23. Hui, S. T. *et al.* Txnip balances metabolic and growth signaling via PTEN disulfide reduction. *Proc. Natl Acad. Sci. USA* **105,** 3921–3926 (2008).
24. Zhang, C. Y. *et al.* Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell* **105,** 745–755 (2001).
25. Rosen, E. D. & Spiegelman, B. M. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* **444,** 847–853 (2006).
26. Sreekumar, R., Halvatsiotis, P., Schimke, J. C. & Nair, K. S. Gene expression profile in skeletal muscle of type 2 diabetes and the effect of insulin treatment. *Diabetes* **51,** 1913–1920 (2002).
27. Tamemoto, H. *et al.* Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* **372,** 182–186 (1994).
28. Muoio, D. M. & Newgard, C. B. Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat. Rev. Mol. Cell Biol.* **9,** 193–205 (2008).
29. Hackenbrock, C. R. Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria. *J. Cell Biol.* **30,** 269–297 (1966).
30. Bonnard, C. *et al.* Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice. *J. Clin. Invest.* **118,** 789–800 (2008).
31. Meglasson, M. D. & Matschinsky, F. M. Pancreatic islet glucose metabolism and regulation of insulin secretion. *Diabetes Metab. Rev.* **2,** 163–214 (1986).
32. MacDonald, M. J. Synergistic potent insulin release by combinations of weak secretagogues in pancreatic islets and INS-1 cells. *J. Biol. Chem.* **282,** 6043–6052 (2007).
33. Oberkofler, H., Klein, K., Felder, T. K., Krempler, F. & Patsch, W. Role of peroxisome proliferator-activated receptor-gamma coactivator-1alpha in the transcriptional regulation of the human uncoupling protein 2 gene in INS-1E cells. *Endocrinology* **147,** 966–976 (2006).
34. Medvedev, A. V. *et al.* Regulation of the uncoupling protein-2 gene in INS-1 beta-cells by oleic acid. *J. Biol. Chem.* **277,** 42639–42644 (2002).
35. Fan, M. *et al.* Suppression of mitochondrial respiration through recruitment of p160 myb binding protein to PGC-1alpha: modulation by p38 MAPK. *Genes Dev.* **18,** 278–289 (2004).
36. Ahsan, M. K., Nakamura, H., Masutani, H. & Yodoi, J. Thioredoxin and thioredoxin-binding protein-2 in cancer and metabolic syndrome. *Free Radic. Biol. Med.* **43,** 861–868 (2007).
37. Yoshihara, E., Chen, Z., Matsuo, Y., Masutani, H. & Yodoi, J. Thiol redox transitions by thioredoxin and thioredoxin-binding protein-2 in cell signaling. *Methods Enzymol.* **474,** 67–82 (2010).
38. Stoltzman, C. A. *et al.* Glucose sensing by MondoA:Mlx complexes: a role for hexokinases and direct regulation of thioredoxin-interacting protein expression. *Proc. Natl Acad. Sci. USA* **105,** 6912–6917 (2008).
39. Peterson, C. W., Stoltzman, C. A., Sighinolfi, M. P., Han, K. S. & Ayer, D. E. Glucose controls nuclear accumulation, promoter binding, and transcriptional activity of the MondoA-Mlx heterodimer. *Mol. Cell Biol.* **30,** 2887–2895 (2010).
40. Kaadige, M. R., Looper, R. E., Kamalanaadhan, S. & Ayer, D. E. Glutamine-dependent anapleurosis dictates glucose uptake and cell growth by regulating MondoA transcriptional activity. *Proc. Natl Acad. Sci. USA* **106,** 14878–14883 (2009).
41. Chen, J. L. *et al.* Lactic Acidosis Triggers Starvation Response with Paradoxical Induction of TXNIP through MondoA. *PLoS Genet.* **6,** e1001093 (2010).
42. Chutkow, W. A. *et al.* Deletion of the alpha-arrestin protein Txnip in mice promotes adiposity and adipogenesis while preserving insulin sensitivity. *Diabetes* **59,** 1424–1434 (2010).
43. Sesti, G. *et al.* Defects of the insulin receptor substrate (IRS) system in human metabolic disorders. *FASEB J.* **15,** 2099–2111 (2001).
44. Ferre, P. The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes* 53 (Suppl 1), S43–50 (2004).
45. Shaked, M. *et al.* Insulin counteracts glucotoxic effects by suppressing thioredoxin-interacting protein production in INS-1E beta cells and in Psammomys obesus pancreatic islets. *Diabetologia* **52,** 636–644 (2009).
46. Masson, E. *et al.* High beta-cell mass prevents streptozotocin-induced diabetes in thioredoxin-interacting protein-deficient mice. *Am. J. Physiol. Endocrinol. Metab.* **296,** E1251–1261 (2009).
47. Lin, C. H., MacGurn, J. A., Chu, T., Stefan, C. J. & Emr, S. D. Arrestin-related ubiquitin-ligase adaptors regulate endocytosis and protein turnover at the cell surface. *Cell* **135,** 714–725 (2008).
48. Hirasawa, T., Ohara, T. & Makino, S. Genetic typing of the mouse ob mutation by PCR and restriction enzyme analysis. *Exp. Anim.* **46,** 75–78 (1997).
49. Sutton, R., Peters, M., McShane, P., Gray, D. W. & Morris, P. J. Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation* **42,** 689–691 (1986).
50. Fujimoto, S. *et al.* The novel insulinotropic mechanism of pimobendan: Direct enhancement of the exocytotic process of insulin secretory granules by increased Ca2+ sensitivity in beta-cells. *Endocrinology* **139,** 1133–1140 (1998).

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Author contributions

E.Y. designed and performed the experiments and wrote the manuscript with the help of S.F., N.I., J.Y. and H.M. E.Y., S.F., N.I and H.M. analysed the data. K.O. performed mass spectrometry analysis. S.M. prepared protein-immobilized beads. I.Y. directed the project as the principal investigator. H.M. supervised the study. All authors discussed the results and commented on the manuscript.

Additional information

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Exendin-4 Suppresses Src Activation and Reactive Oxygen Species Production in Diabetic Goto-Kakizaki Rat Islets in an Epac-Dependent Manner

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OBJECTIVE—Reactive oxygen species (ROS) is one of most important factors in impaired metabolism secretion coupling in pancreatic β-cells. We recently reported that elevated ROS production and impaired ATP production at high glucose in diabetic Goto-Kakizaki (GK) rat islets are effectively ameliorated by Src inhibition, suggesting that Src activity is upregulated. In the present study, we investigated whether the glucagon-like peptide-1 signal regulates Src activity and ameliorates endogenous ROS production and ATP production in GK islets using exendin-4.

RESEARCH DESIGN AND METHODS—Isolated islets from GK and control Wistar rats were used for immunoblotting analyses and measurements of ROS production and ATP content. Src activity was examined by immunoprecipitation of islet lysates followed by immunoblotting. ROS production was measured with a fluorescent probe using dispersed islet cells.

RESULTS—Exendin-4 significantly decreased phosphorylation of Src Tyr416, which indicates Src activation, in GK islets under 16.7 mmol/l glucose exposure. Glucose-induced ROS production (16.7 mmol/l) in GK islet cells was significantly decreased by coexposure of exendin-4 as well as PP2, a Src inhibitor. The Src kinase–negative mutant expression in GK islets significantly decreased ROS production induced by high glucose. Exendin-4, as well as PP2, significantly increased impaired ATP elevation by high glucose in GK islets. The decrease in ROS production by exendin-4 was not affected by H-89, a PKA inhibitor, and an Epac-specific cAMP analog (8CPT-2Me-cAMP) significantly decreased Src Tyr416 phosphorylation and ROS production.

CONCLUSIONS—Exendin-4 decreases endogenous ROS production and increases ATP production in diabetic GK rat islets through suppression of Src activation, dependently on Epac. ***Diabetes* 60:218–226, 2011**

In pancreatic β-cells, glucose metabolism regulates exocytosis of insulin granules through metabolism secretion coupling, in which glucose-induced ATP production in mitochondria plays an essential role (1). Impairment of mitochondrial ATP production causes reduced glucose-induced insulin secretion.

Reactive oxygen species (ROS) is one of the most important factors that impair metabolism secretion coupling in β-cells. Exposure to exogenous hydrogen peroxide (H_2O_2), the most abundant ROS, reduces glucose-induced insulin secretion by impairing mitochondrial metabolism in β-cells (2,3). However, little is known of the role of endogenous ROS in impaired glucose-induced insulin secretion from β-cells. Some studies (4,5) have shown that endogenous ROS is produced in mitochondria by exposure to high glucose. In Zucker diabetic fatty rats, the superoxide content of islets at basal glucose levels is higher than that in Zucker lean control rats (4). Furthermore, we recently reported that high glucose–induced ROS production in islet cells is elevated in diabetic Goto-Kakizaki (GK) rats compared with control Wistar rats (6). Thus, endogenous ROS production is elevated in β-cells under diabetic pathophysiological conditions.

Although the mechanism of endogenous ROS production in β-cells in the diabetic state remains largely unknown, we have reported that Src (c-Src) plays an important role in the signal transduction that produces ROS (6). Src is a nonreceptor tyrosine kinase that is associated with the cell membrane and plays important roles in various signal transductions, and its activity is regulated by intramolecular interactions that depend on tyrosine phosphorylation (7,8). Phosphorylation of Tyr527 (Tyr529 in humans), which is located near the C terminus of Src, is brought about by COOH terminal Src kinase (Csk), a negative regulator of Src (9), and holds the kinase in the inactive form. Dephosphorylation of Tyr527 followed by disruption of the intramolecular interaction allows phosphorylation of Tyr416 (Tyr418 in humans) at the kinase domain, resulting in Src activation. In our previous report (6), PP2, a selective Src inhibitor, decreased high-glucose–induced ROS production in GK islet cells, in contrast to the lack of any effect of the agent in Wistar islet cells, suggesting that Src may be activated in the diabetic condition and cause elevation of ROS production in the presence of high glucose.

Glucagon-like peptide (GLP)-1 is one of the incretin peptides released from the intestine in response to nutrient ingestion that augments glucose-induced insulin secretion from β-cells (10,11). GLP-1 binding to the GLP-1 receptor, a member of the G protein–coupled receptor

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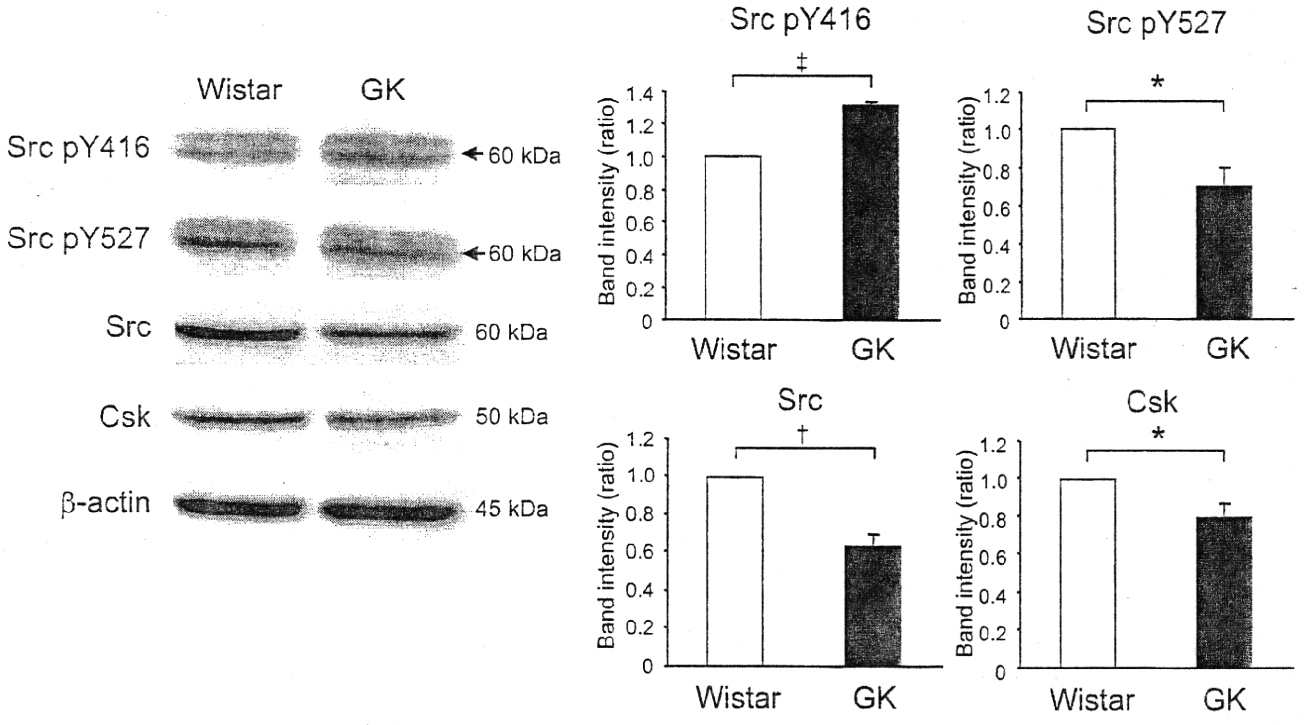

FIG. 1. Comparison of expression of Src between fresh Wistar and GK islets. Fresh islets were lysated and subjected to immunoblot analyses. Blots (50 μg of protein) were probed with anti–phospho-Src (Tyr^{416}), anti–phospho-Src (Tyr^{527}), anti-Src, or anti-Csk. The same blots were stripped and reprobed with anti–β-actin, respectively. Intensities of the bands were quantified with densitometric imager. The bar graphs are expressed relative to Wistar islet value corrected by β-actin level (means ± SE). *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$. Representative blot panels of three to five independent experiments are shown.

(GPCR) superfamily, induces activation of adenylyl cyclase and elevation of intracellular cAMP levels, which elicits protein kinase A (PKA)-dependent signal transduction. Recently, Epac (also known as cAMP-GEF [guanine nucleotide exchange factor]) has been shown to be a novel cAMP sensor in the PKA-independent pathway (12,13). In β-cells, one member of the Epac family, Epac2, has an important role in insulin secretion, especially in regulation of exocytosis of insulin granules (14,15). Previous studies have shown that GLP-1 also has beneficial long-term effects on diabetic β-cells, including induction of β-cell proliferation (16,17), enhanced resistance to apoptosis (17,18), and amelioration of endoplasmic reticulum stress (19). Furthermore, increased ROS in diabetic *db/db* mouse islets is decreased by treatment with an inhibitor of dipeptidyl peptidase IV that delays the degradation of GLP-1 (20).

In the present study, we investigated whether the GLP-1 signal directly ameliorates endogenous ROS production in diabetic GK islets using exendin-4, a GLP-1 receptor agonist. In particular, we focused on clarifying regulation of Src activity by GLP-1 signaling. We describe here both a novel effect and a mechanism of GLP-1 signaling that acutely decreases ROS production by high glucose through suppression of Src activation PKA independently and Epac dependently.

RESEARCH DESIGN AND METHODS

Male Wistar and GK rats were obtained from Shimizu (Kyoto, Japan). All experiments were carried out with rats that were aged ~7–8 weeks. Nonfasting blood glucose levels were ~160–240 mg/dl in the GK rats and ~70–120 mg/dl in the Wistar rats used in the experiments. The animals were maintained and used in accordance with the guidelines of the animal care committee of Kyoto University.

Islet preparation. Pancreatic islets were isolated from Wistar and GK rats by the collagenase digestion technique (6). Isolated islets were washed with Krebs Ringer bicarbonate buffer (KRBB) (in mmol/l: 129.4 NaCl, 5.2 KCl, 2.7 $CaCl_2$, 1.3 KH_2PO_4, 1.3 $MgSO_4$, and 24.8 $NaHCO_3$ [equilibrated with 5% CO_2/95% O_2, pH 7.4]) containing 2.8 mmol/l glucose and cultured for ~20 h in RPMI-1640 medium containing 5.5 mmol/l glucose and 10% FCS. Cultured islets were preincubated for 30 min at 37°C in KRBB supplemented with 0.2% BSA and 10 mmol/l HEPES (KRBB medium) containing 2.8 mmol/l glucose and incubated for the indicated times at 37°C in KRBB medium containing 16.7 mmol/l glucose with or without test materials.

Retroviral-mediated gene transfer. Production of retroviral vectors with pCX4 was performed as previously described (21). Src kinase–negative mutant (K295M) was subcloned into pCX4pur (22). Gene transfer experiments of islets were carried out by an in vivo gene transduction method (23). Briefly, after rats were anesthetized and subjected to laparotomy, the hepatic artery with the portal vein and the splenic artery were ligated. The upper side of the celiac artery that branches from the abdominal aorta was clamped, and 100 μl of retroviral vector suspension was injected into the lower side of the clamped point of the artery. The pancreatic islets were then isolated and cultured for 48 h before the experiment. Gene expression using green fluorescent protein–expressing vector was effective in the inside of the islets, as previously reported (23).

Immunoprecipitation and immunoblotting. Fresh or incubated islets were lysed in ice-cold lysis buffer (10 mmol/l Tris [pH 7.2], 100 mmol/l NaCl, 1 mmol/l EDTA, 1% Nonidet P-40, and 0.5% sodium deoxycholate) containing protease inhibitor cocktail (Complete; Roche, Mannheim, Germany), phosphatase inhibitor cocktail (Calbiochem, Darmstadt, Germany), and 5 mmol/l sodium pyrophosphate. For determination of Src activation, lysates were centrifuged at 560,000*g* for 10 min at 4°C, and the supernatant (~2 mg of protein content/2,500 islets) was mixed with 4 μg mouse monoclonal anti-Src antibody (clone GD11; Upstate, Billerica, MA) and 30 μl washed protein G Sepharose (GE Healthcare, Uppsala, Sweden) followed by gentle rotation for 4 h at 4°C. Immunoprecipitates or islet lysates (50 μg) were subjected to immunoblotting as previously described (23). Primary antibodies used were rabbit anti–phospho-Src (Tyr416) and anti–phospho-Src (Tyr527) from Biosource (Camarillo, CA); rabbit anti-Src, anti-Csk, anti-Epac2, extracellular signal–regulated kinase (ERK) 1/2, and mouse anti–phospho-ERK1/2 (Thr202/Tyr204) from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-Rap1 from Upstate; rabbit anti–phospho-Akt (Ser473) and anti-Akt from Cell

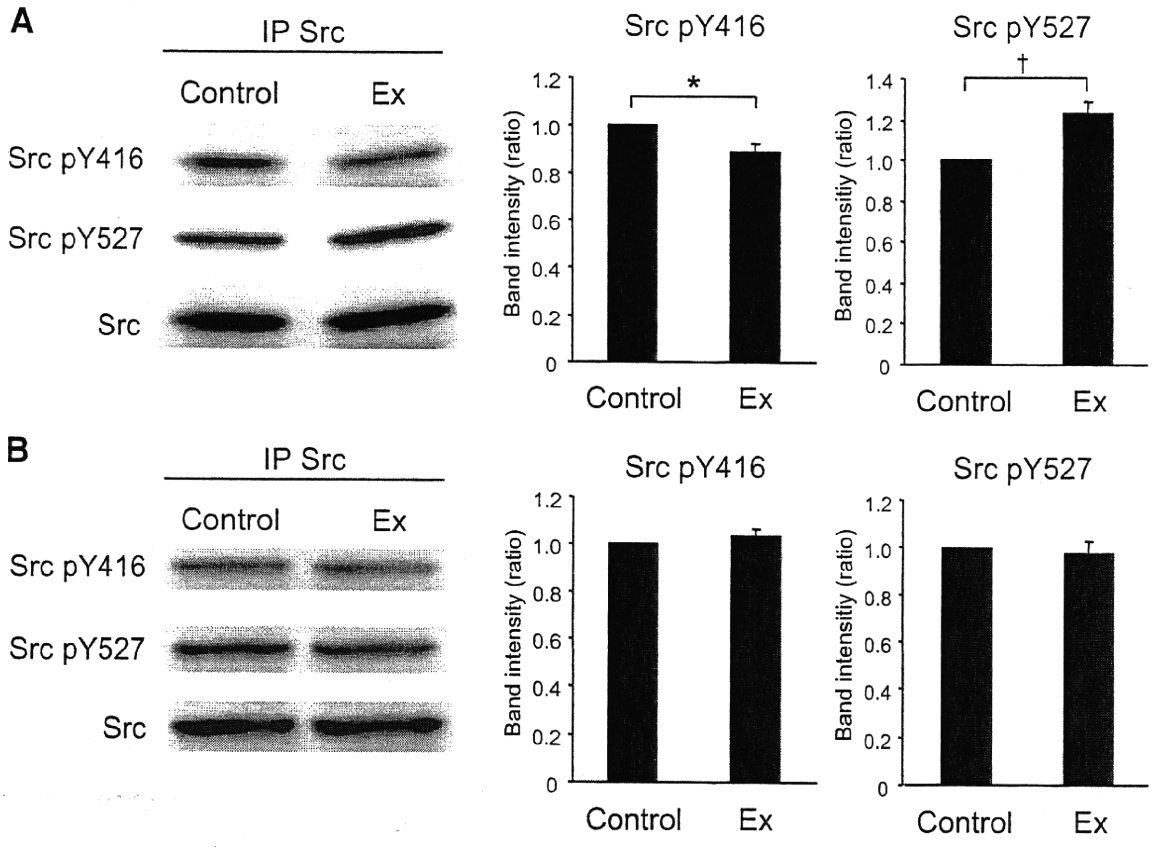

FIG. 2. Exendin-4 suppresses Src activity at high glucose in GK islets. Effects of exendin-4 on Src activity at high glucose in GK (*A*) and Wistar (*B*) islets. After preincubation in the presence of 2.8 mmol/l glucose for 30 min, islets were incubated in the presence of 16.7 mmol/l glucose with or without 100 nmol/l exendin-4 for 10 min. Islet lysates (~2 mg of protein) were immunoprecipitated with anti-Src antibody and subjected to immunoblot analyses. Blots were probed with anti–phospho-Src (Tyr^{416}), anti–phospho-Src (Tyr^{527}), or anti-Src by stripping and reprobing of the same blots. Intensities of the bands were quantified with densitometric imager. The bar graphs are expressed relative to control value corrected by Src level (means ± SE). *$P < 0.05$; †$P < 0.01$. Representative blot panels of four (*A*) or three (*B*) independent experiments are shown.

Signaling (Danvers, MA); and mouse anti–β-actin from Sigma (St. Louis, MO). Secondary antibodies used were horseradish peroxidase–conjugated anti-rabbit and mouse antibody (GE Healthcare). Band intensities were quantified with Multi Gauge software (Fujifilm, Tokyo, Japan).

Measurement of ROS production. ROS production in islet cells was measured by 2′,7′-dichlorofluorescein fluorescence (6). Briefly, cultured islets were dispersed using 0.05% trypsin/0.53 mmol/l EDTA (Invitrogen, Carlsbad, CA) and PBS. Dispersed islet cells were preincubated in KRBB medium containing 2.8 mmol/l glucose and 10 μmol/l 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Invitrogen) for 20 min at 37°C. After a 60-min incubation in 400 μl KRBB medium containing 16.7 mmol/l glucose with or without test materials, fluorescence was measured using a spectrofluorophotometer (RF-5300PC; Shimadzu, Kyoto, Japan), with excitation wavelength at 505 nm and emission wavelength at 540 nm. Fluorescence was corrected by subtracting parallel blanks and represented by fold increases of the value at time zero.

Measurement of ATP content. ATP content in islets was determined by luminometry as previously described (6). Briefly, after preincubation, groups of 10 islets were batch incubated for 30 min in KRBB medium containing 2.8 or 16.7 mmol/l glucose with or without test materials. Incubation was stopped immediately by addition of $HClO_4$ and sonication in ice-cold water for 10 min. They were then centrifuged, and a fraction of the supernatant was mixed with HEPES and Na_2CO_3. The ATP content in the supernatant of islet lysates was measured using ENLITEN luciferase/luciferin reagent (Promega, Madison, WI) with a luminometer (GloMax 20/20n; Promega).

Materials. Exendin-4 and forskolin were purchased from Sigma. PP2 was purchased from Tocris (Ellisville, MO). PP3, H-89, myristoylated PKA inhibitor amide14–22 (PKI), LY294002, wortmannin, PD98059, and AG1478 were purchased from Calbiochem. Dibutyryl cAMP was purchased from Daiichisankyo (Tokyo, Japan). 8-(4-chlorophenylthio)-2′-*O*-methyl-cAMP (8CPT-2Me-cAMP) was purchased from Biolog Life Science (Bremen, Germany).

Statistical analysis. Data are expressed as means ± SE. Statistical significance of difference was evaluated by the unpaired Student *t* test. $P < 0.05$ was considered significant.

RESULTS

Comparison of expression of Src between Wistar and GK islets. To examine whether the expression levels of Src in GK islets differ from those in Wistar islets, immunoblotting using fresh islets was performed. As shown in Fig. 1*A*, the level of Src pY416, which indicates activation of Src, in GK islets was significantly higher than that in Wistar islets. The levels of Src pY527, total Src, and Csk in GK islets were significantly lower than those in Wistar islets. The levels of other Src family kinases (SFKs) were similar in Wistar and GK islets, whereas the expression of Fgr was very low and that of Fyn was undetectable (supplementary Fig. 1 in the online appendix, available at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0021/DC1). Results of immunoblotting using islets cultured for 20 h in the presence of 5.5 mmol/l glucose (supplementary Fig. 2) were similar to those shown in Fig. 1*A*.

Exendin-4 suppresses Src activity in GK islets. To investigate whether exendin-4 regulates Src activity, phosphorylation of Src was examined by immunoprecipitation and immunoblotting. As shown in Fig. 2*A*, Src pY416 was

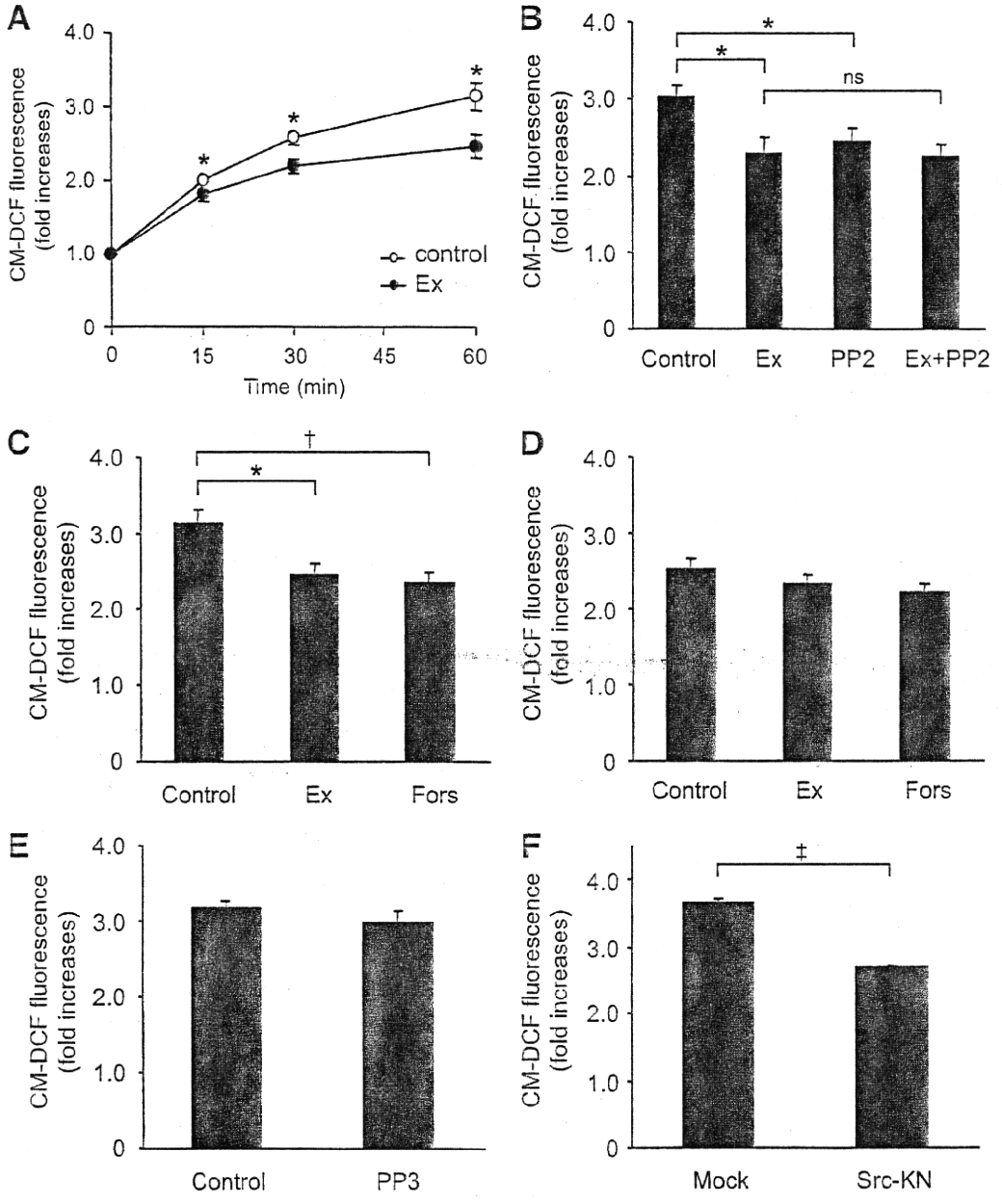

FIG. 3. Exendin-4 decreases ROS production at high glucose in GK islet cells. *A*: Time course of high-glucose–induced ROS production with or without 100 nmol/l exendin-4 in GK islet cells. After preincubation in the presence of 2.8 mmol/l glucose and 10 μmol/l CM-H_2DCFDA for 20 min, dispersed islet cells were incubated in the presence of 16.7 mmol/l glucose with (●) or without (○) 100 nmol/l exedin-4 for 60 min. Fluorescence is represented as fold increases against the value at time zero. Data are expressed as means ± SE (n = 5–7). *P < 0.05 vs. control. *B*: Effects of exendin-4 and PP2 on high-glucose–induced ROS production at 60 min in GK islet cells. Data are expressed as means ± SE (n = 4–6). *P < 0.05. *C*: Effects of exendin-4 and forskolin on high-glucose–induced ROS production at 60 min in GK islet cells. Data are expressed as means ± SE (n = 5–6). *P < 0.05; †P < 0.01. *D*: Effects of exendin-4 and forskolin on high-glucose–induced ROS production at 60 min in Wistar islet cells. Data are expressed as means ± SE (n = 3–4). *E*: Effects of PP3 on high-glucose–induced ROS production at 60 min in GK islet cells. Data are expressed as means ± SE (n = 3). *F*: Effect of Src-KN on high-glucose–induced ROS production at 60 min in GK islet cells. Retroviral (empty vector and Src-KN vector)-mediated gene transfer to islets was carried out by in vivo gene transduction method, as described in RESEARCH DESIGN AND METHODS. Data are expressed as means ± SE (n = 3). ‡P < 0.001.

significantly decreased by 100 nmol/l exendin-4 in the presence of 16.7 mmol/l glucose in GK islets. Exendin-4 also significantly increased Src pY527 in GK islets in the same condition. On the other hand, exendin-4 did not affect Src pY416 or pY527 at high glucose in Wistar islets (Fig. 2*B*). Both Src pY416 and pY527 were not altered by change in glucose concentration in GK or Wistar islets (supplementary Fig. 3).

Exendin-4 decreases ROS production in GK islet cells. We then investigated whether exendin-4 ameliorates endogenous ROS production at high glucose in GK islet cells. A total of 16.7 mmol/l glucose exposure induced ROS production in GK islet cells (Fig. 3*A*). Coexposure of exendin-4 significantly decreased ROS production in the presence of 16.7 mmol/l glucose at 15, 30, and 60 min. A total of 10 μmol/l PP2, a Src inhibitor, significantly de-

creased high-glucose–induced ROS production (Fig. 3*B*), but PP3, the inactive PP2 analog, did not affect it (Fig. 3*E*). Exendin-4 did not further decrease ROS production in the presence of PP2 (Fig. 3*B*), suggesting that the effect of exendin-4 is via the Src signal. The decrease in high-glucose–induced ROS production also was observed in the presence of 10 μmol/l forskolin, an adenylyl cyclase activator (Fig. 3*C*). High-glucose–induced ROS production in Wistar islet cells was lower than that in GK islet cells and was not changed by addition of exendin-4 or forskolin (Fig. 3*D*). To confirm that Src is actually involved in ROS production, we measured ROS production in GK islets expressing a kinase-negative form of Src (Src-KN) by retroviral vector. ROS production in Src-KN–expressing islets was significantly lower than that in control (Fig. 3*F*), demonstrating that Src regulates ROS production in GK islets.

Exendin-4 increases ATP content in GK islets. In Wistar islets, 16.7 mmol/l glucose-exposure significantly increased ATP content compared with that in the presence of 2.8 mmol/l glucose, as shown in Fig. 4*B*. Exendin-4, PP2, or exendin-4 plus PP2 did not affect the ATP content in the presence of 16.7 mmol/l glucose in Wistar islets. The ATP content in GK islets exposed to 16.7 mmol/l glucose was not increased compared with that in the presence of 2.8 mmol/l glucose (Fig. 4*A*). Exendin-4 as well as PP2 significantly increased the ATP content in the presence of 16.7 mmol/l glucose. Further increase of ATP content by combined exendin-4 and PP2 was not observed.

The effects of exendin-4 are dependent on Epac. We then investigated whether the decrease in ROS production by exendin-4 is dependent on PKA. As shown in Fig. 5*A*, decreased ROS production by exendin-4 or forskolin was not affected by 10 μmol/l H-89 or PKI, a PKA inhibitor, indicating that the effect is PKA independent. Not only dibutyryl cAMP, a general cAMP analog, but also 8CPT-2Me-cAMP, an Epac-specific cAMP analog, decreased ROS production (Fig. 5*C*). Epac possesses guanine nucleotide exchange factor activity toward Rap1, a member of the Ras superfamily of small GTPases. Epac2 and Rap1 proteins were expressed similarly in both Wistar and GK islets (Fig. 5*B*). To determine involvement of Epac in Src activation, Src phosphorylation was examined. Src pY416 was significantly decreased by 8CPT-2Me-cAMP (Fig. 5*D*).

A downstream pathway of Src is PI3K/Akt signaling. Src signalings toward downstream proteins are complex, but one of the typical pathways is phosphatidylinositol 3 kinase (PI3K)/Akt signaling (8). We therefore examined the involvement of PI3K/Akt signaling on ROS production. A total of 50 μmol/l LY294002 and 0.5 μmol/l wortmannin, both of which are PI3K inhibitors, significantly decreased ROS production in GK islets (Fig. 6*A*). Exendin-4 and PP2 both significantly decreased phosphorylation of Akt in GK islets (Fig. 6*B*) but not in Wistar islets (Fig. 6*C*). Considering these findings together, PI3K/Akt signaling that produces ROS is located downstream of Src activation. We also examined the involvement of mitogen-activated protein kinase signaling, another downstream pathway of Src. A total of 50 μmol/l PD98059, a MAPK-ERK kinase inhibitor, did not affect ROS production in GK islets (Fig. 6*D*), and neither exendin-4 nor PP2 affected phosphorylation of ERK (Fig. 6*E*). Several GPCR agonists have been shown to induce transactivation of epidermal growth factor receptor (EGFR) (24,25) by a mechanism involving Src (25–27) and frequently subsequent PI3K/Akt signaling (25,28). Therefore, involvement of EGFR transactivation on regulation of ROS production was examined. A total of 0.5 μmol/l AG1478, an EGFR kinase inhibitor, significantly decreased ROS production (Fig. 6*F*).

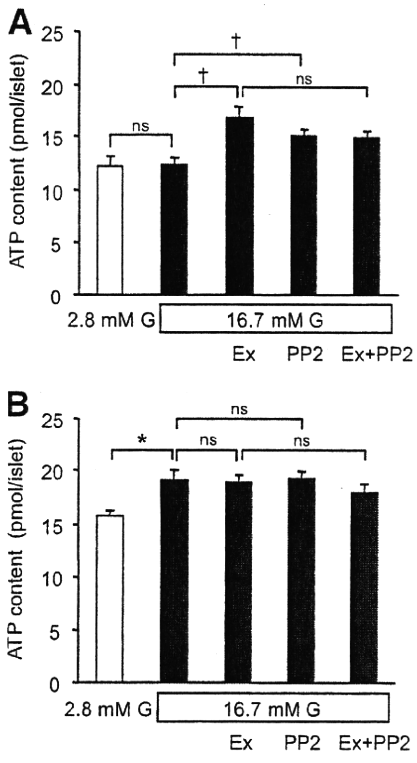

FIG. 4. Exendin-4 increases ATP content at high glucose in GK islets. Effects of exendin-4 and PP2 on ATP content in the presence of high glucose for 30 min in GK (*A*) and Wistar (*B*) islets. After preincubation in the presence of 2.8 mmol/l glucose for 30 min, islets were incubated in the presence of 2.8 or 16.7 mmol/l glucose with or without 100 nmol/l exendin-4, 10 μmol/l PP2, or both for 30 min. Data are expressed as means ± SE (n = 7–8). *P < 0.05; †P < 0.01.

DISCUSSION

We previously reported that endogenous ROS production by high glucose in diabetic GK islets is elevated compared with that in control Wistar islets and is effectively ameliorated by Src inhibition, suggesting that Src may be activated in GK islets (6). In the present study, we first investigated whether Src activity is altered in GK islets. Immunoblotting analysis revealed that the level of Src pY416, which indicates the level of Src activation, is higher in GK islets than that in Wistar islets, despite lower levels of total Src, Src pY527, and Csk. The lower level of total Src seems to be a consequence of Src activation. Targeted degradation of active forms of Src is brought about by ubiquitination (29). The protooncogene c-Cbl, recently found to be an E3 ubiquitin ligase, mediates ubiquitination of activated Src (30). These reports suggest that increased degradation of activated Src may result in a lower level of total Src in GK islets. In addition, a lower level of Csk might cause a lower activity of the kinase in GK islets. However, Src activity is not directly regulated through phosphorylation of Tyr527 by Csk (8), and a subtle decrease in Csk activity is not believed to contribute to regulation of Src activity because of the excess amount of expression of Csk. This is supported by the findings that heterozygous disruption of ubiquitously expressed Csk

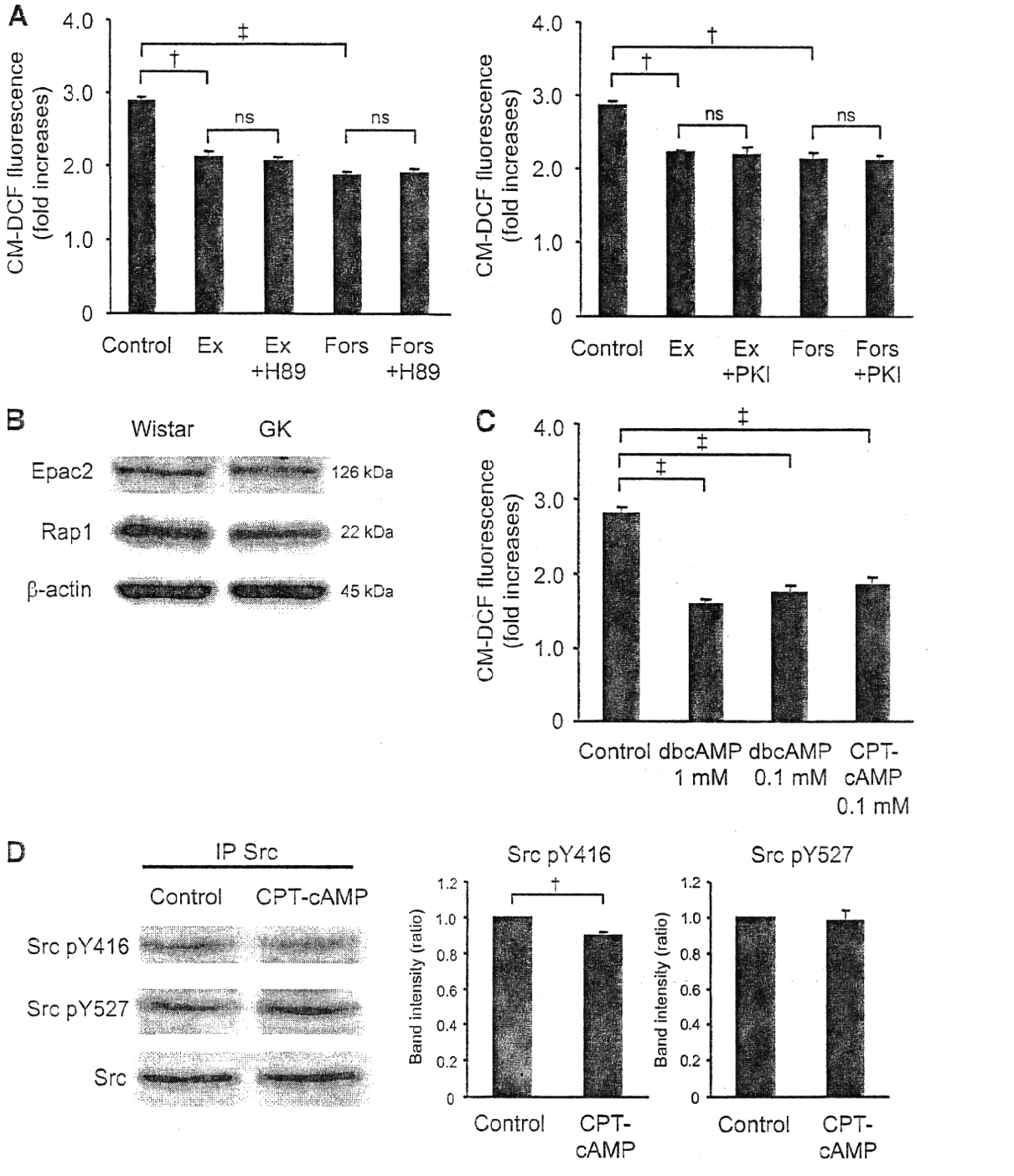

FIG. 5. The effects of exendin-4 are dependent not on PKA but on Epac. *A*: Effects of H-89 or PKI on the decrease in high-glucose–induced ROS production by exendin-4 or forskolin at 60 min in GK islet cells. After preincubation in the presence of 2.8 mmol/l glucose and 10 μmol/l CM-H_2DCFDA for 20 min, dispersed islet cells were incubated in the presence of 16.7 mmol/l glucose with or without 100 nmol/l exendin-4 or 10 μmol/l forskolin with or without 10 μmol/l H-89 or 10 μmol/l PKI for 60 min. Fluorescence is represented as fold increases against the value at time zero. Data are expressed as means ± SE (n = 3). †P < 0.01; ‡P < 0.001. *B*: Expression of Epac2 and Rap1 in Wistar and GK islets. Fresh islets were lysated and subjected to immunoblot analyses. Blots (50 μg of protein) were probed with anti-Epac2 or anti-Rap1. The same blots were stripped and reprobed with anti–β-actin, respectively. Representative blot panels of three independent experiments are shown. *C*: Effects of cAMP analogs on high-glucose–induced ROS production at 60 min in GK islet cells. Data are expressed as means ± SE (n = 3–4). ‡P < 0.001. *D*: Epac-specific cAMP analog suppresses Src activity at high glucose in GK islets. After preincubation in the presence of 2.8 mmol/l glucose for 30 min, islets were incubated in the presence of 16.7 mmol/l glucose with or without 0.1 mmol/l 8CPT-2Me-cAMP for 8 min. Islet lysates (~2 mg of protein) were immunoprecipitated with anti-Src antibody and subjected to immunoblot analyses. Blots were probed with anti–phospho-Src (Tyr^{416}), anti–phospho-Src (Tyr^{527}), or anti-Src by stripping and reprobing of the same blots. Intensities of the bands were quantified with densitometric imager. The bar graphs are expressed relative to control value corrected by Src level (means ± SE). †P < 0.01. Representative blot panels of four independent experiments are shown.

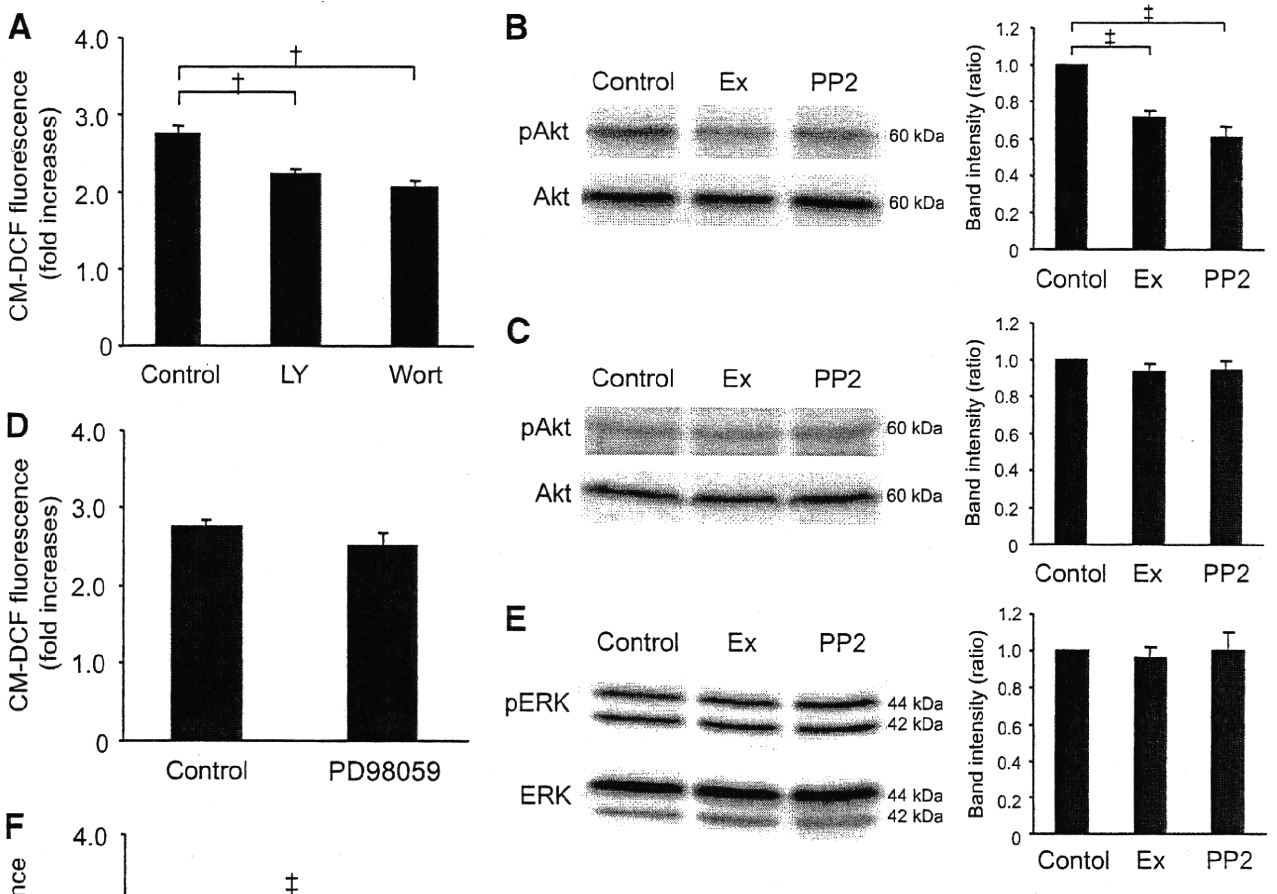

FIG. 6. The downstream pathway of Src is involved in PI3K/Akt signaling. *A*: Effects of LY294002 or wortmannin on high-glucose–induced ROS production at 60 min in GK islet cells. After preincubation in the presence of 2.8 mmol/l glucose and 10 μmol/l CM-H_2DCFDA for 20 min, dispersed islet cells were incubated in the presence of 16.7 mmol/l glucose with or without 50 μmol/l LY294002 or 0.5 μmol/l wortmannin for 60 min. Fluorescence was represented as fold increases against the value at time zero. Data are expressed as means ± SE (n = 3–5). †P < 0.01. *B*: Akt phosphorylation in the presence of high glucose with or without exendin-4 or PP2 in GK islets. After preincubation in the presence of 2.8 mmol/l glucose for 30 min, islets were incubated in the presence of 16.7 mmol/l glucose with or without 10 μmol/l forskolin or 10 μmol/l PP2 for 10 min. Islets were lysated and subjected to immunoblot analyses. Blots (50 μg of protein) were probed with anti–phospho-Akt or anti-Akt by stripping and reprobing of the same blots. Intensities of the bands were quantified with densitometric imager. The bar graphs are expressed relative to control value corrected by Akt level (means ± SE). ‡P < 0.001. Representative blot panels of five independent experiments are shown. *C*: Akt phosphorylation in the presence of high glucose with or without exendin-4 or PP2 in Wistar islets. Representative blot panels of three independent experiments are shown. *D*: Effects of PD98059 on high-glucose–induced ROS production at 60 min in GK islet cells. Data are expressed as means ± SE (n = 4). *E*: ERK phosphorylation in the presence of high glucose with or without exendin-4 or PP2 in GK islets. Blots (50 μg of protein) were probed with anti–phospho-ERK or anti-ERK by stripping and reprobing of the same blots. The bar graphs are expressed relative to control value corrected by ERK level (means ± SE). Representative blot panels of three independent experiments are shown. *F*: Effects of AG1478 on high-glucose–induced ROS production at 60 min in GK islet cells. Data are expressed as means ± SE (n = 5). ‡P < 0.001.

does not affect the phenotype in mice, contrary to neural tube defects and embryonic lethality in homozygous deficient mice (31). Moreover, the localization of Csk in the cytosol before recruitment to the membrane for Src regulation dose not differ in Wistar and GK islets (supplementary Fig. 4). Thus, the lower expression level of Csk found in our results is not likely to play a role in the Src activation in GK islets. Activation of Src as well as elevated endogenous ROS production at high glucose in GK islets was clearly suppressed by exendin-4, which did not affect Src phosphorylation or ROS production in Wistar islets. Thus, the GLP-1 signal might well suppress activation of Src and excessive ROS production under diabetic conditions in addition to other beneficial long-term effects on β-cells.

GLP-1 induces elevation of intracellular cAMP levels and subsequent activation of PKA after binding to the GLP-1 receptor. In the present study, the effect of GLP-1 signaling, which suppresses Src activation and ROS production, was found to be independent of PKA. Epac is a PKA-independent cAMP sensor; Epac2 is expressed mainly in neuroendocrine cells including pancreatic β-cells. Epac2 regulates exocytosis of insulin granules in β-cells by mobilizing intracellular Ca^{2+} and interacting granule-associated proteins (14,15). Although the relationship between Epac and Src is not well known, a recent

report (32) has shown that cAMP protects against hepatocyte apoptosis Epac dependently through Src and PI3K/Akt activation. Further evaluation of the role of cAMP in regulation of Src and PI3K/Akt signaling is required.

In the present study, we have shown that one of these Src signals, the PI3K/Akt signal, regulates ROS production. Furthermore, GLP-1 induces β-cell proliferation through PI3K signaling via Src and EGFR transactivation (33). Our finding that the EGFR kinase inhibitor decreases ROS production suggests that EGFR transactivation may be involved in the ROS-reducing effect of exendin-4 via Src. Under normal conditions, GPCR stimulation generally activates Src toward EGFR transactivation, frequently followed by PI3K activation (25). The present study reveals that Src and PI3K activities are upregulated in islets under diabetic conditions, which are suppressed by the GLP-1 signal. Many studies in oncology have shown that several growth factors including EGF and platelet-derived growth factor induce ROS through PI3K activation (34–36). Thus, EGFR transactivation/PI3K signaling should be activated under pathophysiologically disordered conditions. In the various states between normal and diabetic conditions, the ameliorative effects of the GLP-1 signal may differ (37). Further elucidation of these signals in the pathophysiology of diabetes should be helpful in future development of therapeutic strategies.

Previous studies have shown that the antioxidant capacity in β-cells is very low because of weak expression of antioxidant enzymes in pancreatic islets compared with that in various other tissues (38). The superoxide anion is converted by superoxide dismutase (SOD) into hydrogen peroxide that is eventually removed by glutathione peroxidase (Gpx). The expression level of MnSOD, which is localized in mitochondria, was significantly lower in GK islets than in Wistar islets, and that of of Gpx was similar in Wistar and GK islets (supplementaty Fig. 5*A*). However, an enzymatic assay revealed that MnSOD activity in GK islets was similar to that in Wistar islets and that it was not affected by exendin-4 or PP2 (supplementary Fig. 5*B* and *C*). These results indicate that regulation of MnSOD activity does not play a role in the suppressive effects of ROS production by exendin-4.

One of the important sites of ROS generation in β-cells is the mitochondrial electron transport chain, in which ROS generation increases according to the hyperpolarization of mitochondrial inner membrane derived from accelerated glucose metabolism (39). However, in pathophysiological conditions, NADPH oxidase may play an important role in ROS generation in β-cells. Chronic exposure to proinflammatory cytokines and abundant nutrients including glucose and palmitate augments the expression of a phagocyte-like NADPH oxidase in β-cells (40). Moreover, the expression of NADPH oxidase is increased in islets of diabetic Otsuka Long Evans Tokushima Fatty rats (41). Because Src is involved in regulation of NADPH oxidase activity (42), further examination to elucidate the site of ROS generation related to Src activation in β-cells is needed. On the other hand, previous reports have shown that ROS itself regulates Src activity (43,44) in addition to Src activity regulation of ROS production (45). To clarify this mutual causal relationship between Src and ROS, we examined ROS production in GK islets expressing Src-KN, which was found to cause a distinct decrease in high-glucose–induced ROS production. This finding demonstrates that Src activity regulates ROS production and does not contradict the possibility of a feedback regulation mechanism of ROS on Src activity (45).

The high-glucose–induced increase in ATP production is impaired in GK rats (6,46) as well as in patients with type 2 diabetes (47). In addition, islets in GK rats and human type 2 diabetes are oxidatively stressed (48–50). In the present study, exendin-4 was able to recover this impaired increase in ATP production by high glucose in GK islets as well as to decrease excessive ROS production. Thus, GLP-1 signaling may improve β-cell function in the diabetic state not only because it enhances Ca^{2+} efficacy of the exocytotic system of insulin granules but also because it improves impaired metabolism-secretion coupling. GLP-1 receptor agonists are widely used in treatment of type 2 diabetes for their ability to improve glucose intolerance. Their clinical beneficial effect seems to be provided not only by their insulinotropic action but also by their reduction of β-cell apoptosis and induction of β-cell proliferation (16–18). Further elucidation of endogenous ROS regulation by GLP-1 may help to clarify the mechanism of the various beneficial effects of these agents.

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No potential conflicts of interest relevant to this article were reported.

E.M. researched data, contributed to the discussion, wrote the manuscript, and reviewed/edited the manuscript. S.F. contributed to the discussion, wrote the manuscript, and reviewed/edited the manuscript. H.S., C.O., R.K., Y.S., M.S., and Y.N. researched data. M.O. contributed to the discussion and reviewed/edited the manuscript. N.I. contributed to the discussion and reviewed/edited the manuscript.

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REFERENCES

1. Maechler P, Wollheim CB. Mitochondrial function in normal and diabetic beta-cells. Nature 2001;414:807–812
2. Krippeit-Drews P, Kramer C, Welker S, Lang F, Ammon HP, Drews G. Interference of H2O2 with stimulus-secretion coupling in mouse pancreatic beta-cells. J Physiol 1999;514(Pt 2):471–481
3. Maechler P, Jornot L, Wollheim CB. Hydrogen peroxide alters mitochondrial activation and insulin secretion in pancreatic beta cells. J Biol Chem 1999;274:27905–27913
4. Bindokas VP, Kuznetsov A, Sreenan S, Polonsky KS, Roe MW, Philipson LH. Visualizing superoxide production in normal and diabetic rat islets of Langerhans. J Biol Chem 2003;278:9796–9801
5. Sakai K, Matsumoto K, Nishikawa T, Suefuji M, Nakamaru K, Hirashima Y, Kawashima J, Shirotani T, Ichinose K, Brownlee M, Araki E. Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells. Biochem Biophys Res Commun 2003;300:216–222
6. Kominato R, Fujimoto S, Mukai E, Nakamura Y, Nabe K, Shimodahira M, Nishi Y, Funakoshi S, Seino Y, Inagaki N. Src activation generates reactive oxygen species and impairs metabolism-secretion coupling in diabetic Goto-Kakizaki and ouabain-treated rat pancreatic islets. Diabetologia 2008;51:1226–1235
7. Xu W, Harrison SC, Eck MJ. Three-dimensional structure of the tyrosine kinase c-Src. Nature 1997;385:595–602

8. Martin GS. The hunting of the Src. Nat Rev Mol Cell Biol 2001;2:467–475
9. Nada S, Okada M, MacAuley A, Cooper JA, Nakagawa H. Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. Nature 1991;351: 69–72
10. Baggio LL, Drucker DJ. Biology of incretins: GLP-1 and GIP. Gastroenterology 2007;132:2131–2157
11. Holst JJ. The physiology of glucagon-like peptide 1. Physiol Rev 2007;87: 1409–1439
12. Seino S, Shibasaki T. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. Physiol Rev 2005;85:1303–1342
13. Roscioni SS, Elzinga CR, Schmidt M. Epac: effectors and biological functions. Naunyn Schmiedebergs Arch Pharmacol 2008;377:345–357
14. Ozaki N, Shibasaki T, Kashima Y, Miki T, Takahashi K, Ueno H, Sunaga Y, Yano H, Matsuura Y, Iwanaga T, Takai Y, Seino S. cAMP-GEFII is a direct target of cAMP in regulated exocytosis. Nat Cell Biol 2000;2:805–811
15. Kang G, Joseph JW, Chepurny OG, Monaco M, Wheeler MB, Bos JL, Schwede F, Genieser HG, Holz GG. Epac-selective cAMP analog 8-pCPT-2'-O-Me-cAMP as a stimulus for Ca2+-induced Ca2+ release and exocytosis in pancreatic beta-cells. J Biol Chem 2003;278:8279–8285
16. Xu G, Stoffers DA, Habener JF, Bonner-Weir S. Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. Diabetes 1999; 48:2270–2276
17. Farilla L, Hui H, Bertolotto C, Kang E, Bulotta A, Di Mario U, Perfetti R. Glucagon-like peptide-1 promotes islet cell growth and inhibits apoptosis in Zucker diabetic rats. Endocrinology 2002;143:4397–4408
18. Li Y, Hansotia T, Yusta B, Ris F, Halban PA, Drucker DJ. Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. J Biol Chem 2003;278:471–478
19. Tsunekawa S, Yamamoto N, Tsukamoto K, Itoh Y, Kaneko Y, Kimura T, Ariyoshi Y, Miura Y, Oiso Y, Niki I. Protection of pancreatic beta-cells by exendin-4 may involve the reduction of endoplasmic reticulum stress; in vivo and in vitro studies. J Endocrinol 2007;193:65–74
20. Cheng Q, Law PK, de Gasparo M, Leung PS. Combination of the dipeptidyl peptidase IV inhibitor LAF237 [(S)-1-[(3-hydroxy-1-adamantyl)amino]acetyl-2-cyanopyrrolidine] with the angiotensin II type 1 receptor antagonist valsartan [N-(1-oxopentyl)-N-[[2'-(1H-tetrazol-5-yl)-[1,1'-biphenyl]-4-yl]methyl]-L-valine] enhances pancreatic islet morphology and function in a mouse model of type 2 diabetes. J Pharmacol Exp Ther 2008;327:683–691
21. Akagi T, Sasai K, Hanafusa H. Refractory nature of normal human diploid fibroblasts with respect to oncogene-mediated transformation. Proc Natl Acad Sci U S A 2003;100:13567–13572
22. Florio M, Wilson LK, Trager JB, Thorner J, Martin GS. Aberrant protein phosphorylation at tyrosine is responsible for the growth-inhibitory action of pp60v-src expressed in the yeast Saccharomyces cerevisiae. Mol Biol Cell 1994;5:283–296
23. Mukai E, Fujimoto S, Sakurai F, Kawabata K, Yamashita M, Inagaki N, Mizuguchi H. Efficient gene transfer into murine pancreatic islets using adenovirus vectors. J Control Release 2007;119:136–141
24. Daub H, Weiss FU, Wallasch C, Ullrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature 1996; 379:557–560
25. Rozengurt E. Mitogenic signaling pathways induced by G protein-coupled receptors. J Cell Physiol 2007;213:589–602
26. Eguchi S, Iwasaki H, Inagami T, Numaguchi K, Yamakawa T, Motley ED, Owada KM, Marumo F, Hirata Y. Involvement of PYK2 in angiotensin II signaling of vascular smooth muscle cells. Hypertension 1999;33:201–206
27. Gao Y, Tang S, Zhou S, Ware JA. The thromboxane A2 receptor activates mitogen-activated protein kinase via protein kinase C-dependent Gi coupling and Src-dependent phosphorylation of the epidermal growth factor receptor. J Pharmacol Exp Ther 2001;296:426–433
28. Chiu T, Santiskulvong C, Rozengurt E. EGF receptor transactivation mediates ANG II-stimulated mitogenesis in intestinal epithelial cells through the PI3-kinase/Akt/mTOR/p70S6K1 signaling pathway. Am J Physiol Gastrointest Liver Physiol 2005;288:G182–G194
29. Harris KF, Shoji I, Cooper EM, Kumar S, Oda H, Howley PM. Ubiquitin-mediated degradation of active Src tyrosine kinase. Proc Natl Acad Sci U S A 1999;96:13738–13743
30. Yokouchi M, Kondo T, Sanjay A, Houghton A, Yoshimura A, Komiya S, Zhang H, Baron R. Src-catalyzed phosphorylation of c-Cbl leads to the interdependent ubiquitination of both proteins. J Biol Chem 2001;276: 35185–35193
31. Nada S, Yagi T, Takeda H, Tokunaga T, Nakagawa H, Ikawa Y, Okada M, Aizawa S. Constitutive activation of Src family kinases in mouse embryos that lack Csk. Cell 1993;73:1125–1135
32. Gates A, Hohenester S, Anwer MS, Webster CR. cAMP-GEF cytoprotection by Src tyrosine kinase activation of phosphoinositide-3-kinase p110 beta/alpha in rat hepatocytes. Am J Physiol Gastrointest Liver Physiol 2009;296: G764–G774
33. Buteau J, Foisy S, Joly E, Prentki M. Glucagon-like peptide 1 induces pancreatic β-cell proliferation via transactivation of the epidermal growth factor receptor. Diabetes 2003;52:124–132
34. Zhu QS, Xia L, Mills GB, Lowell CA, Touw IP, Corey SJ. G-CSF induced reactive oxygen species involves Lyn-PI3-kinase-Akt and contributes to myeloid cell growth. Blood 2006;107:1847–1856
35. Baumer AT, Ten Freyhaus H, Sauer H, Wartenberg M, Kappert K, Schnabel P, Konkol C, Hescheler J, Vantler M, Rosenkranz S. Phosphatidylinositol 3-kinase-dependent membrane recruitment of Rac-1 and p47phox is critical for alpha-platelet-derived growth factor receptor-induced production of reactive oxygen species. J Biol Chem 2008;283:7864–7876
36. Binker MG, Binker-Cosen AA, Richards D, Oliver B, Cosen-Binker LI. EGF promotes invasion by PANC-1 cells through Rac1/ROS-dependent secretion and activation of MMP-2. Biochem Biophys Res Commun 2009;379: 445–450
37. Peyot ML, Gray JP, Lamontagne J, Smith PJ, Holz GG, Madiraju SR, Prentki M, Heart E. Glucagon-like peptide-1 induced signaling and insulin secretion do not drive fuel and energy metabolism in primary rodent pancreatic beta-cells. PLoS One 2009;4:e6221
38. Tiedge M, Lortz S, Drinkgern J, Lenzen S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes 1997;46:1733–1742
39. Newsholme P, Haber EP, Hirabara SM, Rebelato EL, Procopio J, Morgan D, Oliveira-Emilio HC, Carpinelli AR, Curi R. Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. J Physiol 2007;583:9–24
40. Morgan D, Oliveira-Emilio HR, Keane D, Hirata AE, Santos da Rocha M, Bordin S, Curi R, Newsholme P, Carpinelli AR. Glucose, palmitate and pro-inflammatory cytokines modulate production and activity of a phagocyte-like NADPH oxidase in rat pancreatic islets and a clonal beta cell line. Diabetologia 2007;50:359–369
41. Nakayama M, Inoguchi T, Sonta T, Maeda Y, Sasaki S, Sawada F, Tsubouchi H, Sonoda N, Kobayashi K, Sumimoto H, Nawata H. Increased expression of NAD(P)H oxidase in islets of animal models of Type 2 diabetes and its improvement by an AT1 receptor antagonist. Biochem Biophys Res Commun 2005;332:927–933
42. Chowdhury AK, Watkins T, Parinandi NL, Saatian B, Kleinberg ME, Usatyuk PV, Natarajan V. Src-mediated tyrosine phosphorylation of p47phox in hyperoxia-induced activation of NADPH oxidase and generation of reactive oxygen species in lung endothelial cells. J Biol Chem 2005;280:20700–20711
43. Giannoni E, Buricchi F, Raugei G, Ramponi G, Chiarugi P. Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. Mol Cell Biol 2005;25:6391–6403
44. Zhang J, Xing D, Gao X. Low-power laser irradiation activates Src tyrosine kinase through reactive oxygen species-mediated signaling pathway. J Cell Physiol 2008;217:518–528
45. Xie Z, Cai T. Na+-K+-ATPase-mediated signal transduction: from protein interaction to cellular function. Mol Interv 2003;3:157–168
46. Hughes SJ, Faehling M, Thorneley CW, Proks P, Ashcroft FM, Smith PA. Electrophysiological and metabolic characterization of single β-cells and islets from diabetic GK rats. Diabetes 1998;47:73–81
47. Anello M, Lupi R, Spampinato D, Piro S, Masini M, Boggi U, Del Prato S, Rabuazzo AM, Purrello F, Marchetti P. Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. Diabetologia 2005;48:282–289
48. Ihara Y, Toyokuni S, Uchida K, Odaka H, Tanaka T, Ikeda H, Hiai H, Seino Y, Yamada Y. Hyperglycemia causes oxidative stress in pancreatic β-cells of GK rats, a model of type 2 diabetes. Diabetes 1999;48:927–932
49. Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S. Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. Diabetologia 2002;45:85–96
50. Del Guerra S, Lupi R, Marselli L, Masini M, Bugliani M, Sbrana S, Torri S, Pollera M, Boggi U, Mosca F, Del Prato S, Marchetti P. Functional and molecular defects of pancreatic islets in human type 2 diabetes. Diabetes 2005;54:727–735

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Difructose Anhydride III Enhances Bioavailability of Water-Insoluble Iron in Anemic Vietnamese Women

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Summary Difructose anhydride III (DFAIII) is an indigestible disaccharide and has been shown to enhance iron absorption in animal studies; however, the effect has not been investigated in anemic subjects. We investigated the efficacy of co-administration of DFAIII with water-insoluble iron in the treatment of iron deficiency anemia in Vietnamese women. One hundred sixty-eight moderately anemic women (80 g/L<hemoglobin (Hb)<120 g/L) participated in a double-blinded, placebo-controlled study with daily supplementation of iron for 6 mo. The volunteers were randomly assigned into four groups, i.e., Group A: received 15 mg Fe as ferric pyrophosphate; Group B: received 15 mg Fe as ferric pyrophosphate and 1.25 g DFAIII; Group C: received 15 mg Fe as ferrous sulfate; Group D: received a placebo. Hb and iron status were measured at baseline and after 2, 4 and 6 mo of intervention. The ratio of transferrin receptor to ferritin was used to estimate stored and functional body iron (BI). One hundred sixteen (69.0%) women completed the trial. After 6 mo, mean (±SE) Hb concentration was higher in Group A (121.6±1.7 g/L), Group B (126.4±1.5 g/L) and Group C (126.8±1.6 g/L) compared to Group D (107.0±1.7 g/L, $p<0.0001$). Mean change in BI was twofold greater in Group B (5.0±0.5 mg/kg) than that in Group A (2.5±0.6 mg/kg, $p=0.008$). The percentage of anemia was significantly reduced in Group B (18.8%) compared to Group D (95.8%, $p<0.0001$) and Group A (39.1%, $p=0.033$). Co-administration of DFAIII enhances Hb concentration and iron stores more than single administration of water-insoluble iron in anemic Vietnamese women.

Key Words difructose anhydride III, iron, anemia, randomized controlled trial

Anemia affects 1.62 billion people, which corresponds to 24.8% of the worldwide population (*1*). Iron deficiency (ID) is estimated to be the most common cause of anemia worldwide and is particularly prevalent in developing nations in Africa and Asia (*2*). In Vietnam, anemia had been widely prevalent and the prevalence of iron deficiency anemia (IDA) in reproductive-age women and children was 40.2 and 45.3%, respectively (*3*). Through the great efforts of the National Program to Control IDA, the prevalence of IDA in reproductive-age women was reduced to 24.3% in the year 2000 (*4*). However, until the present, IDA remains a public health issue in Vietnam, especially in reproductive women, children and workers in some garment factories (*5*). Finding an efficient strategy for controlling IDA has attracted much interest as a part of policy planning in Vietnam.

To combat iron deficiency, food fortification programs are considered the most cost-effective and sustainable approach. However, the success of an iron fortification program depends largely on the careful choice of the iron compound. A cheap and highly bioavailable iron compound that causes no organoleptic changes would be the ideal fortification compound. Ferrous sulfate ($FeSO_4$) which is a water-soluble iron compound is the most bioavailable iron compound, but it often causes unacceptable color or flavor changes in the food vehicle (*6*). Ferric pyrophosphate is a water-insoluble iron compound often used by European food companies to fortify infant cereals and chocolate drink powders and its main advantage is that it causes no adverse color and flavor changes to food vehicles (*6*). However ferric pyrophosphate is only poorly soluble in dilute acid, such as gastric juice, and is thus only of mediocre absorption in humans (*7*). Its low absorption rate needs further improvement.

Indigestible carbohydrates, such as fructooligosaccharide (FOS) (*8*, *9*), water-soluble soybean fiber (*10*), and insoluble dietary fibers (*11*) have received attention in promoting iron absorption. Difructose anhydorate III (DFAIII) is a naturally occurring indigestible disaccharide (Fig. 1) in the root of *Lycoris radiate* (*12*). This disac-

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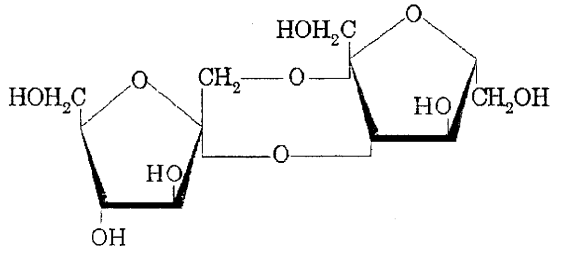

Fig. 1. Structural formula of DFAIII.

charide has recently been processed from inulin with *Arthrobactor* sp. H65-7 inulin fructotransferase [EC 2.4.1.93; Inulinase II (*13*)] for mass-production, and is stable at high temperature under acidic conditions (pH 2.0 at 100°C for 30 min) (*14*). DFAIII has been shown to enhance iron absorption in animal studies (*10*, *15*, *16*); however, the effect has not been investigated in anemic subjects.

The objective of this study was to investigate the efficacy of co-administration of DFAIII with water-insoluble iron on treatment of iron deficiency anemia in Vietnamese women.

SUBJECTS AND METHODS

Study design and participants. This study was conducted from Aug 2006 to Apr 2007 with female factory workers in two garment factories in Hai Duong and Hung Yen Province in Vietnam. This study was designed with two phases. The first phase of the study involved screening of the female factory workers to identify anemic women. All the women aged 20–49 y working in the factories were recruited into the screening study, which was held independently for the study. Phase two involved a randomized, double-blind, placebo-controlled intervention where women consumed iron supplementation every day for 6 mo.

It was calculated that a minimum of 37 women with moderately anemic status would be required in each group to demonstrate a significant difference in hemoglobin (Hb) concentration of 8 g/L at 90% power and 5% significance (two side test). Power calculations were based on a standard deviation for hemoglobin of 10.3 g/L determined from a group of anemic women measured in a pre-conducted study in Vietnam (*17*). Anticipating 16% dropouts, a sample size of at least 42 subjects per group was required at baseline.

Women aged 20–49 y with moderately anemic status (Hb concentration<120 but >80 g/L) (*18*) were included in this study. Subjects were excluded if they were pregnant, breastfeeding, parasite infection positive or if they had any known health problems likely to influence anemic status including a history of gastrointestinal or intestinal resection; blood transfusion; renal disease; genital disease; cancer; malaria infection or severe infection such as dysentery, hepatitis and cholera.

Intervention. One hundred and sixty-eight women who met the study criteria were randomly assigned into four groups, i.e., Group A: received 15 mg Fe as ferric pyrophosphate (IPP), Group B: received 15 mg Fe as IPP and 1.25 g DFAIII; Group C: received 15 mg Fe as $FeSO_4$ as a positive control as they experienced anemia improvement and Group D: received a placebo after being stratified by age and Hb levels.

Each supplement was taken daily twice; after breakfast and after lunch, under the monitoring of trained health staff members for 6 mo. Each participant consumed 10 tablets (2,500 mg)/d and the composition of the tablets for each group was Group A: IPP 187.5 mg (elemental iron 15 mg), lactose 2,282.5 mg, sucrose stearate 30 mg; Group B: IPP 187.5 mg (elemental iron 15 mg), DFAIII 1,250 mg, cellulose 532.5 mg, sucrose stearate 30 mg, lactose 500 mg; Group C: ferrous iron 40.8 mg (elemental iron 15 mg), lactose 2,429.2 mg, sucrose stearate 30 mg; and Group D: lactose 2,470 mg, sucrose stearate 30 mg. All four types of supplements provided were identical in appearance in the form of tablets and were manufactured by FANCL Co. (Kanagawa, Japan). The dose of DFAIII (1.25 g/d) was based on a study that determined the stimulatory effects of DFAIII on calcium (Ca) absorption in humans (*19*) since the dose-stimulate effect of DFAIII on iron absorption has not been determined. The efficacy of DFAIII on Ca absorption has been demonstrated in in vivo balance studies with rats (*20–23*) and humans (*19*, *24*). The safety of oral ingestion of DFAIII in healthy humans was previously determined with repeated 9 g/d of DFAIII ingestion for 4 mo with incidence of diarrhea and some gastrointestinal symptoms (*25*). No serious adverse effects were observed in the study (*25*) and the dose of DFAIII in the present study was under this level. The dose of iron (15 mg/d) was decided by a factor related to production of the tablets. One tablet contained 1.5 mg iron and we supplied 10 tablets, anticipating that study participants would be apprehensive and refuse to take more than 10 tablets; therefore 15 mg iron administration was the maximum feasible dose.

Assessments. Blood samples were collected before breakfast in fasting condition at baseline (T0), and after 2 mo (T2), 4 mo (T4) and 6 mo (T6) intervals. Concentrations of Hb, serum ferritin (SF) and transferrin receptor (TfR) were measured at each sampling. C-reactive protein (CRP) was measured at T0 and T6. Hb was measured by the cyanmethemoglobin method within 12 h. SF was measured by a two-site enzyme-linked immunosorbent assay with monoclonal reagents for both the capture and indicator antibodies (*26*). TfR was measured by using an assay with double monoclonal antibodies against intact TfR purified from human placenta (*27*). CRP was measured by latex Immunoagglutination assay (Sekisui Medical Co. Ltd., Japan). The ratio of TfR to SF was used to estimate stored and functional body iron (BI). The following formula was used for the estimation (*28*):

$$BI\ (mg/kg) = -[\log(TfR/SF) - 2.8229]/0.1207.$$

Anemia was defined as a Hb concentration<120 g/L and ID was defined as a SF concentration <15 μg/L (*18*) or a TfR concentration>8.5 mg/L (*27*). Infectious status was measured by CRP for short-term effects and the ele-

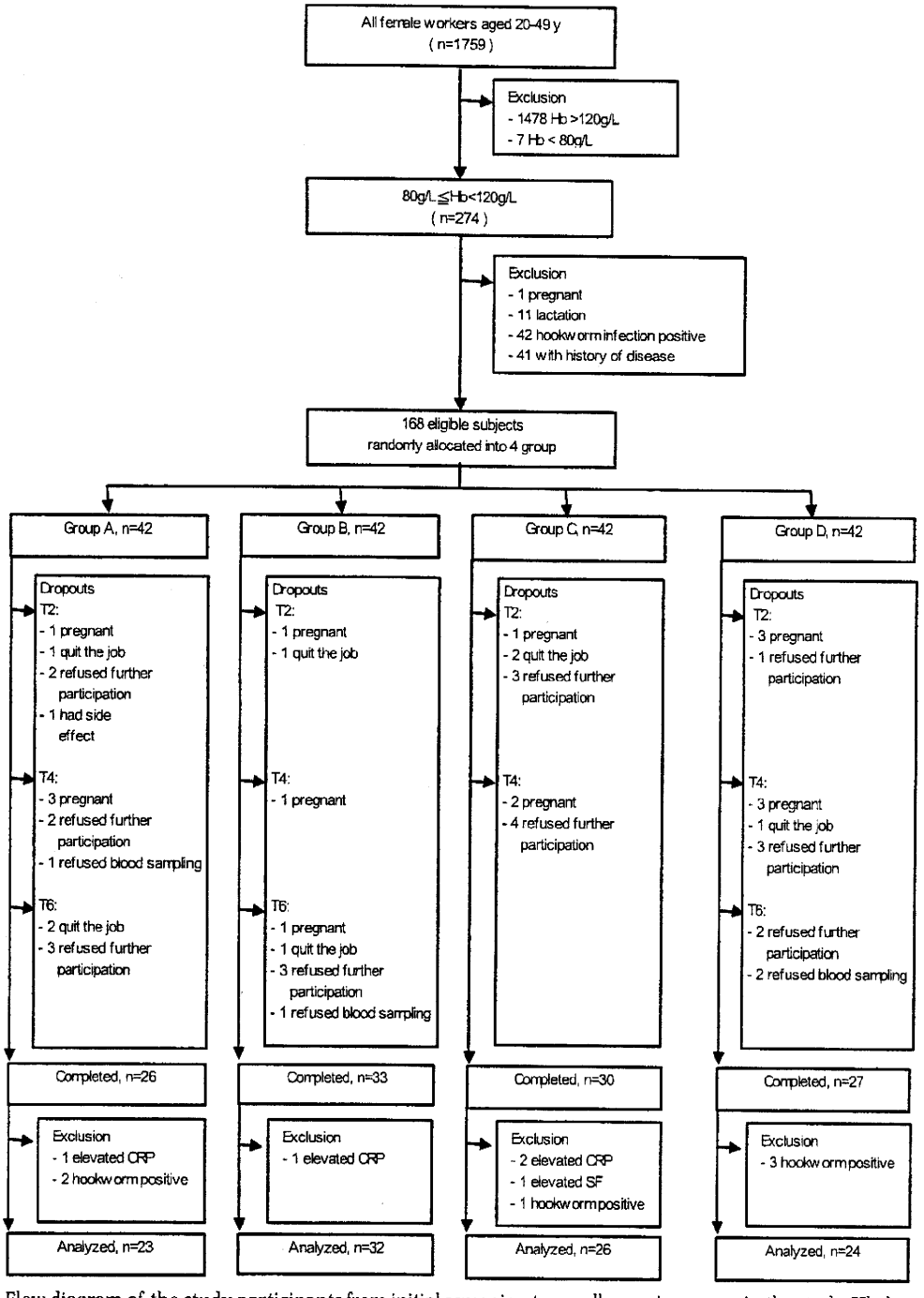

Fig. 2. Flow diagram of the study participants from initial screening to enroll anemic women in the study. Hb, hemoglobin; T0, at baseline: T2, after 2 mo; T4, after 4 mo; T6, after 6 mo.

vation for CRP was considered to be significant at a level of >3.0 mg/L (*29*).

Body height and weight were measured at T0, T2, T4 and T6. Dietary intakes were collected by 24-h food recall for 3 non-consecutive days at T0 and T6. Interviewers were centrally trained and certified in data collection according to standardized methods (*30*). Conversion to nutrient intake was based on food composition tables for Vietnamese foods (*31*). Gastrointestinal parasites were determined at T0 and T6 from fecal specimens using standard Kato-Katz methodology (*32*).

Ethics. Informed written consent was obtained from all participants. The study was approved by the Ethical and Scientific Committee of Vietnam National Institute of Nutrition, and the Ethical Committee of FANCL Co. in Japan. Women with Hb less than 80 g/L at a screening of the study participants were treated with a proper treatment schedule. Women diagnosed with hookworm infection at the screening were treated

Table 1. Baseline characteristics of 111 moderately anemic women.

	Group A (n=23)	Group B (n=32)	Group C (n=26)	Group D (n=24)	p[1]
Age and anthropometric data					
Age (y)	26.3±5.5[2]	25.8±5.4	26.6±5.2	28.2±5.4	0.415
Height (cm)	154.8±4.4	153.4±4.3	152.6±4.0	152.1±3.5	0.128
Weight (kg)	46.5±5.3	44.5±4.9	46.4±6.3	43.8±4.7	0.187
Body mass index (kg/m^2)	19.4±2.0	18.9±1.9	19.9±2.4	18.9±1.8	0.231
Energy and nutritent intakes, excluding iron provided					
Energy (kcal/d)	1,673±324	1,576±244	1,687±313	1,631±315	0.495
Protein (g/d)	64.0±12.5	60.5±10.6	61.2±13.9	63.1±11.7	0.699
Animal protein (g/d)	26.6±10.6	24.6±8.5	22.9±8.9	24.9±9.6	0.581
Iron (mg/d)	11.2±2.9	10.6±2.1	11.7±2.9	12.1±3.0	0.500
Iron from animal food (mg/d)	1.5±0.8	1.3±0.6	1.4±0.8	1.4±0.7	0.846
Vitamin C (mg/d)	94.7±67.2	80.9±39.1	86.8±69.2	105.4±70.8	0.193
Calcium (mg/d)	458±291	436±238	495±395	501±324	0.840
Hemoglobin and iron status					
Hemoglobin (g/L)	106.2±10.2	107.4±6.8	106.4±9.4	108.0±8.7	0.876
Serum ferritin (ug/L)	13.9 (4.6, 51.4)[3]	19.5 (5.8, 40.4)	27.2 (7.8, 60.5)	23.1 (6.6, 95.8)	0.341
Transferrin receptor (mg/L)	10.3±7.8[b]	10.3±5.5[b]	7.9±4.5	5.6±4.9[a]	0.011
Body iron (mg/kg)	0.5±6.3[b]	0.8±5.6[b]	2.9±4.8	5.1±5.4[a]	0.014

[1] p-values of ANOVA. Bonferroni adjustment was made for multiple comparisons.
[2] Mean±SD (all such values).
[3] Median; 25th, 75th percentile in parentheses. For the statistical analysis, the parameter was log transformed.
[a,b] Values in a row with different superscript letters are significantly different, $p<0.05$.

with single dose of Albendazole. At the end of the intervention, all women who were still anemic were referred to the health center and received iron supplements.

Statistical analysis. Statistical analysis was performed with SPSS version 15.0 (Statistical Package for Social Science, Inc.). Normally distributed data were described as means±SDs or SEs, while non-normally distributed data were expressed as geometric means with 95% CI. Data not normally distributed (SF) were log transformed for comparisons. Characteristics of study participants such as age, anthropometrics, nutrient intakes, Hb and iron status were compared across the groups by using analysis of variance (ANOVA). Mean differences of Hb, SF, TfR and BI among groups at T2, T4 and T6 were assessed using a multivariate linear regression model to allow us to account for potential confounders due to differences between groups in their baseline concentrations. Bonferroni's adjustment was used for the multiple comparisons. For non-normally distributed variables (SF) a comparison among groups of the absolute difference between T0 and T6 values was made by the Kruskal-Wallis test: if $p<0.05$, the Mann-Whitney U test (2 comparisons) was used for the multiple comparison. In addition, the effect of the treatments on the prevalence of anemia and ID during the intervention study was evaluated. Logistic regression analyses were performed for the binary response variables such as prevalence of anemia and ID. p values <0.05 were considered significant.

RESULTS

Figure 2 shows the flow diagram of the study participants from initial screening to the data analysis in the present study. The screening study was implemented in 1,759 women. During the screening survey, seven women with a low Hb concentration (<80 g/L), 1,478 women with a Hb concentration≧120 g/L, one pregnant woman, 11 lactating women, 42 hookworm infection-positive women and 41 women with a history of disease likely to influence anemic status unrelated to iron deficiency were excluded from the study; thus 179 (10.2%) women met the inclusion criteria. Of those 179 women, 168 women were randomly selected for the intervention. No one used oral contraceptives or smoked. During the intervention, 52 participants dropped out of the study (Reasons for the dropouts are shown in Table 2). For the analysis, we excluded six participants who had elevated CRP and/or ferritin at T0. One participant who had extremely high SF at T0 (483.0 μg/L), T2 (652.8 μg/L), T4 (687.2 μg/L) and T6 (390.3 μg/L) was excluded. Six participants found to have become hookworm positive at T6 were also excluded. Finally, the data of 105 women were analyzed for the primary outcomes.

Baseline characteristics of participants

Table 1 shows the characteristics of the participants (n=105). Age and anthropometrics did not differ among the four groups. The mean (±SD) iron intakes and iron intakes from animal food were 11.3 (±2.7) and 1.4 (±0.7) mg/d, respectively. There was no significant difference in the iron intake among the four groups. Nutrient intakes that may affect non-heme iron absorption such as animal protein, vitamin C and Ca (*33*) were not significantly different among the four groups, either. Although phytate and polyphenol intakes are known to influence iron absorption (*33*), ther were not assessed because of the lack of food composition data in Vietnam (*31*). These nutrient intakes

Table 2. Effects of interventions on hemoglobin and iron stores.

	Group A (n=23)	Group B (n=32)	Group C (n=26)	Group D (n=24)	p[1]
Hemoglobin (g/L)					
T2	110.3±1.5[2]	111.1±1.3	115.8±1.4	111.7±1.5	0.026
T4	113.1±1.5[b]	117.0±1.3[b]	117.8±1.4[b]	106.4±1.5[a]	<0.0001
T6	121.6±1.7[b]	126.4±1.5[b]	126.8±1.6[b]	107.0±1.7[a]	<0.0001
ΔT0−T6[3]	14.6±1.7[b]	19.3±1.5[b]	19.7±1.6[b]	−0.1±1.7[a]	<0.0001
Serum ferritin (μg/L)					
T2	17.2 (5.5, 51.9)[a,4]	30.5 (9.4, 53.4)[a]	56.5 (26.6, 93.8)[b]	30.6 (7.2, 97.3)[a]	<0.0001
T4	18.4 (10.3, 39.7)[ac]	28.2 (15.8, 65.6)[bc]	55.7 (32.2, 85.4)[b]	22.3 (6.2, 65.9)[a]	<0.0001
T6	25.4 (12.8, 42.6)[b]	39.9 (18.4, 69.9)[bc]	51.0 (33.2, 68.7)[c]	18.6 (4.9, 57.0)[a]	<0.0001
ΔT0−T6	6.3 (−1.0, 19.0)[b]	14.9 (5.1, 39.0)[c]	22.5 (6.2, 33.9)[c]	−5.2 (−25.1, 0.8)[a]	<0.0001
Transferrin receptor (mg/L)					
T2	6.6±0.4	6.0±0.4	6.1±0.4	7.3±0.4	0.067
T4	6.7±0.4[ac]	5.6±0.3[bc]	5.4±0.3[bd]	7.3±0.4[a]	<0.0001
T6	5.8±0.6[bc]	4.3±0.6[bc]	5.4±0.6[bd]	10.9±0.6[a]	<0.0001
ΔT0−T6	−2.8±0.6[b]	−4.4±0.6[b]	−3.3±0.6[b]	2.2±0.7[a]	<0.0001
Body iron (mg/kg)					
T2	3.5±0.5[ac]	4.8±0.4[bc]	6.1±0.5[b]	2.0±0.5[a]	<0.0001
T4	3.7±0.5[b]	5.5±0.4[c]	6.6±0.5[c]	1.6±0.5[a]	<0.0001
T6	4.7±0.6[b]	7.2±0.5[c]	6.6±0.6[bc]	−1.0±0.6[a]	<0.0001
ΔT0−T6	2.5±0.6[b]	5.0±0.5[c]	4.3±0.6[bc]	−3.1±0.6[a]	<0.0001

T0, at baseline; T2, after 2 mo; T4, after 4 mo; T6, after 6 mo.

[1] Linear regression was used after control for baseline values, and a Bonferroni adjustment was made for the multiple comparisons. For non-normally distributed variables (serum ferritin) a comparison among groups of the absolute difference between T0 and T6 values was made by the Kruskal-Wallis test; if $p<0.05$, the Mann-Whitney U test (2 comparisons) was used for the multiple comparison.

[2] Adjusted β coefficient; mean±SE (all such values).

[3] Δ, change.

[4] Median; 25th, 75th percentile in parentheses. For the statistical analysis, the parameter was log transformed.

[a, b, c] Values in a row with different superscript letters are significantly different, $p<0.05$.

were also not different among the four groups at T6 ($p>0.05$).

At baseline, mean Hb and SF did not differ among the four groups; however, TfR was significantly higher and BI was significantly lower in Group A ($p=0.004$ and 0.017, respectively) and Group B ($p=0.010$ and 0.011, respectively) compared to those in Group D. Prevalence of anemia was 100% in all the groups. Prevalence of ID (SF <15 μg/L or TfR >8.5 mg/L) was 64.0, 75.0, 59.3 and 51.9% in Group A, Group B, Group C and Group D, respectively; but there was no significant difference among the groups ($p=0.501$).

Changes in hemoglobin, iron status, anemia and iron deficiency

Table 2 shows concentration of Hb and iron status at T2, T4 and T6, and the differential changes between T0 and T6. At T2, median SF in Group C was the highest among the four groups and was higher than that in Group A ($p<0.0001$) or Group B ($p=0.008$). At T4, mean Hb was increased in all the iron supplemented groups: Group A ($p=0.016$), Group B ($p<0.0001$) and Group C ($p<0.0001$), compared to the placebo group (Group D). Iron status; SF, TfR and BI, were not different between Group B and Group C ($p=0.065$, 1.000 and 0.488, respectively), while Group B had lower TfR ($p=0.028$) and higher BI ($p=0.026$) compared to Group A. At T6, all the iron supplemented groups had higher Hb and iron stores compared to Group D ($p<0.0001$).

The improvements of Hb and BI level in Group B compared to Group A were 132% (Hb: 19.3 g/L compared to 14.6 g/L) and 200% (BI: 5.0 mg/kg compared to 2.5 mg/kg), respectively. Compared to Group C, improvements of Hb and BI were 74% (Hb: 14.6 g/L compared to 19.7 g/L) and 58% (BI: 2.5 mg/kg compared to 4.3 mg/kg) in Group A, while these were 98% (Hb: 19.3 g/L compared to 19.7 g/L) and 116% (BI: 5.0 mg/kg compared to 4.3 mg/kg) in Group B, respectively.

At T6, percentage of anemia was significantly reduced in Group A (39.1%, $p=0.001$), Group B (18.8%, $p<0.0001$) and Group C (11.5%, $p<0.0001$) compared to Group D (95.8%); while percentage of ID was significantly reduced only in Group B (12.5%, $p<0.0001$) and Group C (3.8%, $p<0.0001$) but not in Group A (43.5%, $p=0.113$) compared to Group D (66.7%). Percentage of anemia and ID were significantly lower in Group B ($p=0.033$ and 0.013, respectively) and Group C ($p=0.001$ and 0.007, respectively) compared to Group A.

DISCUSSION

We observed administration of IPP with DFAIII in anemic subjects increased body iron by two times com-

pared to administration of IPP alone and the level of increment was similar to the administration of $FeSO_4$ alone after 6 mo of the supplementation. Intakes of other nutrients such as animal protein, organic acids, phytate and polyphenols are important factors for iron bioavailability (*7*, *33*). In the present study, dietary intakes were assessed at T0 and T6. The distribution of intakes of the other nutrients that may affect non-heme iron absorption such as animal protein, vitamin C and Ca were not different among the four groups. Furthermore, cereals, legumes and vegetables that are main sources of phytate and polyphenol intakes were similar in the four groups (data not shown). These results indicated that intakes of other nutrients might not be confounding factors in the present study.

After 6 mo of iron administration, we confirmed no difference between IPP with DFAIII and $FeSO_4$, the positive control. However, we may not have estimated the efficacy of IPP with DFAIII on anemia or iron deficiency as well as the efficacy of $FeSO_4$ correctly. Relative bioavailability (RBV) and iron absorption have been reported to be variable by food matrices given with the iron components (*34*); furthermore, these are closely related to baseline iron status (*33*, *35*). We used iron supplementation by tablet in the present study. Accordingly, food matrices were not a confounding factor on RBV. We analyzed blood samples with careful consideration; however, there were significant differences in TfR and body iron among the four groups at the baseline. Therefore, to assess the efficacy of IPP with DFAIII on RBV compared to $FeSO_4$, we need a further study with a design of stratified randomization considering TfR and body iron.

In our study, we did not determine the effect of DFAIII on the absorption of water-soluble iron; however, there have been several studies determining the effect with the aim of discovering its mechanism in animal models. By using Sham-operated and totally gastrectomized male Sprague-Dawley rats fed test diets prepared according to the AIN93G formulation, which includes water-soluble iron (*36*), DFAIII feeding was reported to restore gastrectomy-induced iron malabsorption, resulting in complete prevention of iron-deficiency anemia in rats (*10*). The study suggests that cecal fermentation of DFAIII may contribute to the improvement in these gastrectomy-induced defects. Another study using a AIN93G-based diet showed the effects of feeding different types of non-digestible disaccharides on iron absorption in comparison with fructo-oligosaccharide (FOS) in normal and ovariectomized rats and suggested the effects depend on the disaccharide partly associated with the cecal fermentation of these disaccharides (*16*). The mechanism of iron absorption by cecal fermentation of DFAIII have been suggested by the other studies as well: under the conditions of tannic acid (TA) suppression of iron absorption, the feeding of TA with DFAIII decreased pH of the cecal content and increased major organic acid pools in the study (*15*). Shiga et al. (*37*) also showed that feeding DFAIII increased short-chain fatty acid pools and decreased pH of cecal contents in gastrectomized rats. A lately published study showed another mechanism of DFAIII-induced increases in iron absorption in rats fed an AIN93G-based diet: it indicates the effect as a result of increased cecal iron absorptive capacity through expansion of the cecal mucosa maintaining divalent metal transporter-1 (DMT-1) mRNA expression (*38*).

In conclusion, this study indicates that co-administration of DFAIII enhances Hb concentration and iron stores more than administration of water insoluble iron alone in anemic Vietnamese women. Considering the unacceptable issues of water-soluble iron, administration of DFAIII, in conjunction with water-insoluble iron, can be reasonably relied on as a strategy to control anemia or iron deficiency.

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REFERENCES

1) WHO. 2008. Worldwide prevalence of anaemia 1993–2005: WHO Global Database on Anaemia. WHO, Geneva.
2) Stoltzfus RJ. 2003. Iron deficiency: global prevalence and consequences. *Food Nutr Bull* **24** (Suppl 4): S99–103.
3) NIN/UNICEF/Centers for Disease Control and Prevention (CDC)/Program Against Micronutrient Malnutrition (PAMM). 1995. Report of the National Anemia and Nutrition Risk Factor Survey, Vietnam, 1995. NIN/UNICEF/CDC/PAMM, Hanoi.
4) National Institute of Nutrition, Vietnam (NIN)/The United Nations Children's Fund (UNICEF). 2003. Vietnam 2000–2003: A Review of the Nutrition Situation. Medical Publishing House, Hanoi.
5) Ninh NX, Khan NC. 2005. Trends in changing vitamin A deficiency, IDA in Vietnam in recent years. Recommendation for strategies to control these disorders. *Food Nutr Bull Vietnam* **1**: 1–6.
6) WHO/FAO. 2006. Guidelines on food fortification with micronutrients. WHO, Geneva.
7) Hurrell R, Egli I. 2007. Optimizing the bioavailability of iron compounds for food fortification. *In*: Nutritional Anemia (Kraemer K, Zimmermann MB, eds), p 76–98.

Sight and Life Press, Geneva.
8) Ohta A, Ohtsuki M, Uehara M, Hosono A, Hirayama M, Adachi T, Hara H. 1998. Dietary fructooligosaccharides prevent postgastrectomy anemia and osteopenia in rats. *J Nutr* **128**: 485–490.
9) Sakai K, Ohta A, Shiga K, Takasaki M, Tokunaga T, Hara H. 2000. The cecum and dietary short-chain fructooligosaccharides are involved in preventing postgastrectomy anemia in rats. *J Nutr* **130**: 1608–1612.
10) Shiga K, Nishimukai M, Tomita F, Hara H. 2006. Ingestion of difructose anhydride III, a non-digestible disaccharide, prevents gastrectomy-induced iron malabsorption and anemia in rats. *Nutrition* **22**: 786–793.
11) Hayashi K, Hara H, Asvarujanon P, Aoyama Y, Luangpituksa P. 2001. Ingestion of insoluble dietary fiber increased zinc and iron absorption and restored growth rate and zinc absorption suppressed by dietary phytate in rats. *Br J Nutr* **86**: 443–451.
12) Li HY, Hagiwara H, Shu W, Yokoyama C, Harada N. 1997. Isolation and NMR studies of di-D-fructose anhydride III from *Lycoris radiata* Herbert by supercritical extraction with carbon dioxide. *Carbohydr Res* **299**: 301–305.
13) Uchiyama T. 1975. Action of Arthrobacter ureafaciens inulinase II on several oligofructans and bacterial levans. *Biochim Biophys Acta* **397**: 153–163.
14) Saito K, Tomita F. 2000. Difructose anhydrides: their mass production and physiological functions. *Biosci Biotechnol Biochem* **64**: 1321–1327.
15) Afsane K, Shiga K, Ishizuka S, Hara H. 2003. Ingestion of an indigestible saccharide, difructose anhydride III, partially prevents the tannic acid-induced suppression of iron absorption in rats. *J Nutr* **133**: 3553–3560.
16) Asvarujanon P, Ishizuka S, Hara H. 2005. Promotive effect of non-digestible disaccharides on rat mineral absorption depend on the type of saccharide. *Nutrition* **21**: 1025–1035.
17) Thuy PV, Berger J, Davidsson L, Khan NC, Lam NT, Cook JD, Hurrell RF, Khoi HH. 2003. Regular consumption of NaFeEDTA-fortified fish sauce improves iron status and reduces the prevalence of anemia in anemic Vietnamese women. *Am J Clin Nutr* **78**: 284–290.
18) WHO/CDC. 2007. Assessing the Iron Status of Populations, Second Edition: Including Literature Reviews: Report of a Joint World Health Organization/Centers for Disease Control and Prevention Technical Consultation on the Assessment of Iron Status at the Population Level. WHO, Geneva.
19) Shigematsu N, Okuhara Y, Shiomi T, Tomita F, Hara H. 2004. Effect of difructose anhydride III on calcium absorption in humans. *Biosci Biotechnol Biochem* **68**: 1011–1016.
20) Mineo H, Amano M, Shigematsu N, Okuhara Y, Tomita F. 2002. Melibiose, difructose anhydride III and difructose anhydride IV increase net calcium absorption in rat small and large intestinal epithelium by increasing the passage of tight junction in vitro. *J Nutr* **132**: 3394–3399.
21) Mineo H, Amano M, Chiji H, Shigematsu N, Tomita F, Hara H. 2003. Absorption of calcium in the isolated cecal epitherium adaptively increased by 2 weeks feeding of difructose anhydride III in rats. *Biosci Biotechnol Biochem* **67**: 1847–1851.
22) Okuhara Y, Shiomi T, Tamura A, Tomita K, Tamakii N, Shigematsu N, Mineo H, Amano M, Tomita F, Hara H. 2003. Effect of calcium concentration and pH on difructose anhydride III enhance calcium absorption from rat small and large intestinal epithelium in vitro. *Nippon Nogeikagaku Kaishi* **77**: 1004–1006 (in Japanese).
23) Mitamura R, Hara H, Aoyama Y, Chiji H. 2002. Supplementary feeding of difructose anhydride III restores calcium absorption impaired by ovariectomy in rats. *J Nutr* **132**: 3387–3393.
24) Tomita K, Shiomi T, Okuhara Y, Tamura A, Shigematsu N, Hara H. 2007. Ingestion of difructose anhydride III enhances absorption and retention of calcium in healthy men. *Biosci Biotechnol Biochem* **71**: 681–687.
25) Tamura A, Shiomi T, Okuhara Y, Hayamizu K, Shigematsu N, Kikuchi H, Tomita F, Hara H. 2003. The safety of high-dose repeated oral ingestion of difructose anhydride III in healthy humans. *J Jpn Assoc Dietary Fiber Res* **7**: 89–96.
26) Worwood M, Thorpe SJ, Heath A, Flower CH, Cook JD. 1991. Stable lyophilized reagents for the serum ferritin assay. *Clin Lab Haematol* **13**: 297–305.
27) Skikne BS, Flowers CH, Cook JD. 1990. Serum transferrin receptor: A quantitative measure of tissue iron deficiency. *Blood* **75**: 1870–1876.
28) Cook JD, Flowers CH, Skikne BS. 2003. The quantitative assessment of body iron. *Blood* **101**: 3359–3364.
29) Otani H. 1995. C-reactive protein. *Nippon Rinsho* **53**: 233–236 (in Japanese).
30) Thompson FE, Byers T. 1994. Dietary assessment resource manual. *J Nutr* **124**: 2245S–2317S.
31) Ministry of Health (MOH)/National Institute of Nutrition (NIN). 2000. Nutritive Composition Table of Vietnamese Foods. Medical Publishing House, Hanoi.
32) Ash LR, Orihel TC, Savioli L, Sin MA, Montresor A, Renganathan E. 2004. Training Manual on Diagnosis of Intestinal Parasites—Tutor's Guide. WHO, Geneva.
33) Gibson RS, Ferguson E. 1999. An Interactive 24 Hour Recall for Assessing Dietary Adequacy of Iron and Zinc Intakes in Developing Countries. ILSI Press, Washington DC.
34) Moretti D, Zimmermann MB, Wegmüller R, Walczyk T, Zeder C, Hurrell RF. 2006. Iron status and food matrix strongly affect the relative bioavailability of ferric pyrophosphate in humans. *Am J Clin Nutr* **83**: 632–638.
35) Lynch MF, Griffin IJ, Hawthorne KM, Chen Z, Hamzo MG, Abrams SA. 2007. Iron absorption is more closely related to iron status than to daily iron intake in 12- to 48-mo-old children. *J Nutr* **137**: 88–92.
36) Reeves PG, Nielsen FH, Fahey GJ. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**: 1939–1951.
37) Shiga K, Hara H, Okano G, Aoyama Y. 2003. Ingestion of watersoluble soybean fiber prevents gastrectomy-induced malabsorption, anemia and impairment of voluntary running performance in rats. *J Nutr* **133**: 1120–1126.
38) Hara H, Onoshima S, Nakagawa C. 2010. Difrcutose anhydride III promotes iron absorption in the rat large intestine. *Nutrition* **26**: 120–127.

ORIGINAL

Nutritional status, feeding practice and incidence of infectious diseases among children aged 6 to 18 months in northern mountainous Vietnam

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Abstract : *Objective* : To assess the prevalence of undernutrition, incidence of infectious diseases and the situation of feeding practices to determine the risk factors for undernutrition among children aged 6 to 18 months in rural Vietnam. *Design* : A cross-sectional study was conducted among one hundred eighty-eight mother-child pairs in Bac Giang, Vietnam. Weight and height of the children were measured and referred to data from the WHO/CDC/NCHS. Incidence of infectious diseases was diagnosed based on the WHO Recommended Surveillance Standards. Data on socio-demographic variables and feeding practices were obtained through a structured questionnaire. *Result* : The prevalence of underweight, stunting and wasting was 19.7%, 23.4% and 5.3%, respectively. The incidence of diarrhea and acute respiratory infections (ARIs) during the last 14 days of the interview was 12.2% and 20.2%, respectively. Although 99% of the children were breastfed, the prevalence of exclusive breastfeeding in the first 4 mo was 21.3%. Non-exclusive breastfeeding in the first 4 month (OR 3.95, p=0.025) and low birth weight (OR 4.38, p=0.009) were associated with underweight in the children, while incidence of infectious disease was not (OR 1.16, p=0.734). *Conclusion* : Undernutrition is highly prevalent in the study site and non-exclusive breast feeding is one of the risk factors. J. Med. Invest. 57 : 45-53, February, 2010

Keywords **:** *undernutrition, exclusive breastfeeding, infectious disease, Vietnam, infant and child*

INTRODUCTION

Child deaths worldwide have decreased in number from 13·5 (13·4-13·6) million in 1980 to an estimated 9·7 (9·5-10·0) million in 2005 ; however, the decline is less than the target set by Millennium Development Goal 4 (MDG4) (1). Most of the child deaths were caused by preventable and treatable illness despite effective health interventions. At least half of the deaths are caused by undernutrition (2). UNICEF has developed a framework for the basic underlying causes of undernutrition and has demonstrated that a combination of insufficient nutrient intake and infection are the

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primary factors (3). When children consume insufficient nutrients, their immune systems deteriorate, resulting in greater incidence, severity and duration of disease. Disease in turn affects their nutrient intake and also interferes with nutrient absorption, further aggravating the undernutrition. As to the underlying causes of undernutrition, poverty plays a central role with environmental, economic, and sociopolitical factors.

In Vietnam, child undernutrition is one of the major national health problems. It has been reported that the nationwide prevalence of child undernutrition has been remarkably reduced during the last 2 decades : the prevalence of underweight among children aged under 5 y was 51.5% in 1985, but it was reduced to 25.2% in 2005 (4). However, the prevalence of undernutrition varied among ecological regions in Vietnam. A greater proportion of the population in mountanious and remote areas were undernourished than in urban areas. The child undernutrition problem was reported to occur from an early stage of their life in Vietnam. The prevalence of undernutrition increased remarkably during 6 to 18 mo and was sustained over the next 3 y (4). Previous studies indicated that premature complementary feeding (5, 6) and frequent infectious disease (7) were the primary causes. It has also been recognized that stunting occurred even before 6 mo of age : 6.3% of children aged under 6 mo were stunted (4). Poor health care and insufficient food intake during pregnancy were also suggested as high risk factors for childhood undernutrition.

Although approach for general deprivation and inequality would result in substantial reduction in undernutrition and should be global priority, major reduction in undernutrition can also be achieved through programmatic health and nutrition intervention (8-11). The present study was aimed at providing baseline information, including the prevalence of undernutrition, incidence of infectious disease and the situation in feeding practice among children aged 6 to 18 mo for future programs with which to combat child undernutrition in northern mountainous Vietnam.

METHODS

During May and June 2005, in the middle of the dry season, a cross-sectional survey was conducted in 4 communes in Yen The, Bac Giang, Vietnam. Bac Giang province is a rural mountainous region in Vietnam, located in the North-East region and 51 km from Hanoi. The province had a relatively high prevalence of childhood undernutrition among the 8 regions in Vietnam (4). Yen The district is one of the nine districts in the province. The district has a population of approximately 91,000 with 7,500 children under 5 y of age and 2,900 children under 2 y (Dec, 2004). There are 19 communes in the district, out of which four communes were selected. Extremely poor villages which belong to the government's "Phase One 10,000 Poorest Communities" Program and the villages that were already targeted in special intervention programs by the government were not included among the selected communes. All children aged 6 to 18 mo were recruited for the survey. A census was carried out to identify all children aged 6 to 18 mo and 191 eligible children were identified.

Anthropometric measurements comprising height and weight were performed among the children and mothers by four trained field workers using standard procedures (12). Briefly, children were weighed in light cloths on an infant scale. Recumbent length was measured to the nearest 0.1 cm with a portable infant measuring board. The anthropometric indicators for children, Z-scores of weight-for-age (WAZ), height-for-age (HAZ) and weight-for-height (WHZ), were calculated on the basis of growth references developed by the World Health Organization (WHO), the Center Disease for Control (CDC), and the National Center for Health Statistics (NCHS) (13) and were calculated also by using growth references recently developed by WHO (14). Most of the available data on the growth of children in Vietnam were calculated based on WHO/CDC/NCHS growth references at the time that the present study was conducted. Undernutrition was classified according to the cut-off indicated by WHO (15) : underweight was defined as WAZ<-2, stunting as HAZ<-2, wasting as WHZ<-2. Mothers were weighed in light clothes (to the nearest 100 g) on a scale. Height was measured to the nearest 0.1 cm. Chronic Energy Deficiency (CED) among mothers was classified as Body Mass Index (BMI) under 18.5 kg/m^2.

Morbidity data were collected from the mother's description of the infant's symptoms of diarrhea, acute respiratory infections (ARIs), measles and pertussis during the previous two weeks. The descriptions of the infectious diseases were based on the WHO recommended Surveillance Standards (16). Children who had a passage of 3 or more loose or watery stools in the past 24 hours were classified

as suffering from diarrhea. If the diarrhea lasted more than 2 weeks, these children were classified as suffering from chronic diarrhea. If the stool contained blood, these children were classified as suffering from dysentery. Children who had a cough or difficulty breathing were classified as suffering from ARIs. If the cough or difficult breathing was accompanied by frequent breathing (>50 breath/min. for age 2 mo to<1 y, >40 breath/min. for age 1 to<5 y), these children were classified as suffering from pneumonia. Measles was determined if a child had all of the following three symptoms : fever, maculopapular rash, and cough or coryza or conjunctivitis. Pertussis was determined if a child showed both of the following symptoms : cough for at least 2 wks with at least one of the following : paroxysms of coughing, inspiratory "whooping", post-tussive vomiting without other apparent cause.

In this survey, a face-to-face interview was conducted with mothers and children to collect information on socio-demographic status, feeding practices and coverage of local immunization and Vitamin A supplementation. Data collection concerning breast feeding practice was based on WHO guidelines (17) and on a previous study in Vietnam (5). The mothers were asked about their breastfeeding practices during the children's first 6 mo and also about the current breastfeeding status. The following operational definitions were used in the survey : breastfeeding referred to children who were receiving at the time of interview or had ever received breast milk. Breastfeeding status at 4 and 6 mo of age were classified as exclusive breastfeeding (only breast milk plus medical drops and syrups), almost exclusive breastfeeding (only breast milk and water plus medical drops and syrups), predominant breastfeeding (water, herbal teas or fruit juice in addition to breast milk), partial breastfeeding (breast milk plus other types of milk or foods) and weaned (no longer breastfed). All the questions were pre-tested at a study site and revised before initiating the survey. The interviewers, physicians from the NIN and local community health centers, were trained in standardized questionnaire administration and anthropometric measurements through lectures and practice in the field. Four investigation teams were established for the four communities, each one including at least eight members and a supervisor. During the survey, a check system was applied including checking in the field by interviewers, interviewer's checking each other, and checking by supervisors. Personnel in the local community centers and collaborators assisted in the organization of data collection and in the explanation of procedures to the study participants. The study participants were re-interviewed whenever transcription or logical questions arose or missing values were found. The apparatus and method for measurement were checked and/or adjusted for accuracy by the supervisors before each day's work. The precise age of each child was obtained from the Permanent Residence Registration where birth data are recorded.

Before the survey, all eligible mothers in the communities were informed of the procedures and purpose of the study, then informed consent was obtained. The protocol of this study was approved by the Scientific Board of the National Institute of Nutrition of Vietnam and the Ethical Committee of Tokushima University.

A database was established using Epi info version 6 (CDC, Atlanta, GA, USA). All data were checked for missing data and outliers, and cleaned before data analysis. Statistical analysis was performed using SPSS version 11.5J (Statistical Package for Social Science, Inc.). A one-sample Kolmogorov-Smirnov test was used to assess whether the data were normally distributed. Results were presented as mean and standard deviation (SD) or as median and 25th, 75th percentile. Z-scores between children aged 6 to 11.9 mo and those aged 12 to 17.9 mo were compared by unpaired *t*-test. Z-scores calculated by WHO/CDC/NCHS and WHO growth reference were compared by paired *t*-test. The proportion of children classified as underweight, stunted and wasted were compared by chi-square test. Logistic regression analysis was used to analyze the effects of infectious disease and exclusive breastfeeding status, as well as those of socioeconomic and demographic factors, on the nutritional status of children. We selected children's underweight for the analysis since acute malnutrition among children is a key indicator routinely used for describing the presence and magnitude of humanitarian emergencies (18).

RESULTS

Socio-demographic characteristics and coverage of some of local health services

A pair of twins and a child whose mother was suffering from tuberculosis were excluded and the data of 188 children were used for the analysis in the present study. Socio-demographic characteristics of the children are shown in Table 1. Ninety one percent