Nitric Oxide and Arginine-Evoked Insulin Secretion

Cationic amino acids stimulate secretion in exocrine and endocrine tissues. H. H. H. W. Schmidt *et al.* (*1*) suggest that the insulinotropic action of L-arginine may be explained by the formation of nitric oxide (NO) from L-arginine by the action of NO synthase in pancreatic B cells. In support of this hypothesis, they undertook histochemical and immunohistochemical staining for NO synthase in rat islets. The immunohistochemical staining of the endocrine cells as presented [figure 4A in (*1*)] is not clear, however. Furthermore, the fixation condition used with the reduced nicotinamide adenine dinucleotide phosphate (NADPH)–diaphorase histochemistry was not appropriate for the demonstration of NO synthase. When pancreatic tissue was processed with the NADPH-diaphorase histochemical method, which we have shown to be specific for NO synthase (*2*), a different picture emerged (Fig. 1). Darkly stained neurons were found scattered in ganglia in the pancreas, and positive fibers were seen innervating the islets. No staining of the endocrine cells was apparent.

Schmidt *et al.* also show that, in the presence of glucose, the clonal pancreatic B cell line HIT-T15 released nitrate and nitrite in response to arginine. These transformed cells are not normal B cells; they secrete much less insulin than normal cells and have an altered response to glucose. In an earlier study that used rat islets, no difference in nitrite production was seen after treatment with L-arginine (1 mM) or N^G-nitroarginine methyl ester (NAME) (1 mM) without arginine (*3*). Islets accumulate and convert exogenous L-arginine primarily into L-ornithine and urea, not into citrulline, the expected product of a NO synthase (*4*). Also, the response to L-arginine described by Schmidt *et al.* showed a U-shaped dose response that was maximal between 0.1 and 1 mM L-arginine, but 10 mM L-arginine produced no significant effect. However, 10 mM L-arginine is a potent stimulus for insulin secretion from rat islets (*4*). Thus nitrate and nitrite production and insulin secretion from rat islets in response to L-arginine appears to be unrelated.

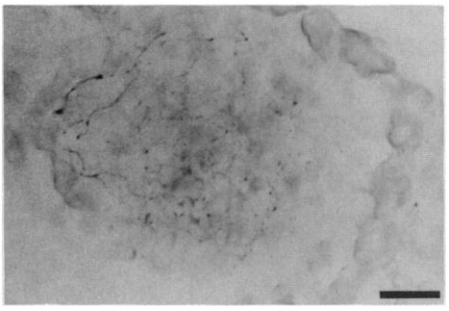

Fig. 1. Histochemical localization of NADPH-diaphorase (NO synthase) in an islet of Langerhans from rat pancreas. A network of thin varicose fibers throughout the islet contains NO synthase, but the endocrine and exocrine cells of the pancreas are unstained. The rat was perfused with paraformaldehyde (4%) in a phosphate buffer (0.1 M), and the pancreas was removed, soaked in 15% sucrose overnight, sectioned at 20 μm, and thaw-mounted onto submerged slides in a cryostat. Sections were stained in 10 ml of 20 mM sodium phosphate buffer (pH 7.4) containing 10 mg of β-NADPH, 1 mg of nitroblue tetrazolium, and 0.3% Triton X-100, at 37°C for 1 hour. They were then rinsed in buffer, air-dried, and coverslipped. Scale bar, 100 μm.

Arginine-induced increases in islet guanosine 3′,5′-monophosphate (cGMP) also appear unrelated to arginine-induced insulin release. Thus, D-arginine is as potent an insulinotropic agent as L-arginine, although it is not a substrate for NO synthase and does not lead to an increase in islet cGMP concentrations (*5*).

Schmidt *et al.* report that N^G-methyl-L-arginine (MeArg) and N^G-nitro-L-arginine (NOArg) inhibited glucose-evoked insulin release from HIT-T15 cells. MeArg and NOArg, however, stimulated insulin release from cultured islets exposed to glucose (*3*). C. Southern *et al.* found that incubation of isolated islets with L-arginine (1 mM) or NAME (1 mM) had no effect on insulin secretion in response to 20 mM of glucose (*6*). Instead, they found that interleukin-1β–induced inhibition of insulin secretion was mediated by L-arginine–dependent NO formation (*6*). NO generation cannot simultaneously mediate both the stimulation and inhibition of insulin secretion from B cells.

Schmidt *et al.* show that intravenous injection of MeArg produced a dose-dependent inhibition of glucose-stimulated insulin secretion in the anesthetized rat. However, no evidence is presented that this effect was mediated within the islets. Indeed, others have found that both arginine and MeArg stimulate insulin release in the perfused rat pancreas (*3*).

Schmidt *et al.* suggest that the observation that insulin secretion is correlated with the intracellular concentration of NADPH (*7*) supports the hypothesis that L-arginine is converted to NO by a type I NO synthase. They do not mention, however, that L-arginine–induced insulin release was not associated with any change in NADPH fluorescence in islets (*7*).

Finally, Schmidt *et al.* suggest that a defect in arginine oxidation may be involved in the pathogenesis of diabetes mellitus. In patients with noninsulin-dependent diabetes mellitus, however, the B cell response to arginine appears normal (*8*). Instead, these patients show a characteristic and selective loss of glucose-induced insulin secretion.

L-lysine, L-ornithine, L-homoarginine, and other cationic amino acids produce an increase in insulin secretion similar to that seen with L-arginine. This insulinotropic action is dependent on amino acid accumulation, but does not require their metabolism (*4*). A similar mechanism probably accounts for the insulin secretion induced by positively charged arginine analogs such as MeArg and NOArg (*3*). Thus, although NO generation in parasympathetic neurons may influence islet function, NO production within B cells cannot explain the secretory action of cationic amino acids.

Steven R. Vincent
Department of Psychiatry,
University of British Columbia,
2255 Wesbrook Mall,
Vancouver, British Columbia,
Canada V6T 1Z3

REFERENCES

1. H. H. H. W. Schmidt, T. D. Warner, K. Ishii, H. Sheng, F. Murad, *Science* **255**, 721 (1992).
2. B. T. Hope, G. J. Michael, K. M. Knigge, S. R. Vincent, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2811 (1991).
3. L. Jansson and S. Sandler, *Endocrinology* **128**, 3081 (1991).
4. F. Blachier, A. Mourtada, A. Sener, W. J. Malaisse, *ibid.* **124**, 134 (1989).
5. S. G. Laychock, M. E. Modica, C. T. Cavanaugh, *ibid.* **129**, 3043 (1991).
6. C. Southern, D. Schulster, I. C. Green, *FEBS Lett.* **276**, 42 (1990).
7. U. Panten and J. Christians, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **276**, 55 (1973).
8. J. L. Leahy, S. Bonner-Weir, G. C. Weir, *Diabetes Care* **15**, 442 (1992); R. H. Unger, *Science* **251**, 1200 (1991).

28 April 1992; accepted 14 July 1992

Response: We detected immunohistochemically type I NO synthase (NOS) in pancreatic B cells with the use of a monospecific antiserum to rat cerebellum type I NOS (*1*). This was supported by protein immunoblot studies with the HIT-T15 pancreatic B cell line. The histochemical NADPH-diaphorase (NADPH-d) stain may, under certain conditions, serve as an additional marker for one or more types of NOS (*2*). S. R. Vincent's figure 1 shows weak NADPH-d staining diffusely over an islet of Langerhans and more intensely in nerve fibers. In rat pancreas tissue stained for NADPH-d (*3*), we observed that islets of Langerhans stain intensely (Fig. 1) and diffusely (*1*) for NADPH-d, suggesting the presence of NOS in the majority of islet

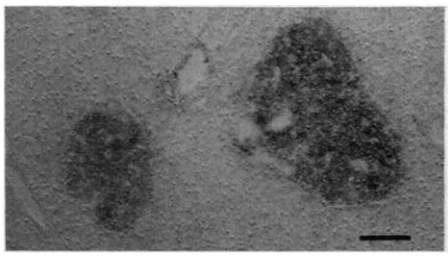

Fig. 1. Histochemical localization of NADPH-d in islets of Langerhans from rat. Preparations of sections, fixing, and staining procedures were as described in (*3*). Scale bar, 0.2 mm.

endocrine cells. J. M. Polak and her co-workers have confirmed our immunohistochemical localization of NOS to endocrine cells of rat pancreatic islets with the use of another antibody to NOS (*4*). Our antibody to NOS type I cross-reacts with nitrinergic nerves and neurons in different organs and species (*5*), and we would expect the same for nitrinergic nerves in islets. However, the strength of the NADPH-d reaction that we observed in the entire islet may have masked labeling of NADPH-d–positive fine nerve fibers described by Vincent.

We do not agree with Vincent's criticisms against the use of HIT-T15 cells as these cells are known to retain the secretory properties of normal pancreatic B cells to glucose, L-arginine, sulfonylureas, and activators of protein kinases; also, both adenosine triphosphate–sensitive K^+ channels and voltage-sensitive Ca^{2+} channels are present in HIT-T15 cells, as required for physiological glucose-stimulated insulin release, (*6*, *7*).

Vincent states that the amount of cGMP in pancreatic islets does not generally correlate with insulin secretion. Nonetheless, it has been shown that exogenous cGMP or stable cGMP—analogs trigger insulin release (*8*). S. G. Laychock *et al.* (*9*) also presented pharmacological evidence (i) that glucose and L-arginine increase cGMP in isolated pancreatic islets and in cultured RINm5F insulinoma cells and (ii) that insulin release induced by glucose and L-arginine is attenuated by the NOS inhibitor N^{ω}-methyl-L-arginine (MeArg) and by the soluble guanylyl cyclase-inhibitors methylene blue and LY83583. Laychock *et al.* (*9*) and we (*1*) have found that about 40% of such insulin release is blocked by NOS inhibitors. Although S. Sandler and co-workers (*10*) showed that the NOS inhibitors MeArg and N^{ω}-nitro-L-arginine (NO_2Arg) stimulate rather than inhibit insulin release, this occurred in the presence of low amounts of glucose that do not cause release of insulin. We demonstrated that insulin release stimulated by glucose (either alone or in synergistic combination with L-arginine) is specifically mediated by NO (*1*). Sandler and co-workers (*11*) found, as we did, that the NOS inhibitors MeArg and NO_2Arg reduce insulin release in response to glucose.

Vincent cites a paper by F. Blachier *et al.* (*12*), which suggests that supraphysiological concentrations of L-arginine and other cationic amino acids stimulate insulin release by a common nonspecific charge effect. However, this view was negated in a later paper from the same laboratory (*13*). We observed a U-shaped concentration-response curve for L-arginine–induced NO release in HIT-T15 cells. A similar decrease in the effectiveness of supraphysiological concentrations of pancreatic B cell stimulators was observed for the insulin and cGMP response to L-arginine (*1*) and the insulin response to glucose (*7*).

Vincent states that "NO generation cannot simultaneously mediate both the stimulation and inhibition of insulin secretion from B cells." However, small amounts of NO can produce a physiological signal, whereas large amounts are cytotoxic (*14*). Depending on the concentration and duration of exposure to NO, both physiological and pathophysiological effects can occur within one organ system (*14*). Interleukin-1β–activated macrophages kill syngeneic islet cells by a process that involves induction of a NOS (presumably type II) and release of large quantities of NO (*15*). The L-arginine:NO pathway we described (*1*) involves a constitutive type I isoform of NOS that produces relatively small quantities of NO, presumably for cell-signaling purposes.

It has been shown (*16*) that the amount of intracellular NADPH, a co-substrate of NOS, is correlated with (glucose-induced) insulin release. Glucose, the trigger for insulin release, is metabolized to produce NADPH. We did not imply an L-arginine–induced change (increase or decrease) in islet NADPH concentrations, nor would we expect it. First, the metabolism of L-arginine to NO consumes, rather than generates, NADPH, and second, the turnover rate of NADPH by a constitutive type I NOS is probably too small relative to that required for an appreciable decrease in the intracellular NADPH.

Vincent remarks that citrulline is not the major product of L-arginine metabolism by pancreatic B cells. However, essentially the same argument can be made for almost every other cell type that contains the L-arginine:NO pathway. The overall contribution of this pathway to intracellular L-arginine metabolism is too small to cause an increase in the amounts of intracellular L-citrulline that are comparable to the amounts of metabolites arising from urea cycle enzymes. Moreover, several cell types regenerate L-arginine from L-citrulline (*17*), and this may also be the case for pancreatic B cells.

The insulinotropic effect of L-arginine depends on the concentration of plasma glucose and is not normal in patients with noninsulin-dependent diabetes mellitus (NIDDM). The seemingly normal insulin response to L-arginine in NIDDM (reference 8 of Vincent's comment) is an artifact of hyperglycemia in these patients (*18*). When nondiabetic subjects are made as hyperglycemic as NIDDM patients, the insulin response to L-arginine is greatly diminished (*19*). Therefore, constitutive NO generation in pancreatic B cells influences islet function and is a mechanism of insulin secretion induced by L-arginine and D-glucose.

H. H. H. W. Schmidt
Medizinische Universitätsklinik, Klinische Biochemie und Pathobiochemie, Versbacher Strasse 5, W-8700 Würzburg, Federal Republic of Germany
T. D. Warner
William Harvey Research Institute, St. Bartholomew's Hospital Medical College, London EC1A-7BE, United Kingdom
K. Ishii
Department of Pharmacology, University of Shizuoka School of Pharmaceutical Sciences, 395 Yada, Shizuoaka-Shi, Skizuoka-ken 422, Japan
H. Sheng
Department of Pharmacology, University of California School of Medicine, Los Angeles, CA 90024
F. Murad
Department of Pharmacology, Northwestern University Medical School, Chicago, IL 60611
Abbott Laboratories, Abbott Park, IL 60064

REFERENCES AND NOTES

1. H. H. H. W. Schmidt, T. D. Warner, K. Ishii, H. Sheng, F. Murad, *Science* **255**, 721 (1992).
2. B. T. Hope, G. J. Michael, K. M. Knigge, S. R. Vincent, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2811 (1991).
3. Pancreata were excised from Sprague-Dawley rats, frozen in hexane–dry ice, and mounted onto microtome chucks with OCT (Tissue-Tek, Elkhart, IN) mounting medium. Sections (8 μm) were immediately cut on a Reichert-Jung Frigocut 2800 cryomicrotome, thaw-mounted onto microscope slides, fixed by immersion in acetone at 4°C for 5 min, air-dried, and stored at 4°C until used. To detect NADPH-d, slides were immersed for 20 to 30 min at 37°C in 50 mM tris-HCl (pH 8.0) containing 1 mM NADPH (8.3 mg/10 ml), 0.5 mM nitroblue tetrazolium (4.1 mg/10 ml), and Triton X-100 (0.2%, v/v). They were washed briefly in phosphate-buffered saline, counterstained with eosin, dehydrated in a graded series of ethyl alcohol solutions, and mounted with Permount.
4. J. M. Polak, personal communication.
5. H. H. H. W. Schmidt *et al.*, *J. Histochem. Cytochem.* **40**, 1439 (1992); C. J. S. Hassal *et al.*, *Neurosci. Lett.*, in press; A. Belai *et al.*, *ibid.*, in press.
6. S. J. Hughes, J. G. Chalk, S. J. H. Ashcroft, *Biochem. J.* **267**, 227 (1990); R. F. Santerre *et al.*,

Proc. Natl. Acad. Sci. U.S.A. **78**, 4339 (1981); R. Regazzi, G. Li, J. Deshusses, C. B. Wollheim, *J. Biol. Chem.* **265**, 15003 (1990); T. Y. Nelson, K. L. Gaines, A. S. Rajan, M. Berg, A. E. Boyd, *ibid.* **262**, 2608 (1987); I. Niki, M. Welsh, P.-O. Berggren, P. Hubbard, S. J. H. Ashcroft, *Biochem. J.* **277**, 619 (1991); R. S. Hill and A. E. Boyd, *Diabetes* **34**, 115 (1985).
7. S. J. H. Ashcroft, P. Hammonds, D. E. Harrison, *Diabetologica* **29**, 727 (1986).
8. N. Voyles *et al.*, *Hormone Res.* **4**, 65 (1973); S. G. Laychock, *Endocrinology* **108**, 1197 (1981); S. G. Laychock, *ibid.* **120**, 517 (1987).
9. S. G. Laychock, M. E. Modica, C. T. Cavanaugh, *Endocrinology* **129**, 3043 (1991).
10. L. Jansson and S. Sandler, *ibid.*, **128**, 3081 (1991).
11. N. Welsh, D. L. Eizirik, K. Bendtzen, S. Sandler, *ibid.* **129**, 3167 (1991).
12. F. Blachier, A. Mourtada, A. Sener, W. J. Malaisse, *ibid.* **124**, 134 (1989).
13. A. Sener, J. Blachier, J. Rasschaert, W. J. Malaisse, *ibid.* **127**, 107 (1990).
14. H. H. H. W. Schmidt, T. D. Warner, F. Murad, *Lancet* **339**, 986 (1992).
15. C. Southern, D. Schulster, I. C. Green, *FEBS Lett.* **276**, 42 (1990); K. D. Kröncke, V. Kolb-Bachofen, B. Berschick, V. Burkart, H. Kolb, *Biochem. Biophys. Res. Commun.* **175**, 752 (1991).
16. U. Panten, J. Christians, E. von Kriegstein, W. Poser, A. Hasselblatt, *Diabetologica* **9**, 477 (1973); U. Panten and J. Christians, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **276**, 55 (1973).
17. G. Wu and J. T. Brosnan, *Biochem. J.* **281**, 45 (1992); R. M. J. Palmer, D. S. Ashton, S. Moncada, *Nature* **333**, 664 (1988); J. A. Mitchell, M. Hecker, J. R. Vane, *Eur. J. Pharmacol.* **176**, 253 (1990); J. A. Mitchell, M. Hecker, E. E. Änggård, J. R. Vane, *ibid.* **182**, 573 (1990).
18. E. Cerasi, *Diabetes Res. Clin. Practice* **14**, S37 (1991).
19. G. D. Dimitriadis, G. B. Pehling, J. E. Gerich, *Diabetes* **34**, 541 (1985); R. Nesher, B. Tuch, C. Hage, J. Levy, E. Cerasi, *Diabetologia* **26**, 142 (1984); W. K. Ward, D. C. Bolgiano, B. McKnight, J. B. Halter, J. Porte D., *J. Clin. Invest.* **74**, 1318 (1984).
20. We acknowledge helpful discussions with S. G. Laychock, S. S. Gross, and R. Weinberg and the assistance of G. D. Gagne and M. F. Miller with photographic reproduction.

3 June 1992; accepted 14 July 1992

Polyamine Depletion and Drug-Induced Chromosomal Damage: New Results

During studies to establish why pretreatment of 9L cells with α-difluoromethylornithine (DFMO) enhanced cell killing induced by 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) (*1*) and reduced cell killing induced by *cis*-platinum (*2*), we reported, in 1982, an enhancement of BCNU-induced sister chromatid exchanges (SCE's) and a reduction in *cis*-platinum–induced SCE frequency with DFMO pretreatment (*3*). Subsequently, we could neither replicate the DFMO enhancement of BCNU-induced SCE's in separate experiments (*4*) nor confirm those findings by rescoring the slides from the original experiments. In 1989, we therefore retracted our report of DFMO enhancement of BCNU-induced SCEs in 9L cells (*4*). The enhancement of BCNU cell killing by DFMO pretreatment had proved certain, however, and the discrepancy was puzzling in view of repeated confirmation of the correlation between reduced cell killing and SCE frequency with *cis*-platinum (*3*).

New experimental results reported by others (*5*) show that when DFMO remains in the cell culture medium during the period of BCNU treatment, the procedure enhances the number of BCNU-induced SCE's in 9L cells. When the DFMO-containing medium is removed and the cells are rinsed before BCNU is added, however, the additional rinsing procedure greatly diminishes or eliminates the enhancement. We believe that our replicate experiments (*4*) deviated from our original protocol (*3*) by the introduction of an additional rinsing procedure, thereby accounting for the discrepant results. There is now evidence that slides containing DFMO-treated cells degrade over time (*6*). Such a degradation over the 5 years between the original scoring of the slides and the rescoring attempts could have contributed to our inability to confirm the original findings.

P. J. Tofilon*
Brain Tumor Research Center, Department of Neurological Surgery, University of California, San Francisco, CA 94143
D. F. Deen
Brain Tumor Research Center, Department of Neurological Surgery, and Department of Radiation Oncology, University of California, San Francisco
L. J. Marton†
Department of Laboratory Medicine and Brain Tumor Research Center, University of California, San Francisco

REFERENCES

1. D. T. Hung, D. F. Deen, J. Seidenfield, L. J. Marton, *Cancer Res.* **41**, 2783 (1981).
2. S. M. Oredsson, D. F. Deen, L. J. Marton, *ibid.* **42**, 1296 (1982).
3. P. J. Tofilon, S. M. Oredsson, D. F. Deen, L. J. Marton, *Science* **217**, 1044 (1982).
4. K. M. Milam, S. Horn, P. J. Tofilon, D. F. Deen, L. J. Marton, *ibid.* **244**, 631 (1989).
5. S. Wolff, L. Feeney, V. Afzal, *Mutat. Res.*, in press.
6. S. Wolff, personal communication.

11 August 1992; accepted 11 September 1992

*Present address: Department of Experimental Radiotherapy, M. D. Anderson Hospital and Tumor Institute, 6723 Bertner Avenue, Houston, TX 77030.
†Present address: Office of the Dean, School of Medicine, University of Wisconsin, 1205 Medical Sciences Center, 1300 University Avenue, Madison, WI 53706.