An insulin-related peptide expressed in 3T3L1 adipocytes is localized in GLUT4 vesicles and secreted in response to exogenous insulin, which augments the insulin-stimulated glucose uptake

Yoko Nakamichi[1], Mica Ohara-Imaizumi[1], Hitoshi Ishida[2] and Shinya Nagamatsu[1,*]

[1]Departments of Biochemistry, [2]Internal Medicine (III), Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan

*Author for correspondence (e-mail: shinya@kyorin-u.ac.jp)

Accepted 18 September 2002
Journal of Cell Science 116, 73-79 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00178

Summary

If an adipocyte is programmed to secrete insulin, then the insulin released may amplify the insulin action by an autocrine manner. To examine this hypothesis in vitro, we investigated the effects of expressing the preproinsulin gene in 3T3L1 adipocytes on (pro)insulin release and glucose uptake. The human preproinsulin gene was transferred into 3T3L1 adipocytes by infecting the cells with recombinant adenovirus Adex1CA human preproinsulin. Immunocytochemical studies showed that (pro)insulin is associated with vesicular structures that colocalize with GLUT4 vesicles but not with GLUT1 vesicles. We then examined insulin-induced proinsulin release from 3T3L1 adipocytes expressing the insulin gene. The exogenously administered insulin stimulated proinsulin release from these cells in a dose-dependent manner. HPLC determination revealed the existence of mature human insulin in these cells, which suggested the release of mature insulin into the medium. Further we monitored the (pro)insulin release from these cells with confocal laser microscopy using the expression of a fusion protein between insulin and green fluorescent protein (GFP). Time-lapse confocal laser-scanning microscopy revealed that the total number of vesicles containing insulin-GFP was decreased by the addition of 10^{-7} M insulin within 1 minute. Finally, we examined the insulin-stimulated glucose uptake by these cells. The data showed that insulin-stimulated glucose uptake increased to about 150% of that of control cells in response to exogenously administered insulin, indicating that the insulin released augmented the insulin-stimulated glucose uptake in an autocrine manner. Thus, the data support our hypothesis, indicating that we could construct the insulin-regulated insulin release system in adipocytes by introducing the preproinsulin gene.

Key words: Gene therapy, Type 2 diabetes, Insulin, Adipocyte, Adenovirus, Glucose transporter

INTRODUCTION

One possible therapeutic strategy for type 2 diabetes is the construction of an insulin production and secretory pathway within non-β cells by transferring relevant genes and utilizing these cells as a substitute for β cells. It is essential, however, that the production and/or the secretion of insulin in such cells is physiologically regulated. Many groups have been seeking to expand or regenerate pancreatic β cells by genetic engineering techniques (Kolodka et al., 1995; Leibowity and Levine, 1999); however, most of the trials have not resulted in clinical applications because a definite way to regulate insulin secretion from those cells has not yet been established (Hughes et al., 1992; Vollenweider et al., 1992; Ferber et al., 2000). In the present study, we took advantage of the characteristic features of adipocytes, that is, that they have the ability to secrete many adipocytokines [leptin, tumor necrosis factor (TNF)-α, plasmignogen activator inhibitor (PAI)-1 etc.] (Mohamed-Ali et al., 1998) and that the regulated exocytotic pathway of GLUT4 molecule can be triggered by insulin stimulation (Suzuki and Kono, 1980; Czech and Corvera, 1999; Hashiramoto and James, 2000). It is notable that adipose tissue has recently been considered to be an endocrine organ (Trayhurn and Beattie, 2001). We hypothesized that if the insulin precursor as spontaneously targeted to the GLUT4-containing intracellular compartments, then (pro)insulin might be secreted in response to exogenously administered insulin via the translocation of GLUT4, which consequently enhances the insulin-stimulated glucose uptake (Fig. 1). Indeed, we had some success in normalizing the blood glucose levels in obese diabetic KKA^y mice by introducing a human insulin gene into adipose tissues (Nagamatsu et al., 2001a). Here, in order to test our hypothesis, we examined in vitro (1) the subcellular localization of (pro)insulin expressed in 3T3L1 adipocytes; (2) insulin-regulated (pro)insulin release and glucose uptake from 3T3L1 adipocytes expressing insulin gene; and (3) proinsulin processing in these cells. The data revealed that, in 3T3L1 adipocytes harboring the insulin gene, (pro)insulin is released in an exogenously administered insulin-dose-dependent manner and glucose uptake is increased, suggesting that the augmentation of insulin-induced glucose uptake is probably mediated by an autocrine mechanism of insulin release from insulin target tissue.

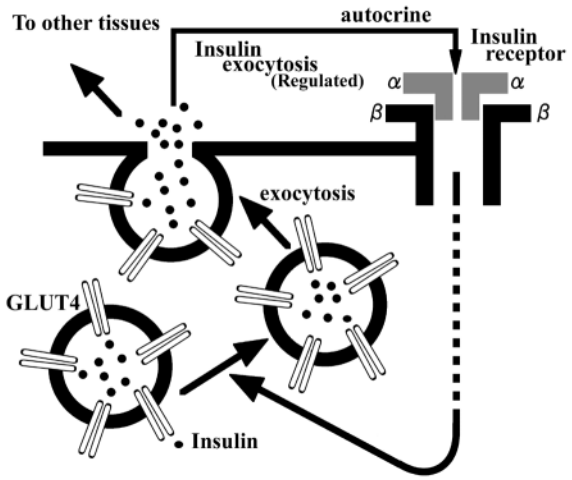

Fig. 1. Scheme illustrating the hypothesis of insulin-induced insulin release system in adipocytes. If (pro)insulin produced in adipocytes is to be included in GLUT4 vesicles, then (pro)insulin would need to be released in parallel with exocytosis of the GLUT4 vesicle in response to exogenous insulin stimulation. This would require the mechanism by which insulin receptor signaling causes movement of the GLUT4 vesicle from the intracellular pool to the plasma membrane and subsequently amplifies the insulin action through its own receptor via the autocrine mechanism.

Materials and Methods

Recombinant adenovirus and plasmid constructs

Adenovirus containing the human preproinsulin cDNA, Adex1CA-pchi, was constructed basically as described before (Nagamatsu et al., 1999). It contains full-length human preproinsulin cDNA (a generous gift from G. I. Bell, University of Chicago) under the control of the modified chicken β-actin promoter with cytomegalovirus immediate early enhancer (CAG) promoter (a gift from I. Saito, Tokyo University Institute of Medical Sciences). Adex1w containing no foreign cDNA was used as a control. We have also produced the recombinant adenovirus encoding the fusion protein of insulin and enhanced green fluorescent protein (EGFP). For appending EGFP to the C-terminus of preproinsulin, human preproinsulin cDNA was fused to pEGFP-N1 vector (Clontech) in-frame (pchi-GFP), then the coding region of this fusion protein was introduced into the adenovirus (Adex1CA pchi-GFP). This construct of insulin-GFP expressed in pancreatic β-cells was shown to be correctly delivered to insulin secretory granules (Ohara-Imaizumi et al., 2002).

Cell culture and adenovirus infection

3T3L1 adipocytes were differentiated by incubation in medium containing 10% fetal bovine serum (FBS), 1.7 μM insulin, 0.5 mM 3-isobutyl-1-methylxantine (IBMX) and 1 mM dexamethasone. Differentiated adipocytes were used for the experiments 10 days after initiation of differentiation. For introducing the recombinant adenovirus, differentiated adipocytes were infected by the indicated adenovirus at multiples of infection (MOI) of 20 plaque forming units (pfu)/cell. 2 days later experiments were performed.

Immunocytochemistry

For immunofluorescence studies of 3T3L1 adipocytes, 3T3L1 cells were differentiated to adipocytes on glass chamber slides (eight wells: Lab-Tek slides, Nunc) and infected with the indicated adenovirus. Two days later cells were fixed with 2% paraformaldehyde, then immunostained with mouse monoclonal anti-insulin antibody (Sigma) and/or rabbit polyclonal anti-GLUT4 antibody and/or anti-GLUT1 antibody (a gift from K. Takata, Gunma University, Maebashi, Japan), using appropriate second antibodies as described previously (Nagamatsu et al., 2001b). Slides were examined using a Carl Zeiss LSM510 laser-scanning confocal microscopy (Carl Zeiss, Co. Ltd., Jene, Germany) at the excitation wavelength of 488 nm for GFP and 543 nm for rhodamine using a band pass filter so as not to overlap the emitted light from GFP and rhodamine as described previously (Ohara-Imaizumi et al., 2002).

Time-lapse confocal microscopy

3T3L1 adipocytes were cultured and infected with Adex1CA pchi-GFP on the glass chamber slide for imaging with confocal microscopy. Prior to imaging, cells were incubated for 1 hour in Krebs-Ringer buffer (KRB) containing 110 mM NaCl, 4.4 mM KCl, 1.45 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.3 mM calcium gluconate, 4.8 mM $NaHCO_3$, 11 mM glucose, 10 mM HEPES (pH 7.4) and 0.3% bovine serum albumine (BSA) without insulin, then transferred to the thermostat-controlled (37°C) stage of confocal microscopy. The cells were, then, stimulated by adding 10^{-7} M bovine insulin. A time-course of the change of GFP-labeled vesicles was obtained from time-lapse images every 1 minute at the excitation wavelength of 488 nm during stimulation. The data were analyzed by Metamorph software (Nippon Roper Co. Ltd.).

Proinsulin release

After 3T3L1 adipocytes expressing human insulin gene were preincubated for 1hour in KRB buffer containing 11 mM glucose without insulin, cells were washed several times, and they were challenged by a various concentration of bovine insulin for 1 hour in a 500 μl of KRBG buffer. At the end of incubation, supernatants were collected, and proinsulin in the medium was measured by proinsulin ELISA assay kit using the human proinsulin standard (DAKO, Denmark), which does not react with bovine insulin at all.

Metabolic labeling and immunoprecipitation

Two days after 3T3L1 differentiated adipocytes were infected with Adex1CA-pchi at an MOI of 20 pfu/cell, cells were pulse-labeled in methionine (Met)-cysteine (Cys)-free RPMI 1640 medium containing 10% (vol/vol) dialyzed FBS with 400 μCi of ^{35}S-Met/Cys (New England Nuclear-Dupont, Boston, MA) for 1 hour at 37°C. After washing out the labeling medium several times, cells were chased for 1 hour. Labeled cells were disrupted by sonication in a lysis buffer (0.1 M Tris-HCl, 0.05 M NaCl, 0.25% (w/v) BSA, 0.1% (v/v) Triton X-100, pH 7.5) containing a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 50 μg/ml trasylol, 10 μg/ml leupeptin, and 5 μg/ml pepstatin A), and cell lysates were immunoprecipitated with a guinea pig anti-insulin antibody (DAKO) by using protein A sepharose beads (Pierce), as described previously (Nagamatsu et al., 1999). The immunoprecipitates were treated with acetic acid, then neutralized and separated on a superdex peptide column (Amersham Pharmacia Biotech, Buckinghamshire, UK) equipped with an HPLC (Beckman instruments, Inc., Fullerton, CA), and radioactivity of each fraction was measured as described previously (Nagamatsu et al., 1999).

HPLC and insulin measurement

For detecting only mature insulin, we used the reverse phase HPLC, a model obtained from Beckman (System Gold), connected to a TSK-GEL ODS-80^{TM} column (length 15.0 cm, internal diameter 4.6 mm) (TOSOH Co. Ltd., Kanagawa, Japan). Ultraviolet absorption was monitored at 210 nm using a Power Chrom (Eicom Co. Ltd., Kyoto, Japan) connected to a Macintosh computer. The two buffers used were

as follows: Buffer A, 0.05% trifluoroacetic acid (TFA) and 10% (v/v) acetonitrile. Buffer B, 0.05% TFA and 80% (v/v) acetonitrile. The flow rate was 1 ml/minute, and 1 minute fractions were collected. The % of B was increased linearly from 10% to 33% for the first 15 minutes, then from 33 to 38% for 15 minutes, finally from 38% to 80% for 30 minutes. The HPLC fraction was neutralized and diluted by insulin assay buffer, and insulin was measured by insulin ELISA kit using a human insulin standard (Molecular Biology Laboratories, Co. Ltd., Nagoya, Japan). Recombinant human insulin and bovine insulin (Sigma) as the standard were dissolved in 0.01 N acetic acid containing 0.05% BSA.

Glucose uptake assay

Two days after 3T3L1 adipocytes were infected with Adex1CA-pchi, cells were placed in Hanks' solution (0.02 g/dl BSA, 136.9 mM NaCl, 5.6 mM KCl, 0.34 mM Na_2HPO_4-$7H_2O$, 0.44 mM KH_2PO_4, 1.27 mM $CaCl_2$ and 4.20 mM $NaHCO_3$, 0.1 mM 2-deoxy-D-glucose, pH 7.4) for 1 hour prior to glucose uptake experiments. The assay was essentially performed as described previously (Nagamatsu and Steiner, 1992). Briefly, after 1 hour of preincubation, cells were washed several times with Hanks' solution, and treated with different concentrations of insulin for 30 minutes at 37°C, and then 0.5 μCi of [^{14}C]-2-deoxy-D-glucose (NEN Life Science Products Inc., Boston, MA) was added to the medium containing 0.1 mM unlabeled 2-deoxy-D-glucose. After 20 minutes of incubation at room temperature, cells were washed several times with ice-cold phosphate buffered saline (PBS), solubilized by the addition of 0.2 M NaOH, and the radioactivity was counted using a liquid scintillation counter.

Statistical analysis

Results are means±s.e.m. from at least three different experiments performed independently unless stated otherwise. Statistical analysis was performed by ANOVA followed by Fisher's test and regression analysis using the Statview software (Abacus Concepts, Inc., Berkeley, CA).

Results

Cellular localization of (pro)insulin expressed in 3T3L1 adipocytes

We first determined the subcellular localization of (pro)insulin expressed in 3T3L1 adipocytes. For this purpose, after 3T3L1 adipocytes were infected with the recombinant adenovirus encoding human preproinsulin gene (Adex1CA-pchi), they were immunostained with both anti-GLUT4 rabbit polyclonal and anti-insulin mouse monoclonal antibodies using the appropriate secondary antibodies conjugated with fluorescein isothiocyate (FITC) and/or rhodamine. They were then examined by confocal laser microscopy. The staining pattern of vesicular structures of insulin (Fig. 2A, insulin, rhodamine) was similar to that observed using an anti-GLUT4 antibody (Fig. 2A, GLUT4, FITC). The yellow color (Fig. 2A, merge) obtained by overlaying the FITC with the rhodamine indicated the colocalization of the expressed (pro)insulin with GLUT4 in the same vesicles. We, further, examined the localization of the introduced (pro)insulin and GLUT1 in 3T3L1 adipocytes. 3T3L1 adipocytes infected with Adex1CA pchi were double-immunostained with both anti-GLUT1 rabbit polyclonal and anti-insulin mouse monoclonal antibodies. As shown in Fig. 2B, GLUT1 (rhodamine) was mainly localized to the plasma membrane, thus there was no colocalization observed between (pro)insulin (FITC) and GLUT1 (rhodamine). Thus, our data demonstrated that (pro)insulin expressed in 3T3L1 adipocytes was mostly delivered to the GLUT4 vesicle, although the precise mechanism is currently unknown.

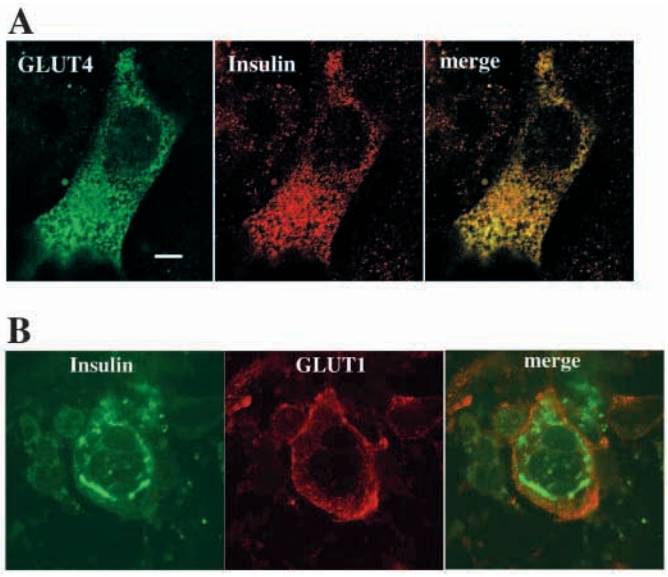

Fig. 2. Cellular localization of (pro)insulin expressed in 3T3L1 adipocytes. (A) Colocalization of (pro)insulin and GLUT4 in the same vesicle. 3T3L1 adipocytes were infected with Adex1CA pchi at an MOI of 20 pfu/cell, then they were fixed with 2% PFA and immunostained with both rabbit polyclonal anti-GLUT4 and mouse monoclonal anti-insulin antibodies followed by respective secondary antibody treatment (GLUT4: FITC, insulin: rhodamine). Immunofluorescent staining was observed by confocal laser microscopy with band-pass filter. Bar, 5 μm. (B) (Pro)insulin and GLUT1 are differently distributed in 3T3L1 adipocytes. After cells were infected and fixed as in A, they were immunostained with both rabbit polyclonal anti-GLUT1 and monoclonal insulin antibodies as described in A. Antibody complexes were visualized with appropriate secondary antibodies coupled to rhodamine (GLUT1) and FITC (insulin). Note the different immunofluorescent staining pattern between the peripheral staining of GLUT1 (rhodamine) and vesicular distribution of (pro)insulin (FITC) in the same cell with confocal laser-microscopy.

Secretion of proinsulin from 3T3L1 adipocytes

If (pro)insulin produced in adipocytes is to be included in GLUT4 vesicles, then the peptide would be released in parallel with the exocytosis of the GLUT4 vesicle and in response to exogenously administered insulin. It would need to use the mechanism by which insulin receptor signaling causes movement of the GLUT4 vesicle from the intracellular pool to the plasma membrane. Therefore, we examined whether adipocyte (pro)insulin is released in response to exogenously administered insulin. We measured the proinsulin release from these cells by adding exogenous bovine insulin from 10^{-9} M to 10^{-7} M. Since we cannot measure the human insulin release from these cells by insulin ELISA assay system, which crossreacts with bovine insulin in the medium, we measured human proinsulin in the medium using a human intact proinsulin ELISA assay system, which does not react with 10^{-7} M bovine insulin (data not shown). As shown in Fig. 3,

proinsulin release was increased in response to exogenously administered insulin (no insulin, 5.1±1.8; 10^{-9} M insulin, 6.7±2.9; 10^{-8} M insulin 22.4±6.2; 10^{-7} M insulin 29.5±7.2 pg/ml/10^5 cells).

Processing of proinsulin and the content of proinsulin and mature insulin in 3T3L1 adipocytes

We next examined whether the produced peptide derived from preproinsulin mRNA in 3T3L1 adipocytes can be processed into mature insulin. For this purpose, 3T3L1 adipocytes were labeled with ^{35}S-Met/cys for 1 hour and chased for 1 hour, then immunoprecipitated materials of cell lysates with an anti-insulin antibody were analyzed by the peptide column. Fig. 4A shows the column-profile of the material, demonstrating that at least a proportion of the proinsulin can be converted to mature insulin even in 3T3L1 adipocytes [% radiolabeled insulin in the cell=18.3±4.3% (*n*=3)]. Proinsulin-converting enzymes, prohormone convertases PC2 and PC3, are known to be expressed in most endocrine and neuroendocrine cells (Halban and Irminger, 1994; Neerman-Arbez et al., 1994), while furin is expressed ubiquitously (Hatsuzawa et al., 1990; Hosaka et al., 1991). Although PC enzymes are not expressed in adipocytes, we actually detected the presence of furin mRNA in adipocytes by RT-PCR analysis (data not shown). Human proinsulin does not have the exact consensus sequence recognized by furin (Yanagita et al., 1993); however, furin is still able to convert proinsulin to insulin (Vollenweider et al., 1995). Therefore, it is conceivable that proinsulin in 3T3L1 adipocytes was processed to insulin by furin, although its efficiency for proinsulin conversion must be low.

We then tried to determine the actual content of proinsulin and insulin in 3T3L1 adipocytes. 3T3L1 adipocytes were disrupted by sonication, and proinsulin content was measured using a proinsulin ELISA assay. The proinsulin content was 512±91 pg/10^5 cells (*n*=10). In order to quantify the mature insulin content, we used the reverse-phase HPLC system. In our system, recombinant human insulin was eluted at 21 minutes, as the % B was linearly increased from 33% to 38% (Fig. 4B). The fraction sample of 3T3L1 adipocyte lysates by HPLC system was measured by insulin ELISA assay, and Fig. 4C shows the mature insulin peak eluted from retention time 21 minutes, which was calculated to be 36±11.2 pg per 10^5

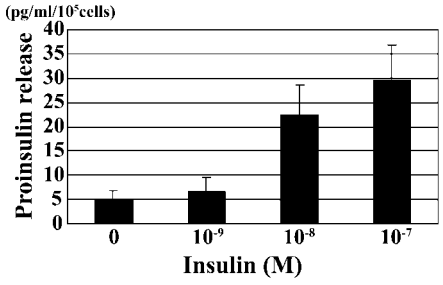

Fig. 3. Proinsulin secretion from 3T3L1 adipocytes harboring the preproinsulin gene. Two days after 3T3L1 adipocytes were infected with Adex1CA pchi, cells were preincubated for 1 hour under KRBG buffer without insulin, and they were challenged by 10^{-9}-10^{-7} M bovine insulin for 1 hour. At the end of incubation, medium was collected and proinsulin was assayed by human proinsulin ELISA.

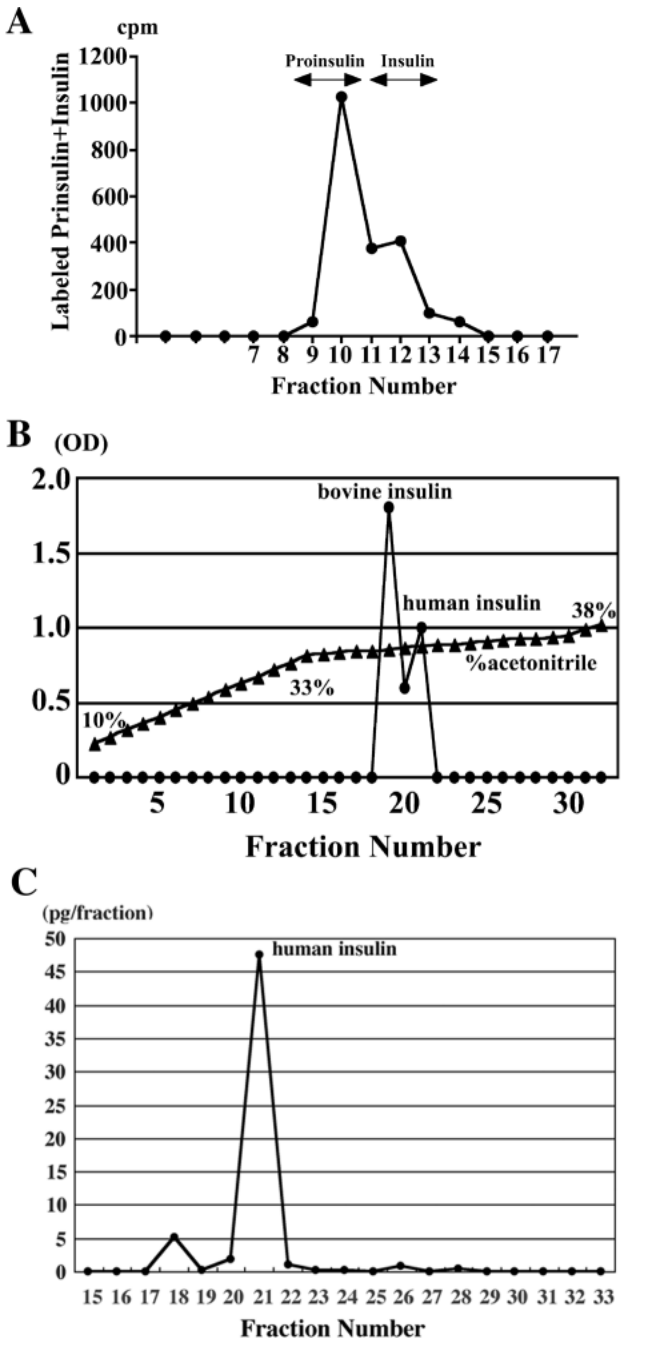

Fig. 4. Processing of proinsulin in 3T3L1 adipocytes. (A) Proinsulin and insulin separated by a peptide column. 3T3L1 adipocytes expressing the human insulin gene were labeled with ^{35}S-Met/Cys for 1 hour, then chased for 1 hour. Cells were disrupted, and cell lysates were immunoprecipitated with an anti-insulin antibody. The immunoprecipitant was separated using a superdex peptide column, and radioactivity in each fraction was counted. (B) Reverse phase HPLC analysis of authentic recombinant human insulin and bovine insulin. The authentic insulin standards (10 μg in 100 μl 3 M acetic acid) were applied to the HPLC system with an acetonitrile gradient and monitored by 210 nm. The elution times for human and bovine insulin are 21 and 19 minutes, respectively. (C) Mature insulin in 3T3L1 adipocytes cells detected by HPLC. 3T3L1 adipocytes expressing the human insulin gene were disrupted by sonication, then cell lysates were analyzed by HPLC. Each fraction from the HPLC was neutralized and assayed using a human insulin ELISA kit.

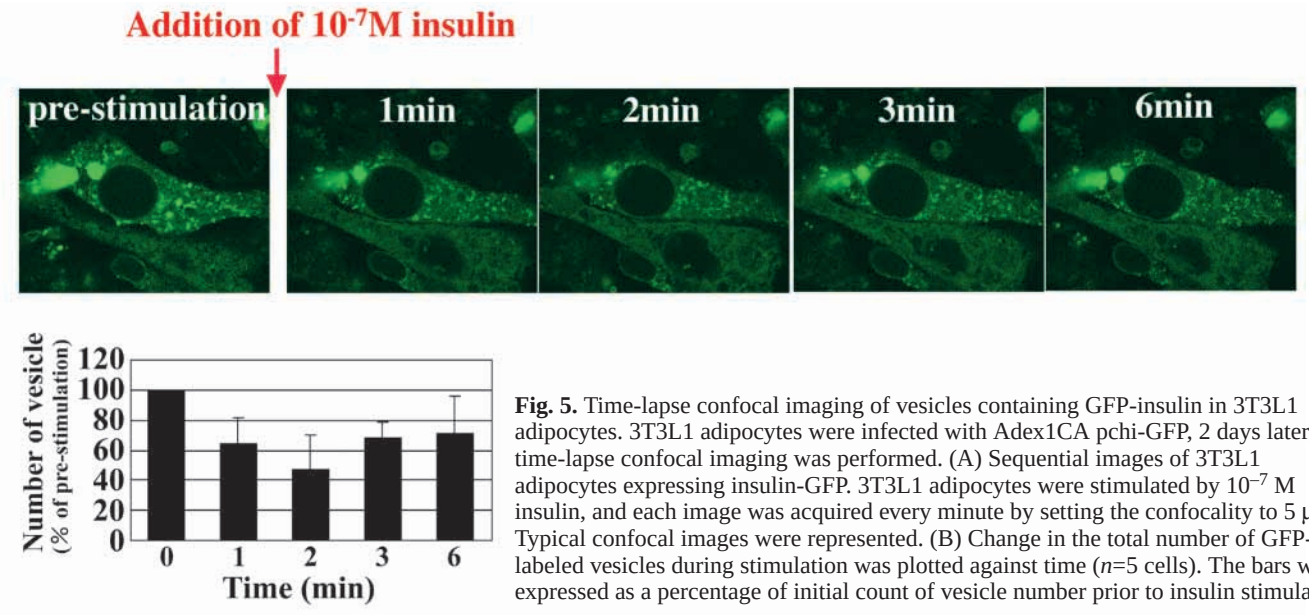

Fig. 5. Time-lapse confocal imaging of vesicles containing GFP-insulin in 3T3L1 adipocytes. 3T3L1 adipocytes were infected with Adex1CA pchi-GFP, 2 days later, time-lapse confocal imaging was performed. (A) Sequential images of 3T3L1 adipocytes expressing insulin-GFP. 3T3L1 adipocytes were stimulated by 10^{-7} M insulin, and each image was acquired every minute by setting the confocality to 5 μm. Typical confocal images were represented. (B) Change in the total number of GFP-labeled vesicles during stimulation was plotted against time (n=5 cells). The bars were expressed as a percentage of initial count of vesicle number prior to insulin stimulation.

cells (n=10). Thus, the molar ratio of proinsulin versus insulin in the cell was about 14:1. As these cells secrete approximately 30 pg proinsulin into 1 ml culture medium per 10^5 cells in response to 10^{-7} M bovine insulin-stimulation as shown in Fig. 3, it is assumed that roughly 2~3 pg insulin is simultaneously released in response to exogenously administered insulin.

Time-lapse images of insulin-induced (pro)insulin release from 3T3L1 adipocytes

In order to directly observe the (pro)insulin release from 3T3L1 adipocytes, we monitored the change in number of GFP-labeled vesicles containing (pro)insulin using time-lapse confocal imaging. 3T3L1 adipocytes infected with Adex1CA pchi-GFP were stimulated by the addition 10^{-7} M insulin. The number of GFP-labeled vesicles in the confocal image of 1 minute post-insulin stimulation was decreased to approximately 64% of that counted in pre-stimulation. (Pre-stimulation; 100%; 1 minute post stimulation, 64±18%; 2 minutes post stimulation; 47±23%; 3 minutes post stimulation, 68±11%; 6 minutes post-stimulation, 71±25%) (Fig. 5B). The decline of the number of GFP-labeled vesicles by insulin stimulation was not due to the photobleaching, because the number of vesicles without insulin stimulation showed no change during the same time course [0 minutes, 100%; 1 minutes, 84±19%; 2 minutes, 94±11% (n=3 cells)]. Thus, the data indicate that (pro)insulin is exocytosed quickly, at least within 1 minute, from 3T3L1 adipocytes expressing the human insulin gene.

Glucose uptake by 3T3L1 adipocytes

Finally, we tested our hypothesis that insulin released from 3T3L1 adipocytes amplifies the insulin action through the endogenous insulin receptor in an autocrine manner by measuring insulin-stimulated ^{14}C-2-deoxy-D-glucose uptake by 3T3L1 adipocytes expressing the human insulin gene. As shown in Fig. 6, these adipocytes led to a progressive increase in glucose uptake in a dose-dependent manner of exogenously administered insulin compared with that by control cells. Glucose uptake by 3T3L1 adipocytes expressing the insulin gene was increased to approximately two-fold that of control cells under 10^{-8} M insulin (10^{-8} M insulin; 21,900±1530 versus 12,100±1020 cpm/dish, P<0.001), and the dose-dependent curve was shifted to the left (no statistical difference under 10^{-7} M insulin); however, only a little difference in glucose uptake was observed under the absence and presence of exogenous 10^{-9} M insulin between insulin expressing and control cells. Thus, it appears that insulin released from 3T3L1 adipocytes augmented the insulin-stimulated glucose uptake by exogenously administered insulin in an autocrine manner.

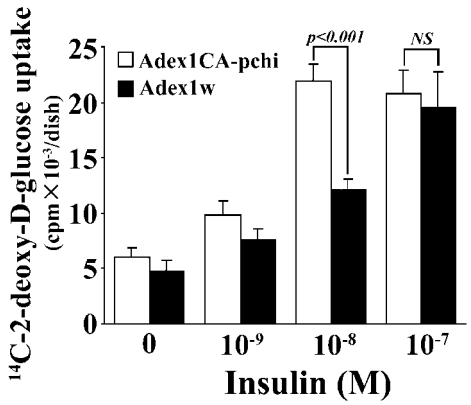

Fig. 6. Glucose uptake by 3T3L1 adipocytes. 3T3L1 adipocytes were infected with either Adex1CA pchi or Adex1w (for control), 2 days later, cells were preincubated for 1 hour under Hanks' solution without insulin and treated with different concentrations of insulin for 30 minutes at 37°C. Then, glucose uptake assay was performed over 20 minutes at room temperature per 1×10^6 cells under 10^{-9} M~10^{-7} M insulin using ^{14}C-2deoxy-D-glucose as a tracer (n=4). Data are means±s.e.m.

Discussion

Type-2 diabetes results from β-cell dysfunction and insulin resistance, or a combination of both, which can lead to diabetic complications such as retinopathy, nephropathy and neuropathy (Lilloja et al., 1993; Ward et al., 1984; Warram et al., 1990). To avoid these diabetic complications, the maintenance of tightly controlled blood glucose levels is required. Although gene therapy is one possible strategy to achieve this, it has not as yet been able to regenerate the entire function of pancreatic-β cells using non-β cells. Here, we have achieved some success in constructing the insulin-regulated insulin secretory pathway by introducing the preproinsulin gene into adipocytes.

In the present study, we first presented evidence that (pro)insulin expressed in 3T3-L1 adipocytes is targeted to GLUT4 vesicles. The signal peptide of preproinsulin seems to be important for its delivery to GLUT4 vesicle in 3T3L1 adipocytes, because the addition of GFP to the N-terminus of preproinsulin disturbed the sorting of the peptide to the GLUT4 vesicle (data not shown). Although the signal peptide of preproinsulin plays a crucial role in sorting the peptide from rough endoplasmic reticulum (RER) to the insulin secretory granules in pancreatic β cells (Rhodes, 2000), there is no report showing the role of preproinsulin signal peptide in adipocytes. Our data indicated that the signal peptide of preproinsulin has a functional role in delivering the peptide to GLUT4 vesicle in adipocytes. Since GLUT4 vesicle is exocytosed by insulin (Oatey et al., 1997; Czech and Corvera, 1999), insulin action on adipocytes may be amplified by the insulin released in parallel with GLUT4 vesicle exocytosis from adipocytes in an autocrine manner (Fig. 1). This autocrine release is probably able to improve the insulin resistance in diabetic adipocytes via a local exposure to progressively increasing concentrations of insulin. Indeed, we have reported that blood glucose levels in diabetic KKA^{y} mice and Zucker *fa/fa* rats, which have insulin resistance in insulin target tissues (Chang et al., 1986; King et al., 1992), were decreased by introducing human insulin gene into adipose tissues (Nagamatsu et al., 2001a).

As (pro)insulin is stored in GLUT4 vesicles, as shown in Fig. 2A, it is conceivable that, in adipocytes, exogenously administered insulin stimulates adipocyte (pro)insulin release via the regulated vesicular transport system. Therefore, we have examined the dose dependency of insulin-induced proinsulin release from 3T3L1 adipocytes. Although it is the best way to directly measure human insulin in the medium, we could not measure mature insulin released from adipocytes into the medium by the standard immunoreactive insulin assay system, because it detects not only human insulin but also bovine insulin, a large amount of which was exogenously administered to the medium. Therefore, we measured human proinsulin released in the medium, using the intact human proinsulin assay system. It is of interest that proinsulin release was increased in a dose-dependent manner with the increase in exogenously administered insulin, suggesting the successful construction of the insulin-regulated (pro)insulin release system in adipocytes. Furthermore, confocal images have clearly shown that the total number of the vesicle containing insulin-GFP was decreased by the addition of 10^{-7} M insulin, confirming the insulin-induced release of (pro)insulin from 3T3L1 adipocytes Thus, we thought that adipocyte (pro)insulin secreted in parallel with GLUT4 vesicle exocytosis might amplify the local insulin action. Indeed, glucose uptake by these cells was almost increased to a maximum response by 10^{-8} M of exogenously administered insulin, showing that insulin-dose-dependent curve for the glucose uptake was shifted to the left (Fig. 6). Of course, it cannot be ruled out that preproinsulin expressed in adipocytes may be sorted to vesicles other than GLUT4 vesicles. Indeed, it is reported that adipsin, a serine protease, and adipocyte complement related protein of 30 kDa (ACRP30) are secreted by insulin stimulation (Robinson et al., 1992; Scherer et al., 1995; Yang and Mueckler, 1999; Bogan and Lodish, 1999). As Bogan and Lodish (Bogan and Lodish, 1999) showed, insulin stimulates exocytosis of a regulated secretory compartment containing ACRP30 in 3T3L1 adipocytes, and some of the preproinsulin expressed in 3T3L1 adipocyte may be delivered to these vesicles. However, it is emphasized that, importantly, in any case, insulin stimulates proinsulin exocytosis in a regulated manner in our system.

The adipocyte insulin but not proinsulin must be released to enhance the insulin-stimulated glucose uptake by these cells, because mature insulin has a potent biological activity (Blundell et al., 1978; Renscheidt et al., 1984). We have attempted to directly measure the mature insulin in the medium using HPLC system; however, it was extremely difficult because it is only present in small quantities. Therefore, we estimated the actual amount of adipocyte insulin in the medium by calculation. The amount of human proinsulin released by the addition of 10^{-8} M bovine insulin was approximately 22 pg/ml, as shown in the results, so that the actual insulin amount in the medium is assumed to be 1~2 pg/ml (approximately 0.2 pM), on the basis of the data of proinsulin/insulin molar ratio (about 14:1) in the cell, which was derived from HPLC results. On the other hand, this concentration of adipose insulin in the medium, less than at least 1 pM, is not enough to evoke the insulin action on the insulin target tissues in general (Ellis et al., 1986). If so, how could insulin released from these cells enhance the insulin action of exogenous administered insulin in an autocrine manner? We assume that the local concentration of adipose insulin around the insulin receptor may be extremely high, just when the vesicles containing insulin are fused to the plasma membrane, which may be able to increase exogenous insulin action. Of course, many mechanisms other than autocrine loop proposed cannot be ruled out; overexpression or release of insulin in 3T3L1 adipocytes may affect the release of leptin, or resistin, which may affect the adipocyte metabolism (Unger et al., 1999; Steppan et al., 2001).

In conclusion, we have succeeded in constructing the regulated insulin secretory pathway within adipocytes. This is a first report of a successful construction of an insulin regulated insulin secretory pathway in non-β cells, and this method could provide potential clues for efficient gene therapy for type 2 diabetes, although many studies are required for the practical application to humans.

We thank for I. Saito for generously donating the adenovirus cosmid vector and parental virus; G. I. Bell for providing cDNAs of human GLUT4 and preproinsulin. This study was supported by a Grant-in-Aid for Scientific Research (Exploratory research) (14657043) from the Japanese Ministry of Education, Science and Culture; by a grant from Japan Private School Promotion Foundation (to S.N.).

References

Blundell, T. L., Bedarkar, S., Rinderknecht, E. and Humbel, R. E. (1978). Insulin-like growth factor. A model for tertiary structure accounting for immunoreactivity and receptor binding. *Proc. Natl. Acad. Sci. USA* **75**, 180-184.

Bogan, J. S. and Lodish, H. F. (1999). Two compartments for insulin-stimulated exocytosis in 3T3-L1 adipocytes defined by endogenous ACRP30 and GLUT4. *J. Cell Biol.* **146**, 609-620.

Chang, A. Y., Wyse, B. M., Copeland, E. J., Peterson, T. and Ledbetter, S. R. (1986). The Upjohn colony of KKAy mice: a model for obese type II diabetes. In *Diabetes* (eds Serrano-Rios, M. and Lefebure, P. J.), pp. 466-470. Amsterdam: Elsevier.

Czech, M. P. and Corvera, S. (1999). Signaling mechanisms that regulate glucose transport. *J. Biol. Chem.* **274**, 1865-1868.

Ellis, L., Clauser, E., Morgan, D. O., Edery, M., Roth, R. A. and Rutter, W. J. (1986). Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell* **45**, 721-732.

Ferber, S., Halkin, A., Cohen, H., Ber, I., Einav, Y., Goldberg, I., Seijffers, R., Kopolovic, J., Kaiser, N. and Karasik, A. (2000). Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. *Nat. Med.* **6**, 568-572.

Halban, P. A. and Irminger, J. C. (1994). Sorting and processing of secretory proteins. *Biochem. J.* **299**, 1-18.

Hashiramoto, M. and James, D. E. (2000). Characterization of insulin-responsive GLUT4 storage vesicles isolated from 3T3-L1 adipocytes. *Mol. Cell Biol.* **20**, 416-427.

Hatsuzawa, K., Hosaka, M., Nakagawa, T., Nagase, M., Shoda, A., Murakami, K. and Nakayama, K. (1990). Structure and expression of mouse furin, a yeast Kex2-related protease. Lack of processing of coexpressed prorenin in GH4Cells. *J. Biol. Chem.* **265**, 22075-22078.

Hosaka, M., Nagahama, M., Kim, W. S., Watanabe, T., Hatsuzawa, K., Ikemizu, J., Murakami, K. and Nakayama, K. (1991). Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. *J. Biol. Chem.* **266**, 12127-12130.

Hughes, S. D., Johnson, J. H., Quaade, C. and Newgard, C. B. (1992). Engineering of glucose-stimulated insulin secretion and biosynthesis in non-islet cells. *Proc. Natl. Acad. Sci. USA* **89**, 688-692.

King, P. A., Horton, E. D., Hirshman, M. F. and Horton, E. S. (1992). Insulin resistance in obese zucker rat (*fa/fa*) skeletal muscle is associated with a failure of glucose transporter translocation. *J. Clin. Invest.* **90**, 1568-1575.

Kolodka, T. M., Finegold, M., Moss, L. and Woo, S. L. C. (1995). Gene therapy for diabetes mellitus in rats by hepatic expression of insulin. *Proc. Natl. Acad. Sci. USA* **92**, 3293-3297.

Leibowity, G. and Levine, F. (1999). Gene therapy for type 1 and type 2 diabetes. *Diabetes Rev.* **7**, 124-138.

Lilloja, S., Mott, D. M., Spraul, M., Ferraro, R., Folry, J. E., Ravussin, E., Knowler, W. C. and Bennett, P. H. (1993). Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. Prospect studies of Pima Indians. *N. Eng. J. Med.* **329**, 1988-1992.

Mohamed-Ali, V., Pinkney, J. H. and Coppack, S. W. (1998). Adipose tissue as an endocrine and paracrine organ. *Int. J. Obes. Relat. Metab. Disord.* **22**, 1145-1158.

Nagamatsu, S. and Steiner, D. F. (1992) Altered glucose regulation of insulin biosynthesis in insulinoma cells: Mouse βTC3 cells secrete insulin-related peptides predominantly via a constitutive pathway. *Endocrinology* **130**, 748-754.

Nagamatsu, S., Nakamichi, Y., Yamamura, C., Matsushima, S., Watanabe, T., Ozawa, S., Furukawa, H. and Ishida, H. (1999). Decreased expression of t-SNARE, syntaxin 1, and SNAP-25 in pancreatic beta-cells is involved in impaired insulin secretion from diabetic GK rat islets: restoration of decreased t-SNARE proteins improves impaired insulin secretion. *Diabetes* **48**, 2367-2373.

Nagamatsu, S., Nakamichi, Y., Ohara-Imaizumi, M., Ozawa, S., Katahira, H., Watanabe, T. and Ishida, H. (2001a). Adenovirus-mediated preproinsulin gene transfer into adipose tissues ameliorates hyperglycemia in obese diabetic KKA^{y} mice. *FEBS Lett.* **509**, 106-110.

Nagamatsu, S., Nakamichi, Y., Watanabe, T., Matsushima, S., Yamaguchi, S., Ni, J., Itagaki, E. and Ishida, H. (2000b). Localization of cellubrevin-related peptide, endobrevin, in the early endosome in pancreatic β cells and its physiological function in exo-endocytosis of secretory granules. *J. Cell Sci.* **114**, 219-227.

Neerman-Arbez, M., Cirulli, V. and Halban, P. A. (1994). Levels of the conversion endoproteases PC1 (PC3) and PC2 distinguish between insulin-producing pancreatic islet beta cells and non-beta cells. *Biochem. J.* **300**, 57-61.

Oatey, P. B., van Weering, D. H. J., Dobson, S. P., Gould, G. W. and Tavar, J. M. (1997). GLUT4 vesicle dynamics in living 3T3L1 adipocytes visualized with green-fluorescent protein. *Biochem. J.* **327**, 637-642.

Ohara-Imaizumi, M., Nakamichi, Y., Tanaka, T., Ishida, H. and Nagamatsu, S. (2002). Imaging exocytosis of single insulin secretory granules with evanescent wave microscopy. *J. Biol. Chem.* **277**, 3805-3808.

Renscheidt, H., Strassburger, W., Glatter, U., Wollmer, A., Dodson, G. G. and Mercola, D. A. (1984). A solution equivalent of the 2Zn…4Zn transformation of insulin in the crystal. *Eur. J. Biochem.* **142**, 7-14.

Rhodes, C. J. (2000). Processing of the insulin molecule. *Diabetes Mellitus-A fundamental and Clinical text* (eds D. LeRoith, S. I. Taylor and J. M. Olefsky), pp. 20-38. Philadelphia: Lippincott Williams & Wilkins.

Robinson, L. J., Pang, S., Harris, D. S., Heuser, J. and James, D. E. (1992). Translocation of the glucose transporter (GLUT4) to the cell surface in permeabilized 3T3-L1 adipocytes: effects of ATP insulin, and GTP gamma S and localization of GLUT4 to clathrin lattices. *J. Cell Biol.* **117**, 1181-1196.

Scherer, P. E., Williams, S., Fogliano, M., Baldini, G. and Lodish, H. F. (1995). A novel serum protein similar to C1q, produced exclusively in adipocytes. *J. Biol. Chem.* **270**, 26746-26749.

Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S. and Lazar, M. A. (2001). The hormone resistin links obesity to diabetes. *Nature* **409**, 307-312.

Suzuki, K. and Kono, T. (1980). Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc. Natl. Acad. Sci. USA* **77**, 2542-2545.

Trayhurn, P. and Beattie, J. H. (2001). Physiological role of adipose tissue: White adipose tissue as an endocrine and secretory organ. *Proc. Nutr. Soc.* **60**, 329-339.

Unger, R. H., Zhou, Y. T. and Orci, L. (1999). Regulation of fatty acid homeostasis in cells: novel role of leptin. *Proc. Natl. Acad. Sci. USA* **96**, 2327-2332.

Vollenweider, F., Irminger, J.-C., Gross, D. J., Villa-Komaroff, L. and Halban, P. A. (1992). Processing of proinsulin by transfected hepatoma (FAO) cells. *J. Biol. Chem.* **267**, 14629-14636.

Vollenweider, F., Kaufmann, J., Irminger, J.-C. and Halban, P. A. (1995). Processing of proinsulin by furin, PC2, and PC3 in (Co) transfected COS (Monkey Kidney) cells. *Diabetes* **44**, 1075-1080.

Ward, W. K., Bolgiano, D. C., Mcknight, B., Halter, J. B. and Porte, D., Jr (1984). Diminished β cell secretory capacity in patients with noninsulin-dependent diabetes mellitus. *J. Clin. Invest.* **74**, 1318-1328.

Warram, J. H., Martin, B. C., Krolewski, A. S., Soeledner, J. S. and Kahn, C. R. (1990). Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. *Ann. Intern. Med.* **113**, 909-915.

Yanagita, M., Hoshino, H., Nakayama, K. and Takeuchi, T. (1993). Processing of mutated proinsulin with tetrabasic cleavage sites to mature insulin reflects the expression of furin in nonendocrine cell lines. *Endocrinology* **133**, 639-644

Yang, C. Z. and Mueckler, M. (1999). ADP-ribosylation factor 6 (ARF6) defines two insulin-regulated secretory pathways in adipocytes. *J. Biol. Chem.* **274**, 25297-25300.