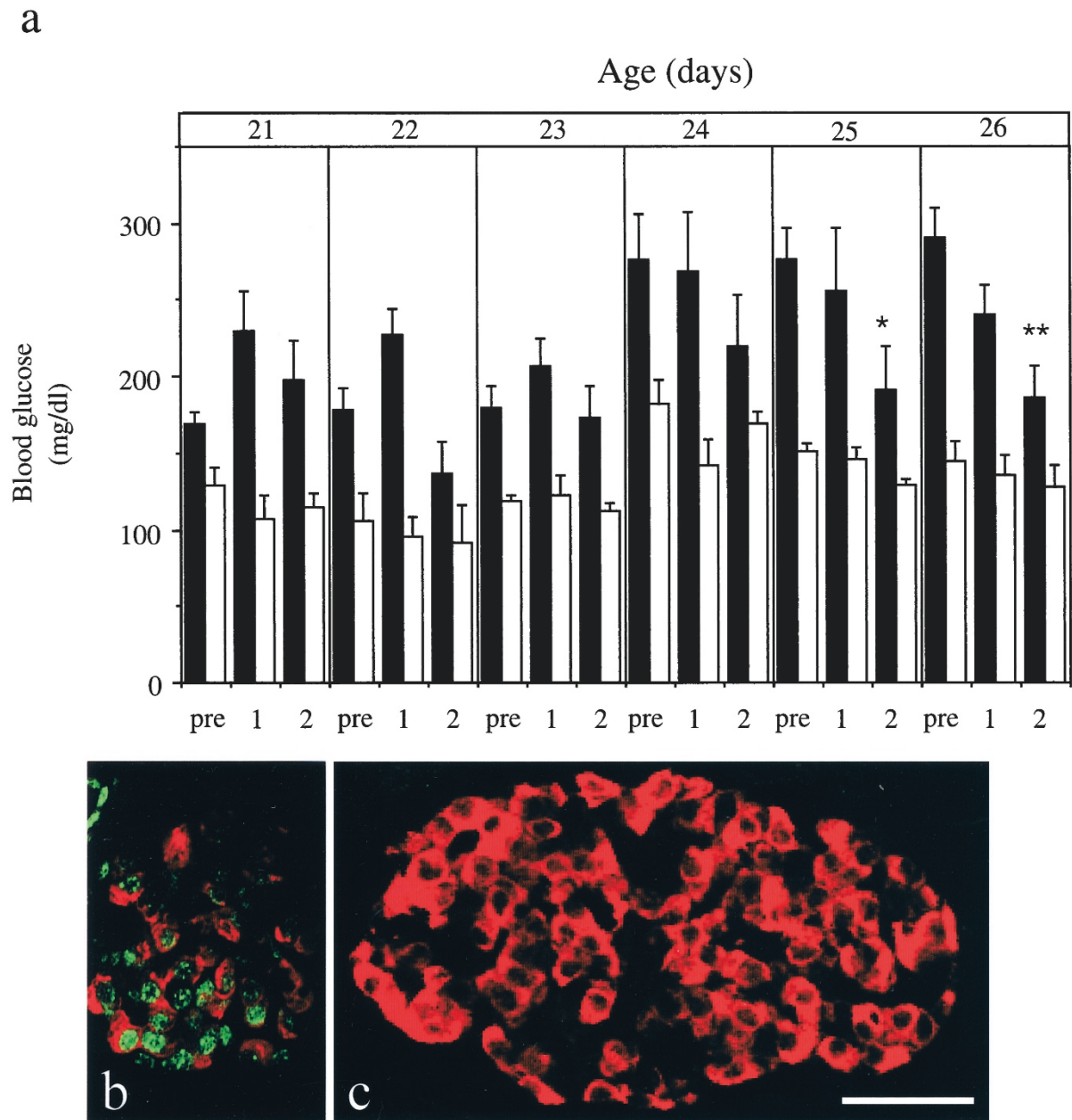


Figure 7.

Tolbutamide-induced apoptosis of β cells in calmodulin-transgenic islets. a, profiles of blood glucose levels of calmodulin-transgenic (closed columns) and nontransgenic (open columns) mice treated with tolbutamide (75 μ g/day ip daily from 21–26 days of age). Blood glucose was monitored daily before (pre), 1 hour after, and 2 hours after tolbutamide injection. * $p < 0.05$, ** $p < 0.005$ as compared with the values before tolbutamide injection on the same day. b and c, pancreata from tolbutamide-treated mice were double stained by a TUNEL method (green) and with anti-insulin antibody (red). b, transgenic; c, nontransgenic. Bar = 50 μ m.

cellular Ca^{2+} is a critical event for insulin exocytosis. Could it be possible that the sulfonylureas also accelerate β -cell loss in type II diabetics by stimulating Ca^{2+} -induced NO production? Certainly, NO has been implicated in glucose-mediated apoptosis of human endothelial cells (Du et al, 1999) and neurons (Lipton and Nicotera, 1998) in culture.

It is also interesting to ponder whether Ca^{2+} -dependent/NO-induced β -cell loss may be implicated as a pathologic component in persistent hyperinsulinemic hypoglycemia of infancy (PHHI). PHHI is a familial disease in which the ATP-sensitive K^+ channel

is closed constitutively because of functional mutations in the genes encoding either of its subunits, Kir6.2 or the sulfonylurea receptor (Aguilar-Bryan and Bryan, 1999). After birth, PHHI patients suffer from persistent hypoglycemia, but in a subset of individuals hyperglycemia with insulin-deficiency eventually develops (Leibowitz et al, 1995). Levels of basal intracellular Ca^{2+} concentrations are elevated in pancreatic β cells from patients with PHHI (Kane et al, 1996), and apoptotic (TUNEL positive) islet cells were found in pancreata from PHHI patients with mutations in the sulfonylurea receptor (Glaser et al, 1999). Perhaps the

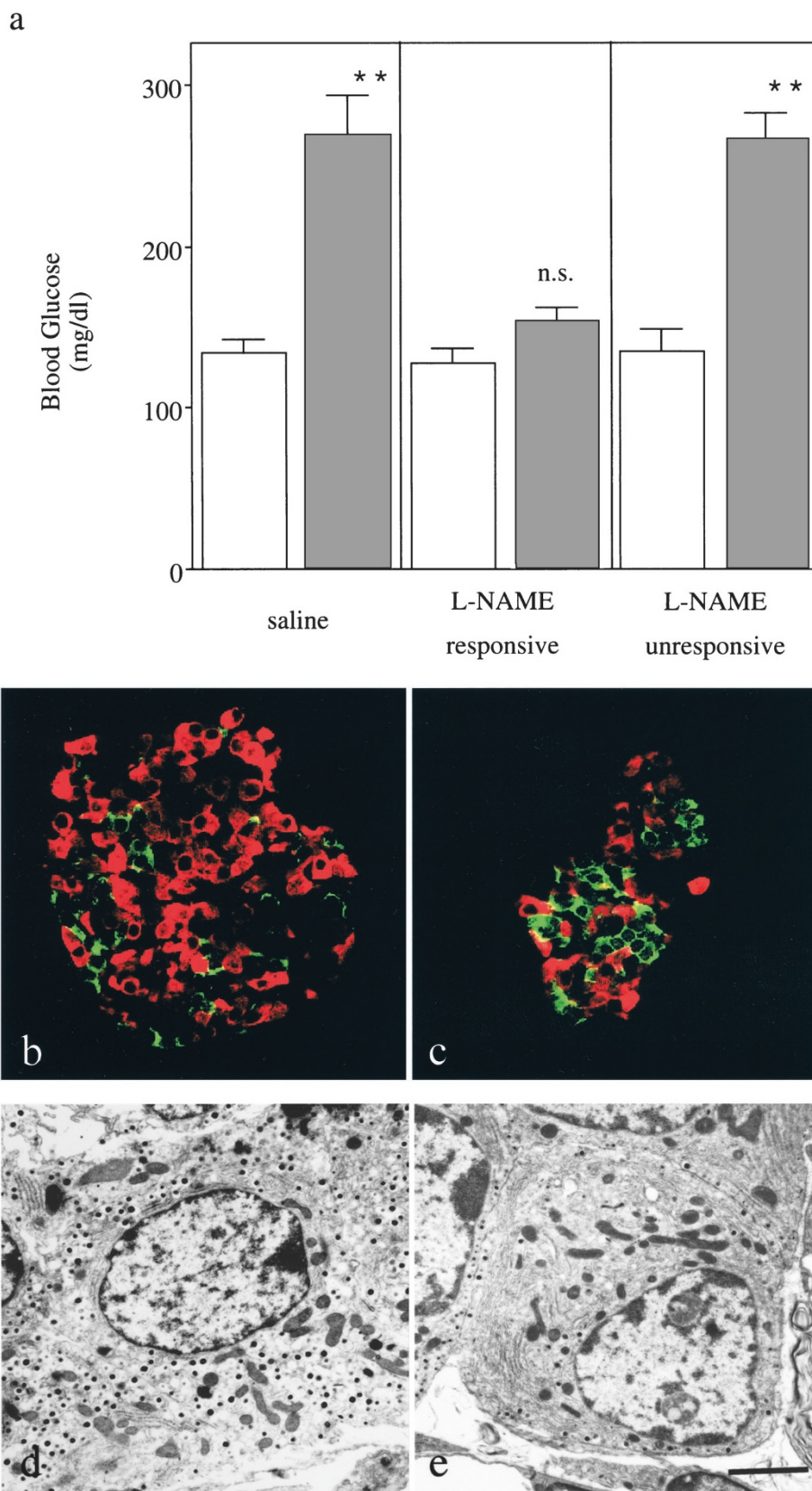


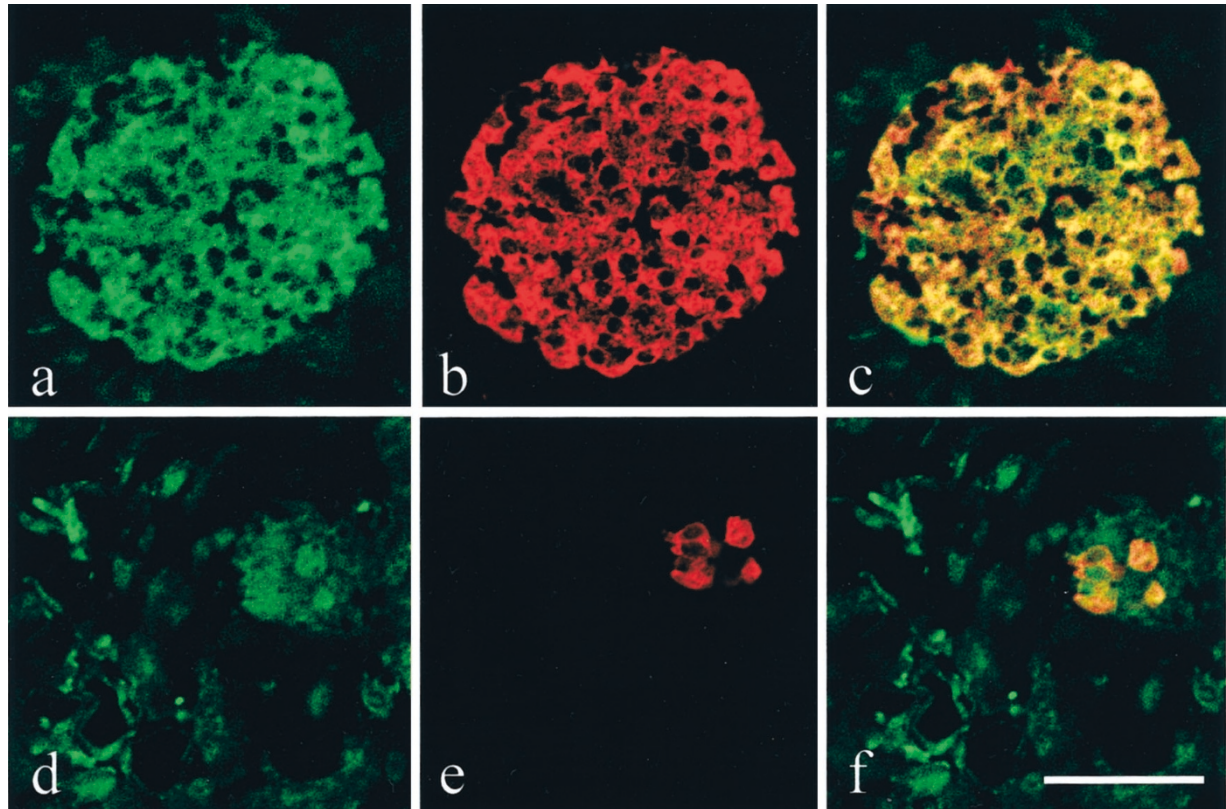
Figure 8.

Prevention of onset of diabetes in calmodulin-transgenic mice by N^ω-nitro-L-arginine methyl ester (L-NAME). L-NAME (0.5 mg/gm body weight ip) was injected daily from the 4th to the 27th day after birth. **a**, Blood glucose levels in 20-day-old (*open columns*) and 27-day-old (*grey columns*) mice are demonstrated. Left, saline-treated transgenic mice (mean ± SE, *n* = 11); middle, L-NAME responsive (*n* = 13); and right, L-NAME unresponsive (*n* = 9). n.s. = not significant. **Significantly higher (*p* < 0.001). **b** and **c**, Pancreata from L-NAME-responsive mice (**b**) and saline-injected ones (**c**) were double stained with antiglucagon and antisomatostatin antibodies (green) and with anti-insulin antibody (red). Bar = 50 μm. **d** and **e**, Electron micrographs of calmodulin-transgenic pancreatic β cells treated with L-NAME (**d**) and saline (**e**). Bar = 2 μm.

Table 3. Effects of L-NAME Injection on β Cell Size, Nuclear Size, and Insulin Granule Numbers in β Cells of Tg Islets

Treatment	Cell size (μm^2)		Nuclear size (μm^2)		Granule number (per β cell)	
	L-NAME	Saline	L-NAME	Saline	L-NAME	Saline
	132.4 \pm 6.5 (6)	106.2 \pm 6.9 (6)	32.6 \pm 2.1 (6)	30.5 \pm 1.0 (6)	161 \pm 12 (6)	47 \pm 9 (6)

The NOS inhibitor L-NAME at a dose of 0.5 mg/gm body weight/day was administered to Tg mice from the 4th to the 27th day after birth. Pancreata from L-NAME-responsive mice were fixed and observed by electron microscopy. The cell size, nuclear size, and granule number of representative β cells were assessed and compared with those in Tg mice treated with saline as a control. Data represent the mean \pm SEM values from six individual cells.

**Figure 9.**

Neural nitric oxide synthase (nNOS) expression in the pancreatic islets. Immunohistochemistry of pancreata from 4-month-old nontransgenic (a to c) and calmodulin-transgenic (d to f) mice. Green, nNOS; red, insulin; c and f are merged images. Bar = 50 μm .

hyperglycemia caused by overexpression of calmodulin in β cells of transgenic mice may mimic the late phase of PHHI in an accelerated manner.

Materials and Methods

Materials

Anti-insulin antibody was from Seikagaku Kogyo (Tokyo, Japan), anti-glucagon antibody was from Zymed (South San Francisco, California), and anti-somatostatin antibody and rhodamine-conjugated anti-guinea pig IgG antibody were from ICN (Costa Mesa, California). FITC-conjugated anti-rabbit IgG antibody was from Vector Laboratories (Burlingame, California). DIG-High Prime reagent and antidigoxigenin-Fab fragments conjugated with horseradish peroxidase were from Boehringer Mannheim (Mann-

heim, Germany). Collagenase (type V), tolbutamide and L-NAME were from Sigma (St. Louis, Missouri). Anti-nNOS antibody was from Euro-Diagnostic (Ideon, Sweden).

Minigene Construct and Production of Transgenic Mice

Construction of a minigene in which the rat insulin II promoter was ligated to the chicken calmodulin cDNA has been previously described (Epstein et al, 1989). The promoter and minigene were inserted into pUC18 plasmid as an *FspI*-*AatII* fragment between the blunt-ended *HindIII* linker and an *AatII* site of the plasmid, amplified, and separated by cleavage with *Bam*HI and *AatII*. The resultant DNA was designated as BIC (ie, Bam-Insulin-Calmodulin). Calmodulin-overexpressing transgenic mice were produced by microinjection of the BIC DNA into mouse embryos derived from female

FVBM mice, followed by implantation of the embryos into pseudopregnant female FVBM mice. Because of the markedly decreased survival of these mice into adulthood, the transgene was subsequently bred into CD1 mice (Ribar et al, 1995a) and later into the ICR strain (Ribar et al, 1995b). Because female transgenic ICR mice are infertile, normal female ICR mice (Slc:ICR; Nippon SLC, Hamamatsu, Japan) were bred with transgenic male ICR mice.

Blood Glucose Measurement and Insulin Assay

Glucose concentrations of the mice were assayed using a compact glucose analyser (MediSafe; Terumo, Tokyo, Japan) by tail sampling. Serum insulin levels were measured by enzyme-linked immunoassay using a commercialized kit (Morinaga, Yokohama, Japan). Statistical analysis was performed by the unpaired Student's *t* test.

Genomic Southern Blotting

Transgenic and nontransgenic mice were readily distinguished by phenotype (blepharostenosis that appeared between 2 and 3 weeks of age and frank hyperglycemia by the end of 4 weeks after birth). However, to verify this and also to distinguish transgenic or nontransgenic mice at an earlier stage, genomic Southern blotting was performed. Mouse tail DNA was extracted using MagExtractor (Toyobo, Osaka, Japan). DNA samples from transgenic or nontransgenic mice were blotted onto Hybond-XL membranes (Amersham Pharmacia Biotech, Chalfont, Bucks, United Kingdom). The BIC DNA probe was labeled with digoxigenin using the DIG-High Prime reagent and hybridized to the membrane. After blocking, the membranes were incubated with antidigoxigenin Fab fragments conjugated with horseradish peroxidase. Chemiluminescent detection was performed using an ECL-plus kit (Amersham Pharmacia Biotech).

Radioimmunoassay of Calmodulin

Pancreatic islets were isolated from transgenic and nontransgenic mice as described by collagenase digestion. A radioimmunoassay was used to quantify calmodulin as previously described (Chafouleas et al, 1979).

Light Microscopy

For stereomicroscopic observations, calmodulin transgenic and nontransgenic mice were perfused with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Pancreatic tails were observed under a stereomicroscope (Olympus S2H10; Tokyo, Japan), and removed pancreata were used for observation by electron microscopy. For hematoxylin and eosin staining, calmodulin transgenic and nontransgenic mice were anesthetized with diethyl ether and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Removed pancreata were immersed in the same

fixative solution for 6 hours, dehydrated in ethanol, and embedded in paraffin. Sections were stained with hematoxylin and eosin and observed with a light-microscope (AX80; Olympus).

Immunofluorescence Microscopy

After fixation as above, pancreata were washed thoroughly in 10%, 15%, and 20% sucrose in PBS, embedded in optimal cutting temperature compound (Sakura, Tokyo, Japan), and frozen. For detection of islet hormones, frozen sections mounted on poly-L-lysine-coated glass slides were incubated in a mixture of anti-insulin guinea pig antibody and a mixture of rabbit antibodies against glucagon and somatostatin. After washing with PBS, they were incubated for 1 hour in a mixture of rhodamine-conjugated anti-guinea pig IgG antibody and FITC-conjugated anti-rabbit IgG antibody. When anti-nNOS antibody was used, frozen sections were treated with anti-nNOS antibody and then processed as described above. Sections were analyzed with a confocal scanning laser microscope (MR-1024; Japan BioRad, Tokyo, Japan).

Electron Microscopy

Anesthetized mice were perfused with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Islet-rich portions of pancreata detected under a stereoscopic microscope (Fig. 2) were excised and fixed in the same fixative solution for 2 hours and subsequently fixed with 1% osmium tetroxide for 1 hour. Tissues were dehydrated in ethanol and embedded in Epon epoxy resin. Ultrathin sections cut with an ultramicrotome (Sorvall, Newtown, Connecticut) were stained with uranyl acetate and lead citrate. Observations were carried out with a transmission electron microscope (H-7100; Hitachi, Tokyo, Japan).

Detection of Apoptotic β Cells by the Modified TUNEL Method

Frozen pancreata were sectioned with a cryostat as described in the immunofluorescence staining section. Apoptotic cells were detected by a modified TUNEL method using Mebstatin Kit Direct (Medical and Biological Laboratories, Nagoya, Japan). After terminal deoxynucleotidyl transferase reaction, sections were further incubated in anti-insulin guinea pig antibody for 1 hour followed by a 1-hour incubation with rhodamine-conjugated anti-guinea pig IgG antibody.

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