Autocrine/Paracrine Function of Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) for Glucose Homeostasis in Pancreatic β-Cells and Adipocytes

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a second messenger for mobilizing Ca\(^{2+}\) from intracellular stores in various cell types. Extracellular application of NAADP has been shown to elicit intracellular Ca\(^{2+}\) signals, indicating that it is readily transported into cells. However, little is known about the functional role of this NAADP uptake system. Here, we show that NAADP is effectively transported into selected cell types involved in glucose homeostasis, such as adipocytes and pancreatic β-cells, but not the acinar cells, in a high glucose-dependent manner. NAADP uptake was inhibited by Ned-19, a NAADP mimic, dipyridamole, a nucleoside inhibitor; or NaN\(_3\), a metabolic inhibitor or under Ca\(^{2+}\)-free conditions. Furthermore, NAADP was found to be released from pancreatic islets upon stimulation by high glucose. Consistently, administration of NAADP to type 2 diabetic mice improved glucose tolerance. We propose that NAADP is functioning as an autocrine/paracrine hormone important in glucose homeostasis. NAADP is thus a potential antidiabetic agent.

background: External application of a Ca\(^{2+}\)-mobilizing messenger, NAADP, to β-cells activates intracellular Ca\(^{2+}\) changes and stimulates insulin secretion.

results: NAADP is transported into adipocytes and β-cells in a glucose-dependent manner and is also released from pancreatic islets upon stimulation by high glucose.

collection: NAADP functions in an auto-/paracrine manner to regulate glucose homeostasis.

significance: NAADP can be exploited as a potential antidiabetic agent.

Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most potent Ca\(^{2+}\)-releasing second messenger that has been shown to regulate many physiological processes, including insulin secretion from pancreatic β-cells (1–5). NAADP induces Ca\(^{2+}\) release through a mechanism distinct from those of other Ca\(^{2+}\)-mobilizing messengers, such as inositol triphosphate and cyclic ADP-ribose. The latter two messengers act on their respective receptors present on the endoplasmic reticulum, whereas NAADP targets acidic Ca\(^{2+}\) stores (4, 5). Interestingly, however, a family of enzymes called ADP-ribosyl cyclases catalyzes the formation of both cyclic ADP-ribose and NAADP via two distinct reactions and using two different substrates, respectively. Cyclic ADP-ribose is formed by the cyclization of NAD, whereas NAADP is synthesized by a base exchange reaction that replaces the nicotinamide group of NADP with nicotinic acid (6–9). The base exchange reaction for NAADP synthesis requires acidic pH (10), consistent with NAADP targeting the lysosome-like acidic organelles (3, 11–13).

Two-pore channels have recently been identified as the NAADP-gated Ca\(^{2+}\) release channels on the endolysosomal membranes (14–16). Consistently, the NAADP-induced current in pancreatic β-cells is abolished in cells prepared from TPC2 (two-pore channel 2) knock-out mice (5). Likewise, Ned-19, a membrane-permeant and selective antagonist of the NAADP receptor, inhibits the glucose-evoked calcium spiking in mouse pancreatic β-cells in a concentration-dependent manner (17). These results indicate a central role for NAADP in the glucose-induced Ca\(^{2+}\) signaling in these cells.

Although endogenous NAADP was first shown to be present in sea urchin sperm (18), the β-cell line MIN6 was the first cell type in which a rise in NAADP was observed in response to a high concentration of glucose (19). Glucagon-like peptide 1 (GLP-1), a gut hormone, also induces an increase in intracellular Ca\(^{2+}\) and insulin secretion in pancreatic β-cells, via stimulation of NAADP production (3). Similarly, in adipocytes, activation of the insulin receptor by insulin elicits an increase in endogenous NAADP that coincides with the Ca\(^{2+}\) signaling, resulting in glucose uptake (20) that was inhabitable by Ned-19.
or bafilomycin A1, an inhibitor of the lysosomal H+ -ATPase (20). These findings indicate that NAADP plays an essential role in glucose homeostasis and insulin signaling in both pancreatic β-cells (3) and adipocytes (20).

In addition to targeting intracellular Ca2+ stores, extracellular application of NAADP has also been reported to induce intracellular Ca2+ changes, possibly via activation of surface adenosine receptors or purinergic receptors (21–23). That external application can induce a pattern of Ca2+ signals similar to that stimulated by GLP-1 (3).

In the present study, we have investigated the issue of NAADP transport and found that it indeed efficiently occurs in both pancreatic islets and adipocytes in a glucose-dependent manner, resulting in stimulation of insulin release and glucose uptake, respectively. Thus, external application of submicromolar concentrations of NAADP elicited Ca2+ signals that are characteristic of Ca2+ mobilization from the Ned-19-sensitive acidic organelles. NAADP uptake was blocked by Ned-19, a NAADP mimic; dipyridamole, a nucleoside inhibitor; or NaN3, a metabolic inhibitor or under Ca2+-free conditions. Furthermore, NAADP was found to be released from pancreatic islets following glucose stimulation via a different route. Our results indicate that NAADP is an autocrine/paracrine signal that can be exploited as a potential antidiabetic agent.

**EXPERIMENTAL PROCEDURES**

**Measurement of [Ca2+]i.—**For [Ca2+]i measurements, cells were plated on confocal dishes and loaded with 1 μM Fluo-3 AM (Molecular Probes) at 37 °C for 20 min. After washing with K-R buffer containing 0.1% BSA with 2.8 or 12 mM glucose, changes in fluorescence were determined at 488-nm excitation/530-nm emission by an air-cooled argon laser system (Nikon, Tokyo, Japan) equipped with a temperature-controlled stage (TOKAI HIT, Tokyo, Japan). The emitted fluorescence at 530 nm was measured with a photomultiplier, and time series were acquired with a frame interval of 4 s. For the calculation of [Ca2+]i, the method of Tsien et al. (25) was applied with a Kd of 325 nM for Fluo-3. Each tracing was calibrated for maximal intensity (Fmax) by the addition of 8 mM ionomycin and for minimal intensity (Fmin) by the addition of 50 mM EGTA at the end of each measurement.

**Glucose Tolerance Test and Histology Experiments—**Male Leprdb/Lepth (db/db) mice (6 weeks old) were obtained from Jackson Laboratory (Japan). Mice were acclimated to the facility for 5 days and fed ad libitum with regular chow. The Animal Research Committee of Chonbuk National University approved the animal study in accordance with the guidelines of the National Institutes of Health. Long term continuous release pellets with NAADP (50.7 μg for 7 days/pellet) or placebo were custom-made by Innovative Research of America (Sarasota, FL) and were used according to the manufacturer’s instructions.

For the intraperitoneal glucose tolerance test, db/db mice were fasted for 18 h before the test and then given a glucose solution (0.5 g/kg) intraperitoneally. Blood samples were taken from the tail vein with a micropipette under anesthesia at 30 min before and at 15, 30, 60, and 120 min after glucose loading. Blood glucose levels were measured by the glucose oxidase method using a glucose analyzer (Lifescan, Inc., Milpitas, CA), and serum insulin levels were determined using a radioimmunoassay kit (Linco Research Inc., St. Charles, MO). For histological experiments, mice treated with NAADP for 4 weeks were anesthetized with diethyl ether, and the peritoneal region was incised. Pancreas was then removed from the mice and fixed overnight in a cold 10% (v/v) formalin solution. Fixed tissues were processed routinely for paraffin embedding, and 5-μm sections were used for immunohistochemistry staining. Immunohistochemistry for insulin was performed with an antibody to insulin (DAKO, CA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (DAKO, CA). Stained morphology was analyzed using a microscope (Leica, Wetzlar, Germany), and insulin-positive islets were counted in five randomly selected areas from each section.

**Isolation of Pancreatic Islets and β-Cells—**Pancreatic islets were isolated according to the collagenase method (26) with some modifications. The pancreas of an ether-anesthetized mouse was digested with infusion of Krebs-Ringer (KR) buffer (pH 7.4) containing 1.5 mg/ml type V collagenase (Sigma-Aldrich) via the common bile duct. After removing vascular connections of the pancreas by washing with the same buffer, intact islets were handpicked with a Pasteur micropipette under a dissecting microscope and stabilized in a low glucose (5-8 mM) culture medium overnight in a CO2 incubator. For β-cell isolation, islets were triturated with a pipette, and the dispersed β-cells were seeded on dishes suitable for confocal microscopy and cultured in the medium described previously (3).

**Insulin Secretion Assay from Pancreatic Islets—**Islets were incubated with KR buffer containing 0.01% (w/v) bovine serum albumin (BSA), 16 mM HEPES, and 2.8 mM glucose for 60 min at 37 °C. After washing with the above buffer, the islets were further incubated with GLP-1 or NAADP (Sigma-Aldrich) in KR buffer containing 0.01% (w/v) BSA, 16 mM HEPES, and 12 mM glucose for 30 min. Released insulin in the supernatant was measured by radioimmunoassay using an insulin assay kit.

**Amylase Secretion Assay from Pancreatic Acini—**Pancreatic acini were prepared as described with some modifications (27). Briefly, chopped pancreases of mice were digested with digestion buffer (KR buffer, pH 7.4, 10 unit/ml collagenase type IV (Sigma-Aldrich), 1% (w/v) BSA) and washed with KR buffer, 1% (w/v) BSA. The cell suspension was gently pipetted and then washed with the same buffer. Isolated acini were stimulated with CCK-8 (Sigma-Aldrich) and NAADP in 1-ml aliquots in Eppendorf tubes for 30 min. Samples were then centrifuged for 30 s in a centrifuge, and the supernatant was assayed for amylase activity with Phadebas reagents (Magle Life Science, Lund, Sweden). Amylase release was calculated as a percentage of initial acinar amylase content.

**Glucose Uptake into Isolated Adipocytes—**Epididymal adipocytes were isolated using a collagenase method (28) with some modification. Epididymal fat pads were excised and pooled in 5 ml of KR buffer, 3.5% (w/v) BSA with 1 mg/ml collagenase type II (Sigma-Aldrich). The fat pads were chopped with scissors, and the suspension was shaken in a water bath at
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75 rpm for 20 min at 37 °C. The cells were washed through 200-μm sterile mesh, and the cells were washed twice with KR buffer. The cells were gently resuspended in KR buffer, 1% (w/v) BSA. Glucose uptake was assayed as described (29). Isolated adipocytes were incubated with insulin for 30 min at 37 °C in a gyratory bath rotator. 2-Deoxy-D-[3H]glucose (final 1 mM 2-deoxy-D-glucose; 2 μCi/tube) was added with or without NAADP in 50 μl, and after 5 min, cells were harvested by adding 250 μl of the cell suspension to 100 μl of di-isonyl phthalate in a microcentrifuge. The cells (top layer) were taken from the buffer (bottom layer) and put in scintillation tubes, and radioactivity was determined by a scintillation counter. Basal radioactivity was counted by cells exposed to radioactivity and then immediately harvested. Each sample was assayed in duplicate.

Synthesis of Radioactive NAADP—Radioactive NAADP was synthesized as described in previous reports (30, 31). Briefly, [32P]NAD was synthesized from [32P]NAD (2 μM; specific activity 1000 Ci/mmol; Amersham Biosciences) by incubation with 50 units/ml NAD kinase (Sigma-Aldrich) and 10 mM MgATP for 2 h at 37 °C in a buffer containing 5 mM HEPES (pH 7.5). The remaining ATP and ADP was converted into AMP by incubation with apyrase (2 units/ml; Sigma) for 1 h at 37 °C. The reaction was then diluted 5-fold into a medium containing 10 mM MES (pH 5.0), 19 mM nicotinic acid, and 2 μg/ml ADP-ribosyl cyclase (Sigma) and was further incubated for 2 h in order to convert [32P]NAD into [32P]NAADP by base exchange. The final mixture was separated by anion exchange HPLC on an inner diameter 4.6 mm × 100-mm column packed with AG-MP1 (Bio-Rad). Elution was performed at a flow rate of 4 ml/min using a gradient of trifluoroacetic acid (TFA) that increased linearly from 0 to 100% (150 mM TFA) at 25 min. Fractions were collected every quarter minute and were neutralized by the addition of Tris-base (final concentration 75 mM), and their radioactivities were determined by Cerenkov counting. [3H]NAADP was synthesized with [5,6-3H]nicotinic acid (16.67 μM, specific activity 60 Ci/mmol; ARC Inc.) by the same methods.

[32P]NAADP Transport in Vivo—[32P]NAADP (0.25 nmol, 2.16 μCi of NAADP, 100 μl) was intraperitoneally injected into overnight fasted mice that were preinjected with saline or 2 g/kg glucose-saline 30 min before injection (n = 10). After 30 min, mice were anesthetized, and blood was acutely drawn from the vena cava. Tissues were then washed and taped with ice-cold PBS after chopping (2–3 mm) to remove blood. The isolated tissues were weighed and transferred to conical tubes. Tissue samples were homogenized (T25, IKA Co.) and digested with absolute H2SO4, and the radioactivity was measured with a liquid scintillation counter (PerkinElmer Life Sciences). Similar experiments with db/db mice were performed after checking blood glucose levels under 4-h fasted or non-fasted conditions (n = 10). Blood glucose levels of fasted and non-fasted db/db mice were 103 ± 11 mg/dl (5.7 ± 0.61 mm) and 285 ± 21 mg/dl (15.8 ± 1.16 mm), respectively.

NAADP Transport in Vitro—NAADP transport experiments were performed as described earlier (3), with little modification. Briefly, overnight stabilized islets, acini, and adipocytes were incubated with 50 nm (50 fmol of [32P]NAADP or [3H]NAADP, 100 μl) NAADP for the indicated time at 30 °C, and cells were separated by oil stop method (32). Transported NAADP was measured with a liquid scintillation counter (PerkinElmer Life Sciences) and then converted to NAADP concentration.

Measurement of NAADP—The assay used to measure NAADP was described elsewhere (33, 34) with minor modifications. All assays were simultaneously processed in duplicates with standards. To measure intracellular NAADP, nucleotide-rich supernatant was collected by 0.6 M perchloric acid treatment and neutralized. To measure plasma NAADP, blood (0.5 ml) was collected by cardiac puncture into a sodium citrate solution (30 μl as 4% sodium citrate (w/v)) at the indicated time, and plasma was isolated by centrifugation with 15,000 × g at 4 °C for 5 min. To measure extracellular NAADP, supernatants were collected with oil stop method (32) and freeze-dried. For concentration and purification of NAADP released from the MIN6 β-cell line, proteins or other macromolecules in culture supernatants were removed by ultrafiltration with Centriprep YM-3 (Millipore, MA), and the supernatants were diluted 10-fold with deionized water. The diluents were applied to an AG-MP1 column (0.5 × 5 cm) and washed with cold distilled water. Fractions were eluted by a gradient of 0–150 mM TFA, collected, freeze-dried (AES1010, Savant Instrument Inc.), and reconstituted with saline.

Statistics—Data are expressed as mean ± S.E. Statistical comparisons were performed with analysis of variance. Significant differences between groups were determined using the unpaired Student’s t test. Statistical significance was set at p < 0.05.

RESULTS

Glucose-dependent NAADP Uptake in the Glucose-metabolizing Organs—To examine whether exogenously administered NAADP might be metabolized in vivo, we measured the
concentration of NAADP in the blood plasma following administration of NAADP. We found that NAADP was increased with a peak at 5–10 min and then slowly decreased (supplemental Fig. S1), suggesting that exogenously administrated NAADP was retained as an intact form in the blood, which could be transported into the cells. To assess NAADP uptake in various organs, we administered \[^{32}P\]NAADP into the intraperitoneum of mice, and the amount of \[^{32}P\]NAADP taken up by various organs was assessed by measuring the radioactivity per wet weight of the organ. The radioactivity of \[^{32}P\]NAADP was notably high in the kidney (0.367 ± 0.027 fmol/g), heart (0.228 ± 0.017 fmol/g), and liver (0.219 ± 0.017 fmol/g), as
compared with other organs, such as adipose tissue (0.015 \pm 0.001 \text{ fmol/g}) and skeletal muscle (0.017 \pm 0.004 \text{ fmol/g}) (Fig. 1, inset). To determine the glucose dependence of NAADP uptake in various organs, we compared the [\textsuperscript{32}P]NAADP uptake before and after glucose feeding (2 g/kg glucose). NAADP uptake was significantly increased in the adipose tissue and the pancreas and to a lesser degree in the liver in the presence of glucose, compared with in the absence of glucose (Fig. 1). Together, these data showed that NAADP was mainly distributed in major organs that have a relatively large blood reservoir. However, glucose-dependent increase of NAADP uptake was selectively occurring in the glucose-metabolizing organs, suggesting that the NAADP uptake system may play a physiological role in glucose homeostasis.

Kinetics and Pharmacological Properties of the NAADP Uptake in Pancreatic Islets—The kinetics of NAADP uptake were examined in pancreatic \( \beta \)-cells; exogenous NAADP was transported in a concentration- and a time-dependent manner only in the presence of high glucose (Fig. 2, A and B). HPLC analyses showed that \( \sim 80\% \) of the NAADP taken up by the islets remained as NAADP until 30 s (control; 2.3\% versus 15 s; 12.6\% versus 30 s; 23.1\% of total AUC) (Fig. 2C), suggesting that NAADP was taken up by the islets as an intact form but was progressively hydrolyzed to nicotinic acid. That the NAADP taken up by the islets functions as an intracellular Ca\(^{2+}\) signaling messenger was shown by the fact that externally applied NAADP was highly effective in elevating intracellular Ca\(^{2+}\) in a high glucose-dependent man-
ner (Fig. 2D, top panels). In contrast, NAADP-AM, a cell-
permeable NAADP analog, induced a robust Ca\(^{2+}\) signal in a
glucose-independent manner, suggesting that the NAADP
uptake through the NAADP transporter is glucose-depen-
dent (Fig. 2D, bottom panels).

We next investigated the pharmacological properties of the
uptake mechanism for NAADP by using various inhibitors. We
found that DPR, a nucleoside inhibitor; NaN\(_3\), an ATP-deplet-
ing agent; Ca\(^{2+}\)-free conditions; and Ned-19, a structural
mimic of NAADP, all completely blocked NAADP uptake,
whereas carbenoxolone, an inhibitor of connexin 43 (35) was
ineffective (Fig. 2E). Ned-19 was well known as a blocker of
NAADP-mediated mobilization of intracellular Ca\(^{2+}\) stores.
Therefore, we tested the efficacy of the reagent to block the
NAADP uptake and NAADP-mediated Ca\(^{2+}\) mobilization. It
blocked NAADP uptake with an IC\(_{50}\) of \(~50\, \text{nm}\), which was
\(~40\)-fold lower than its inhibition on the NAADP-AM-in-
duced mobilization of intracellular Ca\(^{2+}\) stores (Fig. 2F), indi-
cating that Ned-19 inhibited strongly the uptake process at low
concentrations.
NAADP-induced Ca\(^{2+}\) Signals in Pancreatic \(\beta\)-Cells, Acinar Cells, and Adipocytes—We next analyzed NAADP-induced Ca\(^{2+}\) signals in the adipose tissue and the pancreas. Consistent with our earlier observation (3) and the present data, NAADP-induced Ca\(^{2+}\) signals in \(\beta\)-cells under high glucose conditions (Fig. 3A) were completely blocked by pretreatment of Ned-19 or a V-ATPase inhibitor, bafilomycin A1 (Fig. 3, B and C). Intriguingly, acinar cells did not respond to exogenous NAADP (Fig. 3D). However, these cells appeared to be normal because cholecystokinin, a well known physiological agonist using NAADP as a Ca\(^{2+}\) mobilizing messenger (11), induced Ca\(^{2+}\) signals, which were completely blocked by pretreatment of Ned-19 (Fig. 3, E and F). In adipocytes, NAADP induced Ca\(^{2+}\) signals, which were completely blocked by pretreatment with Ned-19 or bafilomycin A1, as seen in pancreatic \(\beta\)-cells (Fig. 3, G–I). These data indicate that only cells involved in the glucose metabolism, the adipocytes and the pancreatic \(\beta\)-cells, and not exocrine cells elicit Ca\(^{2+}\) signals in response to exogenous NAADP, due to the difference in their NAADP uptake ability.

Differential Effect of NAADP in Different Cell Types Due to the Differences in NAADP Uptake—We examined the functional consequence of NAADP uptake in islets. Consistent with our earlier observation (3), when 5–500 nM NAADP was applied in the presence of 12 mM glucose, insulin secretion was effectively stimulated. In fact, at 50 nM, NAADP was as effective as 10 nM GLP-1 in stimulating insulin secretion (Fig. 4A). However, at low glucose (2.8 mM), 50 nM NAADP did not show any effect (data not shown). We next examined the effects of NAADP on pancreatic acinar cells and adipocytes. In mouse pancreatic acinar cells, where NAADP is known to be a Ca\(^{2+}\)-mobilizing messenger (2, 11), NAADP failed to induce amylase release (Fig. 4B). The cells, however, did respond effectively to cholecystokinin (100 pm) with enhanced amylase release. Similar to islets, adipocytes were responsive to NAADP (Fig. 4C), which induced an increase in glucose uptake with an optimum concentration of 50 nM. Based on these results, we reasoned that the differential effect of NAADP in different cell types might be due to the differences in NAADP uptake across the plasma membrane. Indeed, NAADP was well taken up by pancreatic islets and adipocytes in a glucose-dependent manner, whereas it was not taken up by pancreatic acinar cells, irrespective of the presence of glucose (Fig. 4D).

In order to exclude any possibility that the stimulatory effects of NAADP in the islets and adipocytes are via the activation of surface purinoreceptors, we examined the effects of equimolar concentrations of NAD, NADP, and ATP. However, these purinergic agonists induced neither insulin secretion from the islets nor glucose uptake in adipocytes (Fig. 5, A and B). These results indicate that exogenously applied NAADP on the islets and adipocytes is transported into the cells and acts on its specific receptors as an intracellular Ca\(^{2+}\) second messenger.

Administration of NAADP Ameliorates Glucose Tolerance, Insulin Secretion, and Islet Insulin Contents in a Type 2 Diabetic Mouse Model—Because external NAADP can induce not only insulin secretion in pancreatic \(\beta\)-cells but also glucose uptake in adipocytes, we next examined whether the administration of NAADP can alleviate diabetic symptoms in a type 2 diabetic mouse model. Two different methods were used to administer NAADP to \(db/db\) mice. Glucose tolerance in the mice was significantly ameliorated, either acutely by intraperitoneal injection of a single dose of NAADP 20 min before glucose administration (Fig. 6A) or chronically by using slow releasing NAADP pellets for 2 weeks (Fig. 6B), as compared with control mice treated with just the vehicle or NAADP (Supplemental Fig. S2). After glucose challenge, the serum levels of insulin in NAADP-treated mice were significantly higher than those of control mice (Fig. 6C). We and others have observed that treatment of pancreatic islets or \(\beta\)-cells with insulin can stimulate insulin production, resulting in an increase in cellular insulin content (13, 36). Immunochemistry staining revealed that the NAADP treatment increases the number of insulin-positive islets as compared with those of the control group (Fig. 6D), suggesting that the increase of insulin content in the islets was due to an autocrine action of insulin secreted by exogenous NAADP in \(db/db\) mice.

We examined whether the NAADP treatment produces other side effects in the mice. Thus, we compared the weights of the whole body, adipose tissues, and liver and the serum levels of amylase and hepatic enzymes of \(db/db\) mice before and after treatment with NAADP. No statistically significant changes in body weight and serum amylase level were observed, although adipose tissue weight increased after applying NAADP to \(db/db\) mice (Fig. 6, E–G). Toxicology parameters, such as elevation in serum levels of hepatic enzymes or hepatomegaly, were not observed during the whole experiment period (data not shown).

NAADP Release from Pancreatic Islets or \(\beta\)-Cells and Its Reuptake—That cells possess a high affinity NAADP uptake mechanism suggests that NAADP should be present extracellularly under physiological conditions. We, therefore, investigated the possibility of NAADP being released from the pancreatic islets in a stimulus-dependent manner. We directly measured NAADP released into the culture medium from pancreatic islets or MIN-6 cells (a \(\beta\)-cell line) after stimulation with high glucose. We could not detect NAADP release even in high glucose conditions (Fig. 7A, open circle). We surmised that the released NAADP might be rapidly taken back up by the cells. Therefore, we preincubated the cells with DPR to prevent the
reuptake of NAADP. Indeed, under this condition, we measured NAADP release with a lag time of 1 min following an addition of high glucose (Fig. 7A, closed circle). Treatments with two other NAADP uptake inhibitors, NaN₃ and Ca²⁺/H₁₁₀₀₁⁻ free, were not able to reveal the glucose-induced NAADP release (Fig. 7B), suggesting that both blocked the release process as well. However, Ned-19 did not block the release (Fig. 7B). These findings suggest that transporters for the uptake and release of NAADP are different. The observed glucose-induced release of NAADP and its rapid reuptake suggest a potential autocrine/paracrine role for NAADP.

Paracrine Effect of MIN-6 Cell-released NAADP on Adipocytes—To strengthen the hypothesis of the paracrine role for NAADP, we performed an experiment using different cellular systems for NAADP uptake (adipocytes) and release (MIN-6 β-cells) by challenging the adipocytes with supernatants from high glucose-treated β-cells. The supernatants from β-cells induced a sustained Ca²⁺ rise in adipocytes, which was significantly blocked by Ned-19 (supplemental Fig. S3), demonstrating the paracrine function of NAADP between different cells.

DISCUSSION

In this report, we demonstrated that NAADP was selectively transported in both pancreatic β-cells and adipocytes in the presence of high glucose and Ca²⁺. Application of NAADP can induce insulin secretion and stimulate glucose uptake via long lasting Ca²⁺ signals in these respective cells. Furthermore,
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**A**

![Graph A](image)

**B**

![Graph B](image)

**FIGURE 7. NAADP release was induced by high glucose.** A, high glucose induces the release of NAADP from pancreatic islets. Islets (200 islets, 30 min) were preincubated with 2.8 mM glucose-KR buffer with or without 50 \( \mu \)M DPR and then exchanged with 2.8 or 12 mM glucose-Krebs-Ringer (KR), and supernatants were collected by oil stop method. Released NAADP was measured at different time periods after glucose stimulation with 2.8 mM glucose without DPR (open triangle), 2.8 mM glucose with DPR (closed triangle), 12 mM glucose without DPR (open circle), and 12 mM glucose with DPR (closed circle) (n = 4). B, effects of various inhibitors on NAADP release. MIN-6 cells (1 × 10^6 cells) were preincubated with the indicated inhibitors and/or DPR for 10 min at room temperature and incubated in the glucose conditions indicated for 2 min at room temperature. After the removal of supernatant by oil stop method, released NAADP was measured by enzymatic cycling methods. *, \( p < 0.05 \) versus zero time group with 2.8 mM glucose and DPR. \#, \( p < 0.05 \) versus 12 mM glucose group without DPR. Error bars, S.E.

physiological stimuli, such as high glucose, can activate NAADP release from pancreatic islets, whereas application of NAADP to live diabetic mice can effectively improve their glucose tolerance. These results are consistent with NAADP functioning as an autocrine/paracrine hormone. That the mechanisms for NAADP uptake and release are present only in the glucose metabolism-related cells, adipocytes, and pancreatic islets but not the acinar cells suggests that NAADP could be utilized specifically and selectively for glucose homeostasis. Indeed, we and others have shown previously that NAADP is the \( \text{Ca}^{2+} \) messenger responsible for mediating the autocrine effects of insulin in islets (36) and \( \beta \)-cells (13). These data, together with our current demonstration of its release from these cells and its activity of stimulation of insulin secretion, further document its physiological role in amplifying the insulin signals. Together with the finding that NAADP itself is the most potent \( \text{Ca}^{2+} \) second messenger, with an optimum concentration of as low as 50 nM in adipocytes and pancreatic \( \beta \)-cells (Figs. 2 and 3) (3), NAADP transport systems are likely to function as a physiological positive feedback tool for glucose homeostasis. That an intracellular messenger molecule can also have extracellular signaling functions is a novel concept but not unprecedented. A case in point is ATP, which not only serves an energetic role intracellularly but also is released to function as a neurotransmitter.

The pharmacological characterization of the NAADP release and uptake systems presented in this study indicates that they are mediated by different entities. Although both are dependent on high glucose and on the presence of \( \text{Ca}^{2+} \) in the extracellular medium and are sensitive to metabolic inhibition (NaN_3), the uptake system but not the release system is sensitive to Ned-19 and DPR (cf. Fig. 7B). The remarkable effectiveness of Ned-19 in blocking the uptake process (Fig. 2E) strongly suggests that it is mediated by a transporter with a binding site specific for NAADP, because Ned-19 was developed based on its structural similarity with NAADP (17). Indeed, the potency of Ned-19 for the transporter appears to be more than an order of magnitude higher than for the intracellular NAADP receptor mediating \( \text{Ca}^{2+} \) mobilization. The fact that the transporter should be on the cell surface having ready access to the applied Ned-19 may contribute to its effectiveness also. The transporter may be related to the nucleoside transporter commonly present on cell surface because it is likewise inhibited by DPR. As for the NAADP release mechanism, its insensitivity to these site-specific inhibitors suggests that it may be mediated by a nonspecific channel, such as connexin 43, that has been shown to be able to mediate the release of NAD from cells (35).

Previous studies have reported that NAADP is cell-impermeable. However, as shown in this study, NAADP is selectively transported in certain tissues in a glucose- and \( \text{Ca}^{2+} \)-dependent manner. This is consistent with the findings of Billington et al. (24), showing that NAADP is efficiently transported in a Na^+- and \( \text{Ca}^{2+} \)-dependent manner in RBL-2H3 cells, resulting in an elevation in intracellular \( \text{Ca}^{2+} \). That extracellular application of NAADP is effective in evoking \( \text{Ca}^{2+} \) responses in glial and neuronal cells (21–23) has also been reported, although the response may have been mediated by the adenosine receptors or by purinergic receptors. The impermeability reported previously could be due to a non-conducive condition used.

The therapeutic potential of NAADP is clearly indicated in this study. Indeed, we show here that NAADP administration ameliorates glucose tolerance in the diabetic mice by enhancing insulin secretion in pancreatic \( \beta \)-cells and glucose uptake in adipocytes. Although intensive insulin therapy is required to minimize the development of long term complications of the disease, hypoglycemic shock caused by excess insulin is the most common complication in insulin therapy, occurring in over 90% of patients (37). In contrast, in vivo application of NAADP would have no risk in inducing a hypoglycemic shock when used as an antidiabetic agent, because its action is
dependent on the presence of a glucose concentration higher than normal. Moreover, the dual role of NAADP in targeting systems expressed in both the islets and adipose tissues could be an effective tool for lowering glucose levels and may also avoid many potential side effects from non-selective drugs. Finally, NAADP is an intrinsic second messenger molecule that can be readily metabolized into harmless products. Because many currently prescribed drugs, including insulin, become less effective during treatment, the demand for the development of new antidiabetic drugs is high. This study provides the first evidence for direct application of intracellular Ca\(^{2+}\) second messenger as a potential antidiabetic agent.

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