BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 34–38 (1998)
ARTICLE NO. RC989601

Endogenous Nitric Oxide Inhibits Glucose-Induced Insulin Secretion by Suppression of Phosphofructokinase Activity in Pancreatic Islets

Yoshiyuki Tsuura, Hitoshi Ishida,* Tetsutaro Shinomura,†
Masayoshi Nishimura, and Yutaka Seino

*Department of Metabolism and Clinical Nutrition and †Department of Anesthesia, Kyoto University Faculty of Medicine, Kyoto; and *Third Department of Internal Medicine, Kyorin University Faculty of Medicine, Tokyo, Japan*

Received October 1, 1998

The physiological role of nitric oxide (NO) on the mechanism of insulin secretion is unknown, but some studies suggest that NO affects glucose metabolism in pancreatic β-cells. We have aimed at clarifying the physiological role of endogenous NO and its target in the glucose metabolism of β-cells. The expression of brain-type NO synthase (bNOS) was detected in pancreatic islets by Western blotting. Under the condition of elevated intracellular Ca^{2+} concentration induced in the β-cells by high glucose and forced depolarization by 40 mM K^+, the generation of NO from the islets was enhanced. This increase was suppressed by the NOS blockers, *N*-iminoethyl-L-ornithine (L-NIO), and exposure to Ca^{2+}-free extracellular solution. In addition, the NOS blockers L-NIO and 7-nitro indazole (7-NI) enhanced glucose-induced but not glyceraldehyde- or KIC-induced insulin secretion. In an *in vitro* enzyme study, the NO donor sodium nitroprusside (SNP) suppressed phosphofructokinase activity and activated glucokinase and glucose-6-phosphate isomerase activity, but SNP significantly inhibited the combined activity of the enzymes. This suggests that endogenous NO has an inhibitory role on insulin release induced by glucose and that its underlying mechanism is the suppression of phosphofructokinase activity in glycolysis. © 1998 Academic Press

It is well known that the glucose metabolism in pancreatic β-cells which produces ATP and leads to inhibition of the ATP-sensitive K^+ channels (K_{ATP} channels) is the crucial factor in glucose-induced insulin release (1), but the mechanism of intracellular signaling for the secretion remains to be clarified. Several research groups have reported that nitric oxide (NO), which has been recognized to be an important mediator of the intracellular signaling, inhibits glucose-induced insulin secretion from β-cells (2–6). It has also been reported that exogenous NO derived from NO donors such as sodium nitroprusside (SNP) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) increases $^{86}Rb^+$ efflux (2), and by electrophysiological study that it reopens the K_{ATP} channel activity inhibited by glucose in rat pancreatic β-cells (3). Furthermore, in mouse β-cells, exogenous NO is known to lead to membrane repolarization resulting from the activation of K_{ATP} channels (4). Since NO has been shown not to affect the sensitivity to intracellular ATP of K_{ATP} channels (3), it may be thought that it impairs intracellular glucose metabolism, and lead to the activation of the K_{ATP} channels and inhibition of insulin secretion by glucose. On the other hand, many reports have shown that NO synthase (NOS) inhibitors enhance glucose-induced insulin secretion (5,6). Accordingly, it is possible that endogenous NO generated by NOS in the pancreatic islets plays an inhibitory role in insulin secretion under physiological conditions. However, the physiological target of endogenous NO in its inhibitory effect on glucose-induced insulin secretion is not known.

Our previous report suggested that exogenous NO inhibits glucose metabolism at the phosphofructokinase level in the early pathway of glycolysis, leading to inhibition of glucose-induced insulin release, using a moderately permeabilized open-cell attached patch-clamp technique in which intracellular metabolism is physiologically preserved (3). However, this result might have only pharmacological implications, because a relatively larger amount of exogenous NO than under physiological conditions was applied in the experiments. We examine in this study whether physiological levels of endogenous NO can affect the metabolic pathway in β-cells. It is likely that endogenous NO inhibits a proximal step of glycolysis in β-cells, since it is known that regulation at this step affects total glucose metabolism considerably (7). We investigated whether endogenous NO elicits the same effect under physiological

0006-291X/98 $25.00

conditions by measurement of NO generated from islets using a NO analyzer. We also examined whether fuel-induced insulin secretion can be influenced by NOS inhibitors such as N-iminoethyl-L-ornithine (L-NIO) (8) and 7-nitro indazole (7-NI) (9). In addition, the effect of NO donor sodium nitroprusside (SNP) on *in vitro* enzyme activity at early steps in glycolysis was examined.

MATERIALS AND METHODS

Animals. Male Wistar rats weighing 200 ~ 250g were used in all experiments. The rats were fed on standard laboratory chow *ad libitum* and allowed free access to water in an air-conditioned room with 12-h light/dark cycle before experiments.

Assessment of insulin secretory capacity. Insulin secretory capacity was determined by the batch incubation method as previously reported with freshly isolated islets (10). In brief, the islets were preincubated in Krebs-Ringer bicarbonate buffer (KRBB) medium composed of (in mM) 129.4 NaCl, 4.0 KCl, 2.7 $CaCl_2$, 1.2 KH_2PO_4, 1.3 $MgSO_4$, and 24.8 $NaHCO_3$ containing 2.8 mM glucose and 0.2% bovine serum albumin (BSA) at 37°C for 30 min. Afterward, batches of 5 islets were incubated in 0.7 ml KRBB medium, supplemented with test materials and 0.2% BSA at 37°C for 30 min. L-NIO and 7-NI were prepared as 10 mM stocks in water and 100 mM in dimethylsulphoxide (DMSO), and were diluted immediately before incubation. An aliquot (0.1 ml) from each batch was drawn at the end of the incubation for measurement of immunoreactive insulin (IRI) by RIA.

Measurement of nitric oxide. The concentration of NO was determined by a chemiluminescence method, using a highly sensitive NO analyzing system (FES-450, Scholar-Tec Co. Ltd., Osaka, Japan) (11,12). Measurement of NO in this system is based on detecting chemiluminescence (660-900 nm) emitted by the following reaction: $NO + O_3 \rightarrow NO_2 + O_2$ where NO_2 emits light. This reaction in the analyzer was performed at 0°C. Generated NO was stripped from the sample solution to a gaseous phase by bubbling the solution with argon gas after adding 1M ascorbic acid, a strong reducer, for 60s, under vacuum condition, in a reaction vial (Reacti-Vial Small Reaction Vials 10ml, Pierce, Rockford, IL). The NO released from islets was determined using 100 islets incubated in 1 ml KRBB medium with test materials and 0.1 mM arginine at 37°C for 30 min. When necessary, NaCl and KCl in solution were replaced to 110 mM and 40 mM, respectively. In the case of the Ca^{2+}-free solution, 0.1 mM EGTA was added, instead of $CaCl_2$. The NO generation was calculated by computer (9801VX, NEC, Tokyo, Japan) interfaced with the NO analyzer. The standard curve was drawn up with nitrite ion standard solution (NO_2^- 1000ppm), and it was well fitted linearly as follows: Y = 1.85X, where Y is NO concentration (pM) and X is signals (mm_2) within the range of 1 ~ 10 ppb NO_2^- solutions.

Western blotting. For preparation of protein samples for Western blotting, rat fresh islets, cerebellum, aorta and NG-108 cells, a glioblastoma cell line, were homogenated in a solution which was composed of (in mM) 50 Tris, 1 ethylenediaminetetraacetic acid (EDTA), and 1 EGTA (pH 7.40 with HCl), and then were centrifuged at 2000 rpm at 4°C for 10 min. The extracted supernatants were stored at −80°C until the experiments. Protein contents of each supernatant were determined by the method of Lowry et al. (13). Proteins (20 μg/lane) were fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (14), and gels were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) electrophoretically (15). PVDF membranes were blocked with a 3% (w/v) BSA solution in phosphate buffered saline (PBS) consisting of (in mM) 8.1 Na_2HPO_4, 1.5 KH_2PO_4, 2.7 KCl, and 137 NaCl (pH 7.4 with NaOH) and incubated overnight at 4°C. The filters were washed in PBST (PBS and 0.02% Tween 20), and incubated with primary antibody for 2 hours at room temperature. After being washed three times with PBST, they were incubated for 1 hour with biotinylated horseradish peroxidase in the presence of avidin (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). Immunoreactive proteins were visualized by using a horseradish peroxidase-catalyzed chemiluminescence reaction. Biotinylated SDS-PAGE Standard Kit (Bio-Raboratories, Hercules, CA) was used as molecular weight marker. In order to determine the expression of NOS isozymes in the islets, rat cerebellum for bNOS (16), rat aorta for eNOS (17), and the rat glioblastoma cell line NG-108 (18) for iNOS were used as positive control.

Measurement of enzyme activity in vitro. The activity of glucokinase, glucose-6-phosphate isomerase, phosphofructokinase and fructose diphosphate aldolase was assessed by fluorimetric measurement, as previous reported (19–22). Islets were homogenized in the solution consisting of 50 mM triethanolamine and 250 mM saccharose (pH7.5) on ice. Supernatants were obtained from the homogenates by centrifugation at 4°C at 2000rpm for 10 min. Protein content in the supernatant was adjusted to 20 μg/ml by method of Lowry et al (13). Enzyme reaction was performed in the solution consisting of 50 mM triethanolamine and 1.2 mM $MgCl_2$ (pH7.5) at room temperature for 10 min. Glucokinase activity was estimated as increase of NADPH through the reaction glucose-6-phosphate + NADP → 6-phosphoglucono- δ-lactone + NADPH by glucose-6-phosphate dehydrogenase. The other three enzyme activities were assessed as decrease of NADH through the reaction dihydroxyacetone-phosphate + NADH → glycerol-3-phosphate + NAD by glycerophosphate dehydrogenase. Glucose, instead of fructose-6-phosphate, was used as substrate for measurement of combined enzyme activity of glucokinase, glucose-6-phosphate isomerase and phosphofructokinase. The enzyme activity from three separate experiments was expressed as the percentage of each control.

Statistical analysis. The statistical analyses of results and the significance of the difference were evaluated by unpaired Student's *t* test, and $p<0.05$ was considered significant. Numerical data were expressed as mean±SE.

Chemicals. Nitric ion standard solution were obtained from Wako Junyaku (Osaka, Japan). L-NIO and 7-NI were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Anti-bNOS, Anti-eNOS, and Anti-iNOS antibodies were obtained from Affinity BioReagents (Neshanic Station, NJ). Glucose-6-phosphate dehydrogenase, aldolase, glycerol phosphate dehydrogenase, phosphofructokinase and ATP dipotassium salt were obtained from Sigma. Co (St. Louis, MO). SNP and other chemicals were purchased form Nacalai Tesque (Kyoto, Japan).

RESULTS

Identification of an isozyme of NOS in pancreatic islets. Western blotting revealed a band identical to the molecular weight (about 16kD) of bNOS in extracts from islets, but which was slightly weaker than that obtained from rat cerebellum. On the other hand, eNOS and iNOS bands were found in rat aorta and the glioblastoma NG-108 cell line, respectively, but were not detected in rat islets (Fig. 1).

Endogenous NO generation from pancreatic islets. Endogenous NO generation in the presence of the basal concentration of 2.8 mM glucose was a lower level of 0.43±0.06 pM/islet/30min. A higher concentration of 11.1 mM glucose, which is known to inhibit K_{ATP} channels and lead to Ca^{2+} influx (1), significantly enhanced NO generation. NOS blocker of 10 μM L-NIO signifi-

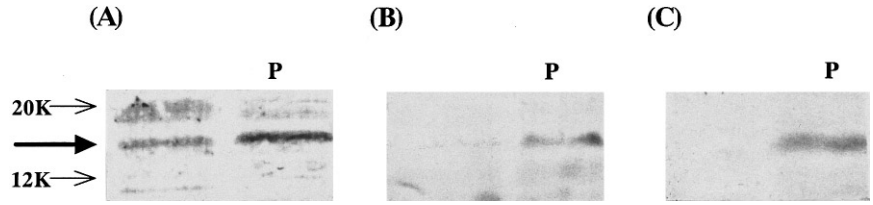

FIG. 1. Identification of NOS isozymes (A) bNOS, (B) eNOS and (C) iNOS. P indicates positive controls; rat cerebellum for bNOS, rat aorta for eNOS and NG-108 cell lines for iNOS. Small arrows at the left side of panel in (A) indicate molecular weight. Large arrow in (A) shows bands. Results were representative of 3 individual experiments.

cantly decreased the NO generation induced by 11.1 mM glucose. On the other hand, in the case of forced membrane depolarization by 40 mM potassium in the presence of 2.8 mM glucose, NO generation was significantly increased to the same higher level as that with 11.1 mM glucose ($P<0.01$). However, this increase was not found with the Ca^{2+}-free extracellular solution (Table 1).

The effect of NOS blockers on nutrient-induced insulin secretion. Two NOS blockers, 100 μM 7-NI and 10 μM L-NIO, were used in the experiments. Both drugs significantly enhanced insulin secretion induced by 11.1 mM glucose in the presence of 0.1 mM L-arginine. On the other hand, both 5 mM glyceraldehyde- and 5 mM KIC-induced insulin release with 0.1 mM L-arginine were not influenced by these drugs (Fig. 2A). Other NOS blockers, 100 μM aminoguanidine and 100 μM S-methylisothiourea also enhanced 11.1 mM glucose-induced insulin secretion, but not that induced by 5 mM glyceraldehyde (data not shown). As shown in Fig.2B, 10 μM L-NIO increases glucose-stimulated insulin secretion but not basal, particularly significantly in the case of 11.1 and 16.7 mM glucose.

The effects of SNP on enzyme activity in the early steps of glycolysis. NO concentrations liberated from 0.1 and 1 mM SNP were 46.5±2.0 pmol/30min (n=3) and 338.9±17.7 pmol/30 min (n=3), respectively.

TABLE 1

Endogenous NO Formation under the Condition of Glucose and High Concentration of Potassium

Glucose (mM)	Extracellular Ca^{2+}	NO generation (pmol/islet/30 min)	*n*
2.8	(+)	0.43 ± 0.06	12
11.1	(+)	0.88 ± 0.08*	14
11.1	(+) + 10 μM L-NIO	0.37 ± 0.02**	4
2.8	(+) + 40 mM K^+	0.85 ± 0.08*	8
2.8	(−) + 40 mM K^+	0.05 ± 0.04☆	8

* $P<0.01$ versus value in 2.8 mM glucose.
** $P<0.01$ versus value in 11.1 mM glucose only.
☆ $P<0.01$ versus value in 2.8 mM glucose and 40 mM K^+ in the presence of extracellular Ca^{2+}.

These ranges were close to NO level formed from 100 islets (40~90 pmol/30 min). SNP significantly activated glucokinase and glucose-6-phosphate isomerase activity in a concentration-dependent manner. On the other hand, phosphofructokinase activity was significantly inhibited by SNP. The combined enzyme activity was also suppressed by SNP in a concentration-dependent manner. Fructose diphosphate aldolase was not affected by SNP (Table 2). Phosphofructokinase activity just after incubation with 2.8 mM was 7.08±0.17 U/mg protein (100±2.5%). On the other hand, the activity just after the activation of NO synthesis by a physiological stimulation (glucose 11.1 mM) was 6.77±0.20 U/mg protein (96±3.0%). There is no significant difference between both groups.

DISCUSSION

In pancreatic β-cells, it has been known since 1981 that the NO donor SNP inhibits the insulin release induced by glucose (23). Recently, in studies of the mechanism underlying this suppressive effect, there have been reports that $^{86}Rb^+$ efflux from pancreatic β-cells under 16.7 mM glucose is increased after adding SNP (2), and that SNP reopens K_{ATP} channels which have already been inhibited by glucose (3,4). This strongly suggests that SNP plays an inhibitory role in the mechanism of insulin secretion induced by glucose at a step in the mechanism prior to K_{ATP} channel closure.

There have been some reports that constitutive NOS is equipped in pancreatic islets, in addition to the iNOS which can be induced by interleukin-1 β, however, it is not known where these isozymes are located in the islets. Vincent has found by immunohistochemical study that bNOS is present only at paraganglion terminals, but not in endocrine cells (24). On the other hand, Schmidt and collaborators reported that by Western blotting bNOS is found in the cytosolic fraction of HIT-T15 β-cell cell lines (25). The results of endogenous NO formation in the present study are consistent with that of bNOS location in β-cells. The enhanced endogenous NO generation was observed when Ca^{2+} influx via voltage-dependent Ca^{2+} channels

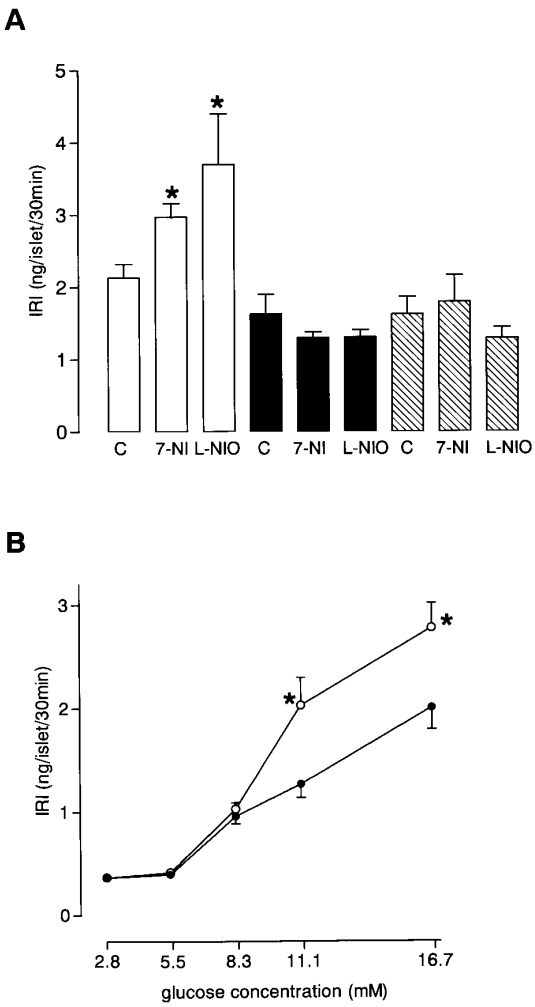

FIG. 2. The effect of NOS blockers on glucose-, glyceraldehyde- and KIC-induced insulin secretion. (A) This panel shows the effect of 10 μM L-NIO and 100 μM 7-NI on 11.1 mM glucose (open columns), 5 mM glyceraldehyde (black columns) and 5 mM KIC (oblique columns). All solutions contains 0.1 mM L-arginine. C indicates insulin secretion without drugs. ✶ shows $P<0.01$ versus insulin secretion without drugs. (B) This illustrates the effect of 10 μM L-NIO on insulin secretion in response to various concentrations of glucose with 0.1 mM L-arginine. Closed circles (●) and open circles (○) indicate the absence or presence of L-NIO, respectively. ✶ shows $P<0.01$ versus insulin secretion without drug. Values are means of 5 batches±SE. The experiments of the same protocol were performed three times.

was induced by a high concentration of glucose, which could depolarize the plasma membrane by closing the K_{ATP} channels in the β-cells, and when the membrane depolarization was forced by high potassium. This enhancement is probably mediated by bNOS and elevated intracellular Ca^{2+}, since it was inhibited by the NOS blocker and under extracellular Ca^{2+}-free conditions, and bNOS activity is known to be Ca^{2+} dependent. In addition, its presence in the pancreatic β-cells could account for the many reports that the various NOS blockers enhance insulin secretion induced by glucose (5,6). It should be expected that endogenous NO plays some physiological role in the insulin secretory regulatory mechanism in β-cells, which is known to be triggered by intracellular Ca^{2+} elevation.

In the present study, we observed that various type of NOS blockers commonly enhance glucose-induced, but not glyceraldehyde- or KIC-induced insulin secretion in the presence of 0.1 mM L-arginine. This suggests that the functional site of NO is located in glycolysis before glyceraldehyde-3-phosphate. Because of the difficulties of demonstrating the exact site of No *in vivo, in vitro* enzyme study was performed in the present study. The finding that phosphofructokinase activity was inhibited by SNP is consistent with our previous patch-clump study (3). SNP enhanced the activity of glucokinase and glucose-6-phosphate isomerase, but it suppressed phosphofructokinase activity and the combined activity of glucokinase, glucose-6-phosphate isomerase and phosphofructokinase. In addition, we observed that ATP concentration in islets was reduced by SNP (unpublished data). They suggest that nitric oxide inhibits glucose metabolism and glucose-induced insulin secretion by suppression of phosphofructokinase activity.

Because constitutive NOS is expressed functionally in pancreatic β-cells, the intracellular exposure is continuous. Our previous patch clamp study showed that K_{ATP} channels, which had been closed by glucose, were reopened within 1 min after application of SNP, which was reversible only a short period after wash out of the drug (3). This phenomenon strongly suggests that NO or its related mediator can directly regulate intracellular glucose metabolism. Since bNOS activity is thought to be enhanced by intracellular Ca^{2+} elevation via Ca^{2+} influx through voltage-dependent Ca^{2+} channels after glucose stimulation, endogenous NO probably plays a physiological role in the putative negative feedback mechanisms in insulin secretion induced by glucose, similarly to its role in the Pasteur effect, in which increasing ATP inhibits phosphofructokinase activity directly (7). Moreover, continuous exposure to endogenous NO could extend the range of total glucose metabolism toward a higher level by preventing an early step of glycolysis from reaching its maximum.

In conclusion, A constitutive NOS, bNOS is present in functional pancreatic β-cells, and its activity is thought to be dependent on the Ca^{2+} influx via voltage-dependent Ca^{2+} channels. The endogenous NO generated through bNOS probably suppressed phosphofructokinase activity and inhibits the insulin secretion induced by glucose, and so plays a physiological role in the putative negative feedback mechanism in glucose-induced insulin release in pancreatic β-cells.

TABLE 2

The Effect of SNP on the Enzyme Activity of Glucokinase, Glucose-6-phosphate Isomerase, Phosphofructokinase, Fructose Diphosphate Aldolase, and the Combined Activity of Glucokinase, Glucose-6-phosphate Isomerase, and Phosphofructokinase

Enzyme	Control (%)	0.1 mM SNP (%)	1 mM SNP (%)
Glucokinase (GK)	100 ± 8	125 ± 7*	145 ± 9*
Glucose-6-phosphate isomerase (ISO)	100 ± 7	135 ± 9*	155 ± 12*
Phosphofructokinase (PFK)	100 ± 3	61 ± 11*	55 ± 12*
Fructose diphosphate aldolase	100 ± 2	104 ± 10	103 ± 11
Combined activity (GK+ISO+PFK)	100 ± 3	85 ± 4**	77 ± 4*

* and ** indicate $P<0.01$ and $P<0.05$ versus each control.

ACKNOWLEDGMENTS

The authors thanks Mr. H. Imamura for his technical assistance. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture and from the Research Committee of Experimental Models for Intractable Disease of the Ministry of Health and Welfare.

REFERENCES

1. Ashcroft, F. M., and Rorsman, P. (1989) *Prog. Biophys. Molec. Biol.* **54,** 87–143.
2. Antoine, M. H., Hermann, M., Herchuelz, A., and Lebrun, P. (1993) *Biochim. Biophys. Acta.* **1175,** 293–301.
3. Tsuura, Y., Ishida, H., Hayashi, S., Sakamoto, K., Horie, M., and Seino, Y. (1994) *J. Gen. Physiol.* **104,** 1079–1098.
4. Krippeit-Drews, P., Kr. T., Lang, F., and Drews, G. (1995) *Endocrinology* **136,** 5363–5369.
5. Panagiotidis, G., Åkesson, B., Rydell, E. L., and Lundquist, I. (1995) *Bri. J. Pharmacol.* **114,** 289–296.
6. Salehi, A., Carlberg, M., Henningson, R., and Lundquist, I. (1996) *Am. J. Physiol.* **270,** C1634–C1641.
7. Malaisse, W. J., Rasschaert, J., Zner, D., and Sener, A. (1988) *Diabetes Res.* **7,** 53–58.
8. Knowles, R. G., Palacios, M., Palmer, R. M. J., and Moncada, S. (1990) *Biochem. J.* **269,** 207–210.
9. Moore, P. K., Babbedge, R. C., Wallace, Z., Gaffen, Z. A., and Hart, S. L. (1993) *Bri. J. Pharmacol.* **108,** 296–297.
10. Tsuura, Y., Ishida, H., Okamoto, Y., Kato, S., Sakamoto, K., Horie, M., Ikeda, H., Okada, Y., and Seino, Y. (1993) *Diabetes.* **42,** 1446–1453.
11. Isaacson, T. C., Hample, V., Weir, E. K., Nelson, D. P., and Archer, S. L. (1994) *J. Appl. Physiol.* **76,** 933–940.
12. Kita, Y., Hirasawa, Y., Maeda, K., Nishino, M., and Yoshida, K. (1994) *Eur. J. Pharmacol.* **257,** 123–130.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
14. Laemmli, U. K. (1971) *Nature (Lond)* **227,** 680–685.
15. Kyhse-Andersen, J. (1984) *J. Biochem. Biophys. Methods.* **10,** 203–209.
16. Bredt, D. S., Glatt, C. E., Hwang, P. M., Fotuhi, M., Dawson, T. M., and Snyder, S. H. (1991) *Neuron.* **7,** 615–624.
17. Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6348–6352.
18. Hokari, A., Zeniya, M., and Esumi, H. (1994) *J. Biochem.* **116,** 575–581.
19. Gracy, R. W., Tilley, B. E. (1975) *Methods Enzymol* **41,** 392–400.
20. Shimizu, T., Kono, N., Mineo, I., Sumi, S., Nonaka, K., Tarui, S., Koyama, W. (1984) *J Inherited Metab Dis* **7,** 107–111.
21. Trus, M. O., Zawalich, W. S., Burch, D. T., Berner, D. K., Weill, V. A., Matschinsky, F. M. (1981) *Diabetes* **30,** 911–922.
22. Willnow, P. (1984) *in* Methods of Enzymatic Analysis (Bergmeyer, H. V., Ed.), pp. 346–353, Verlag Chemie, Weinheim.
23. Laychock, S. G. (1981) *Endocrinology* **108,** 1197–1205.
24. Vincent, S. R. (1992) *Science.* **258,** 1376.
25. Schmidt, H. H. H. W., Warner, T. D., Ishii, K., Sheng, H., and Murad, F. (1992) *Science* **255,** 721–723.