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ORIGINAL ARTICLE

Palmitate induces reactive oxygen species production and β-cell dysfunction by activating nicotinamide adenine dinucleotide phosphate oxidase through Src signaling

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Keywords
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ABSTRACT

Aims/Introduction: Chronic hyperlipidemia impairs pancreatic β-cell function, referred to as lipotoxicity. We have reported an important role of endogenous reactive oxygen species (ROS) overproduction by activation of Src, a non-receptor tyrosine kinase, in impaired glucose-induced insulin secretion (GIIS) from diabetic rat islets. In the present study, we investigated the role of ROS production by Src signaling in palmitate-induced dysfunction of β-cells.

Materials and Methods: After rat insulinoma INS-1D cells were exposed to 0.6 mmol/L palmitate for 24 h (palmitate exposure); GIIS, ROS production and nicotinamide adenine dinucleotide phosphate oxidase (NOX) activity were examined with or without exposure to10 μmol/L 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), a Src inhibitior, for 30 or 60 min.

Results: Exposure to PP2 recovered impaired GIIS and decreased ROS overproduction as a result of palmitate exposure. Palmitate exposure increased activity of NOX and protein levels of NOX2, a pathological ROS source in β-cells. Palmitate exposure increased the protein level of $p47^{phox}$, a regulatory protein of NOX2, in membrane fraction compared with control, which was reduced by PP2. Transfection of small interfering ribonucleic acid of *$p47^{phox}$* suppressed the augmented $p47^{phox}$ protein level in membrane fraction, decreased augmented ROS production and increased impaired GIIS by palmitate exposure. In addition, exposure to PP2 ameliorated impaired GIIS and decreased ROS production in isolated islets of KK-A^y mice, an obese diabetic model with hyperlipidemia.

Conclusions: Activation of NOX through Src signaling plays an important role in ROS overproduction and impaired GIIS caused by chronic exposure to palmitate, suggesting a lipotoxic mechanism of β-cell dysfunction of obese mice.

INTRODUCTION

In pancreatic β-cells, glucose metabolism regulates exocytosis of insulin granules through metabolism-secretion coupling[1]. Reactive oxygen species (ROS) is one of the most important factors that impair glucose-induced insulin secretion (GIIS) 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]. ROS are normal byproducts of glucose metabolism, including glycolysis and mitochondrial oxidative phosphorylation[4]. In β-cells, ROS

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production through non-mitochondrial and mitochondrial pathways has been proposed. In the mitochondrial pathway, ROS is generated in the electron transport chain associated with the mitochondrial membrane potential[5]. However, in pathophysiological conditions, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), an important non-mitochondrial ROS source, could play an important role in ROS generation in β-cells[6].

Deleterious effects of chronic hyperlipidemia on β-cell function are referred to as lipotoxicity[7]. Chronic exposure to palmitate, a long-chain saturated fatty acid, impairs GIIS with an increase in production of ROS in β-cells[8,9]. Recently, an important role of NOX in ROS production in β-cells by palmitate exposure has been proposed[10]. In addition, both oxidative stress markers and NOX expression are increased in islets of obese diabetic rodents with hyperlipidemia[11,12]. We have proposed that endogenous overproduction of ROS involving activation of Src, a non-receptor tyrosine kinase, plays an important role in impaired metabolism-secretion coupling in islets of diabetic Goto–Kakizaki (GK) rats[13–15]. An important role of Src in activation of NOX has been reported in various cells[16,17]. In the present study, to elucidate the mechanism of lipotoxicity in β-cells more precisely, we investigated involvement of Src in ROS production derived from NOX and impaired GIIS caused by chronic exposure to palmitate.

MATERIALS AND METHODS

Materials

Palmitate obtained from Nacalai (Kyoto, Japan) was dissolved in 95% ethanol at stock concentration of 100 mmol/L. The specific Src inhibitor, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2), was purchased from Tocris (Ellisville, MO, USA).

Cell Culture and Mouse Islet Isolation

Rat insulinoma cell line INS-1D cells were cultured as previously described[18] with or without palmitate in the presence of 0.5% bovine serum albumin (BSA) for 24 h.

Male KK-A^y mice and control C57/BL6 mice (Clea Japan, Tokyo, Japan) were maintained and used in accordance with the guidelines of the animal care committee of Kyoto University. All experiments were carried out with mice aged 8–10 weeks. Pancreatic islets were isolated as previously described[19].

Insulin Secretion

Insulin secretion from INS-1D cells was determined as previously described[18]. INS-1D cells cultured on 24-well plates coated with 0.001% poly-L-ornithine were washed with Krebs–Ringer bicarbonate HEPES (KRBH) buffer composed of (in mmol/L) 140 NaCl, 3.6 KCl, 0.5 $MgSO_4$, 0.5 NaH_2PO_4, 1.5 $CaCl_2$, 2 $NaHCO_3$ and 10 HEPES (pH 7.4) with 0.1% BSA and 2 mmol/L glucose, pre-incubated at 37°C for 30 min in KRBH with 2 mmol/L glucose, and then incubated at 37°C for 30 min in KRBH with 2 mmol/L glucose and 10 mmol/L glucose. Insulin release from intact islets was measured using batch incubation using KRBH supplemented with 0.2% BSA as previously described[19].

ROS Measurements

ROS was measured according to the method previously described[20]. INS-1D cells and isolated islets were incubated in KRBH medium containing 2 mmol/L glucose and 10 μmol/L 5-(and 6-) chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Invitrogen, Carisbad, CA, USA) for 60 min at 37°C, and then were rapidly frozen, stored at −80°C and thawed. Fluorescence of the supernatant was quantified using a reader (Powerscan HT; DS Pharma Biomedical, Suita, Japan) with excitation wavelength at 485 nm and emission at 530 nm, which was corrected by subtracting parallel blanks.

Isolation of Total Ribonucleic Acid and Quantitative Reverse Transcription Polymerase Chain Reaction

Total ribonucleic acid (RNA) was isolated from INS-1D cells using RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was prepared by reverse transcriptase (Superscript II; Invitrogen) with an oligo (dT) primer. The rat sequences of forward and reverse primers to *NOX2/gp91^{phox}* and *β-actin* (as an inner control) were as follows: (*NOX2/gp91^{phox}*: 5′-TGA CTC GGT TGG CTG GCA TC-3′, 5′-CGC AAA GGT ACA GGA ACA TGG G -3′, *β-actin*: 5′- CAA TGA GCG GTT CCG ATG CC -3′, 5′- AAT GCC TGG GTA CAT GGT GG -3′). AmpliTaq Gold (Applied Biosystems, Foster, CA, USA) was used as a DNA polymerase for reverse transcription polymerase chain reaction (RT–PCR). SYBR Green PCR Master Mix (Applied Biosystems) was prepared for quantitative RT–PCR run. The thermal cycling conditions were denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 30 s and 60°C for 30 s.

Immunoblot Analysis

For immunoblotting, cells were washed with phosphate-buffered saline (PBS) containing protease inhibitor cocktail (Complete; Roche, Mannheim, Germany) and phosphatase inhibitor cocktail (Calbiochem, Darmstadt, Germany), suspended in 1 mL of PBS containing protease inhibitor and phosphatase inhibitor, and homogenized as previously described[18]. Membrane fraction was prepared as described previously[21]. INS-1D cells were washed three times with PBS, suspended in buffer A consisting of 50 mmol/L Tris (pH 7.5), 1 mmol/L ethylenediaminetetraacetic acid, and containing protease inhibitor cocktail, phosphatase inhibitor cocktail and 5 mol/L sodium pyrophosphate, homogenized and then centrifuged at 10,000 *g* at 4°C for 1 h. The pellets were resuspended in 500 μL of buffer A and stored at −80°C until immunoblot analysis or NOX activity assay. Protein (20 μg per sample) was separated on a 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. After blocking with Tris-buffered saline

(10 mmol/L Tris/HCl and 100 mmol/L NaCl, pH 7.5) containing 0.1% Tween 20 and 5% BSA (blocking buffer) at room temperature (25°C) for 2 h, blotted membranes were incubated overnight at 4°C with anti-p418 Src antibody (Biosource, Camarillo, CA, USA) at 1:1000 dilution, anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Millipore, Billerica, MA, USA) at 1:1000 dilution, anti-NCF1 ($p47^{phox}$) antibody (Abcam, Cambridge, UK) at 1:1000 dilution, anti-NOX2/$gp91^{phox}$ antibody (Abcam) at 1:1000 dilution, anti-flotillin-1 antibody (Cell Signaling Technology, Danvers, MA, USA) at 1:1000 dilution in blocking buffer and subsequently with anti-rabbit or anti-mouse immunoglobulin G horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Little Chalfont, UK) diluted at 1:5000 at room temperature for 2 h before detection using ECL prime™ (GE Healthcare). Band intensities were quantified with Multi Gauge software (Fujifilm, Tokyo, Japan).

NOX Activity Assay

NOX activity was measured by a luminescence assay in a cuvette containing 50 mmol/L phosphate buffer (pH 7.0), 1 mmol/L ethylene glycol tetraacetic acid, 150 mmol/L sucrose, 500 μmol/L lucigenin as the electron acceptor and 100 μmol/L NADPH as the substrate (total volume 900 μL) as previously described[22]. No activity was measured in the absence of NADPH. In some experiments, PP2 (final concentration 10 μmol/L) was added to the cuvette 10 min before readings. The reaction was started by the addition of 100 μL of membrane fraction (50–300 μg protein). Photon emission was measured every 15 s for 15 min in a luminometer (GloMax 20/20n

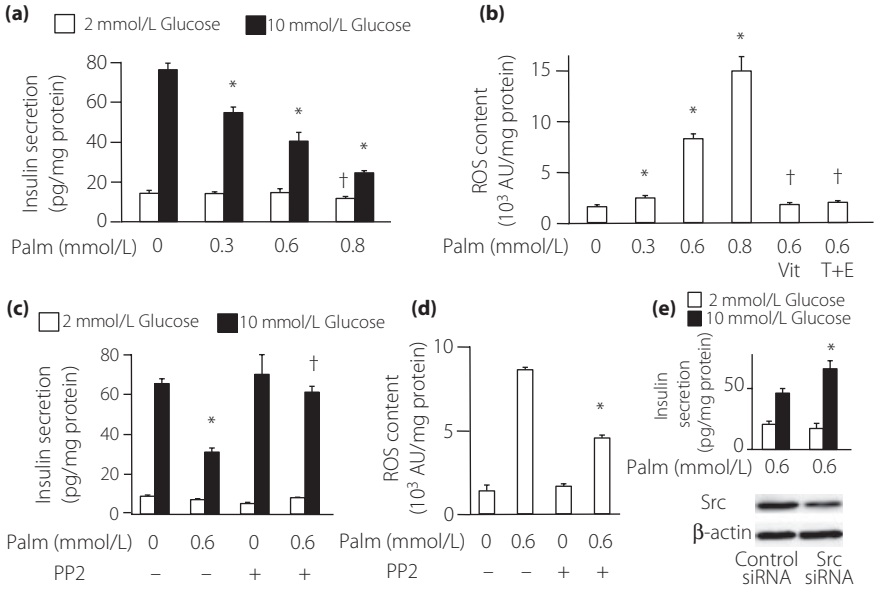


Figure 1 | Effect of palmitate exposure on glucose-induced insulin secretion (GIIS) and reactive oxygen species (ROS) production in INS-1D cells. Values are mean ± standard error of the mean (n = 4 in each bar). After INS-1D cells were cultured with or without various concentrations of palmitate (Palm) for 24 h, GIIS and ROS production were measured. (a) Effect of palmitate exposure on GIIS. GIIS was examined in the presence of 2 mmol/L (white bar) and 10 mmol/L glucose (black bar) for 30 min (n = 4 in each bar). *P < 0.01 vs 10 mmol/L glucose, culture without palmitate; †P < 0.01 vs 2 mmol/L glucose, culture without palmitate. (b) Effect of palmitate exposure on ROS production. After INS-1D cells were incubated in medium containing 2 mmol/L glucose and 10 μmol/L CM-H_2DCFDA for 60 min, ROS production was measured. ROS production was also measured using INS-1D cells cultured with 0.6 mmol/L palmitate plus ROS scavengers (0.1 mmol/L vitamin E + 0.2 mmol/L vitamin C [Vit]) or anti-oxidant mimics (10 mmol/L tempol [superoxide dismutase mimic] + 10 μmol/L ebselen [glutathione peroxidase mimic] [T + E]) for 24 h. Culturing with these agents suppressed enhanced ROS production by exposure to 0.6 mmol/L palmitate (n = 5 in each bar). *P < 0.01 vs culture without palmitate; †P < 0.01 vs culture with 0.6 mmol/L palmitate. (c) Effect of Src inhibitor on impaired GIIS by 0.6 mmol/L palmitate exposure. GIIS was measured in the presence of 2 mmol/L (white bar) and 10 mmol/L glucose (black bar) with or without 10 μmol/L 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) for 30 min (n = 4 in each bar). *P < 0.01 vs 10 mmol/L glucose without PP2, culture without palmitate; †P < 0.01 vs 10 mmol/L glucose without PP2, culture with 0.6 mmol/L palmitate. (d) Effect of Src inhibitor on augmented ROS production by 0.6 mmol/L palmitate exposure. After INS-1D cells were incubated in medium containing 2 mmol/L glucose and 10 μmol/L CM-H_2DCFDA with or without 10 μmol/L PP2 for 60 min, ROS production was measured (n = 5 in each bar). *P < 0.01 vs without PP2, culture with 0.6 mmol/L palmitate. (e) Effect of *Src* small interfering ribonucleic acid (siRNA) on protein expression and GIIS in INS-1D cells cultured with palmitate. After INS-1D cells transfected with control and *Src* siRNA were cultured with 0.6 mmol/L palmitate for 24 h, protein levels and GIIS were measured. Representative immunoblots were presented.

Luminometer; Promega, Fitchburg, WI, USA), which was corrected by a subtracting blank.

Small Interfering RNA Transfection

Stealth™ small interfering (si)RNAs were synthesized by Invitrogen. The sequences of siRNAs specific for rat *NCF1* (*p47^{phox}*) were as follows: 5′-GGU GAA GCC AUC GAG GUC AUU CAU A-3′, 5′-UAU GAA UGA CCU CGA UGG CUU CAC C-3′. The sequences of siRNAs specific for rat Src were as follows: 5′-GGG AGC GGC UGC AGA UUG UCA AUA A-3′, 5′-UUA UUG ACA AUC UGC AGC CGC UCC C-3′. The sequences of control siRNAs were as follows: 5′-ACC AAC AAC AGU UUG GGA AUA GGG A-3′, 5′-U CCC UAU UCC CAA ACU GUU GUU GGU -3′. Cultured INS-1D cells were trypsinized, suspended with RPMI 1640 medium without antibiotics, mixed with Opti-MEM (Invitrogen) containing siRNA and Lipofectamine 2000 (Invitrogen), plated on dishes or wells and then incubated at 37°C. The final contents of INS-1D cell, RPMI 1640, Opti-MEM, siRNA and Lipofectamine 2000 were 1 × 106 cells/mL, 75% v/v, 25% v/v, 80 nmol/L and 0.3% v/v, respectively. The medium was changed to RPMI 1640 3–4 h after transfection. All experiments using siRNA-transfected INS-1D cells were carried out 72 h after transfection.

Statistical Analysis

The data are expressed as mean ± standard error of the mean. Statistical significance was calculated by the unpaired Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Src Inhibition Recovers Impaired Glucose-Induced Insulin Secretion and Decreases Augmented ROS Production as a Result of Exposure to Palmitate

Exposure to palmitate (C16:0) concentration dependently decreased GIIS and increased ROS production (Figure 1a,b). Oleic acid (C18:1) slightly increased ROS production, but arachidonic acid (C20:4 n-6) did not increase ROS production

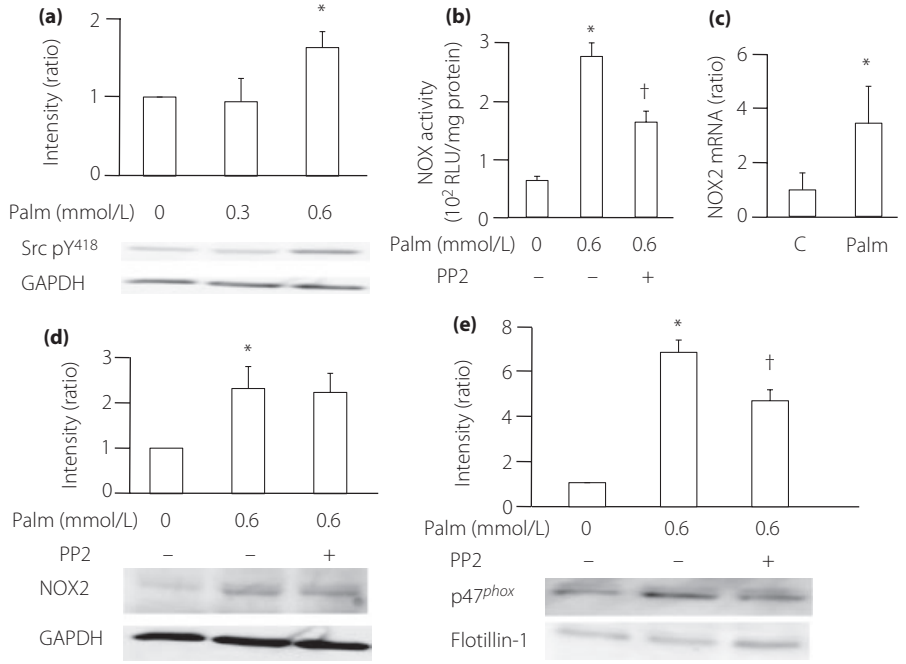
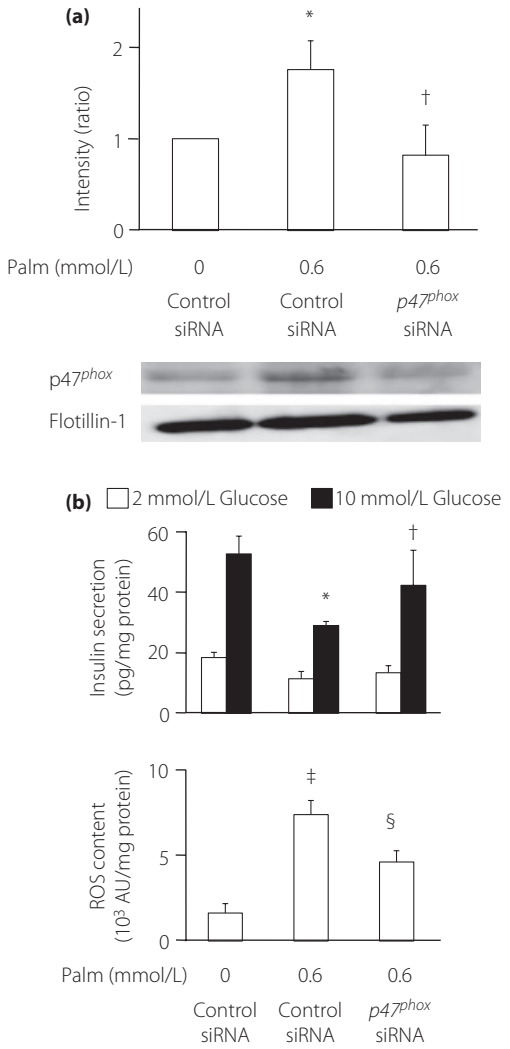


Figure 2 | Effect of palmitate exposure on Src and nicotinamide adenine dinucleotide phosphate oxidase (NOX). Values are mean ± standard error of the mean. After INS-1D cells were cultured with or without palmitate (Palm) for 24 h, and incubated with Krebs–Ringer bicarbonate HEPES with or without 10 μmol/L 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) for 30 min, messenger ribonucleic acid (mRNA), protein levels and NOX activity were measured. Immunoblot was carried out using (a,d) whole cell and (e) membrane fraction. (a,d,e) Representative immunoblots are presented. *$P < 0.01$ vs cultured without palmitate; †$P < 0.01$ vs without PP2, culture with 0.6 mmol/L palmitate. (a) Effect of palmitate exposure on Src activation. Src activation was detected by Tyr^{418}-phosphorylated Src. Data are expressed relative to control values without palmitate corrected by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) level ($n = 5$ in each bar). (b) Effect of palmitate exposure on NOX activity. NOX activity was measured using membrane fraction ($n = 5$ in each bar). (c) Effect of palmiate exposure on expression of *NOX2/gp91^{phox}* mRNA. Data were normalized by the expression of *β-actin* ($n = 4$ in each bar). C, control without palmitate; Palm, 0.6 mmol/L palmitate. (d) Effect of palmiate exposure on expression of NOX2/gp91^{phox} protein. Data are expressed relative to control values without palmitate corrected by GAPDH level ($n = 5$ in each bar). (e) Effect of palmiate exposure on the level of p47^{phox} protein. Data are expressed relative to control values without palmitate corrected by flotillin-1 level ($n = 4$ in each bar).

(Figure S1). Exposure to 10 μmol/L PP2 for 30 min recovered impaired GIIS caused by exposure to 0.6 mmol/L palmitate for 24 h (palmitate exposure), but did not affect GIIS in the control condition without palmitate exposure (Figure 1c). Increased ROS production by palmitate exposure was reduced by 60-min exposure to 10 μmol/L PP2 (Figure 1d). Src downregulation ameliorated glucose-induced insulin secretion of INS-1D cells cultured with palmitate (Figure 1e).

Palmitate Exposure Causes NOX2 Activation by Src Activation

Palmitate exposure caused Src activation, shown by an increased protein level of Tyr^{418}-phosphorylated Src (Figure 2a). NOX activity was prominently increased by palmitate exposure (Figure 2b). Palmitate exposure also increased the messenger RNA level and protein level of NOX2 (Figure 2c,d). Exposure to 10 μmol/L PP2 for 30 min reduced augmented ROS production (Figure 2b), but did not affect the protein level of NOX2 (Figure 2d). Palmitate exposure caused an increase in the protein level of $p47^{phox}$ in membrane fraction that was reduced by exposure to PP2 for 30 min (Figure 2e). Palmitate exposure caused a decrease in the protein level of $p47^{phox}$ in cytosol fraction that was increased by exposure to PP2 for 30 min (Figure S2).



$p47^{phox}$ Knockdown Ameliorates Impaired Insulin Secretion and ROS Overproduction by Palmitate Exposure

The increased protein level of $p47^{phox}$ in membrane fraction by palmiate exposure was reduced by transfection with $p47^{phox}$ siRNA (Figure 3a). Downregulation of the $p47^{phox}$ level was also observed without palmitate exposure by $p47^{phox}$ knockdown (Figure S3). $p47^{phox}$ Knockdown ameliorated impaired GIIS, and decreased augmented ROS production by palmitate exposure (Figure 3b).

Src Inhibition Ameliorates Glucose-Induced Insulin Secretion and Decreases ROS Production in Isolated Islets of KK-A^y Mice

Impairment of GIIS from islets of KK-A^y mice was ameliorated by exposure to PP2 for 30 min (Figure 4a). ROS production and the level of $p47^{phox}$ protein in membrane fraction in the islets of KK-A^y mice was reduced by exposure to PP2 (Figure 4b,c). The protein level of Tyr^{418}-phosphorylated Src was increased in KK-A^y mice islets compared with that in control islets (Figure S4).

DISCUSSION

Exposure to palmitate, a saturated non-esterified fatty acid decreased GIIS and increased ROS production (Figure 1a,b), as

Figure 3 | Effect of $p47^{phox}$ siRNA on protein expression, glucose-induced insulin secretion (GIIS) and reactive oxygen species (ROS) production. Values are mean ± standard error of the mean. After INS-1D cells transfected with control and $p47^{phox}$ small interfering ribonucleic acid (siRNA) were cultured with or without 0.6 mmol/L palmitate (Palm) for 24 h, protein levels, GIIS and ROS production were measured. (a) Effect of $p47^{phox}$ knockdown on enhanced expression of $p47^{phox}$ protein by palmitate exposure. Immunoblot was carried out using membrane fraction. Data are expressed relative to control values transfected with control siRNA and cultured without palmitate corrected by flotillin-1 level (n = 5 in each bar). *P < 0.01 vs transfected with control siRNA, cultured without palmitate; †P < 0.01 vs control siRNA transfected, cultured with 0.6 mmol/L palmitate. Representative immunoblots are presented. (b) Effect of $p47^{phox}$ knockdown on impaired GIIS (upper panel) and increased ROS production (lower panel) by palmitate exposure. Upper panel: GIIS was examined in the presence of 2 mmol/L (white bar) and 10 mmol/L glucose (black bar) for 30 min (n = 4 in each bar). *P < 0.01 vs transfected with control siRNA, culture without palmitate, 10 mmol/L glucose. †P < 0.01 vs transfected with control siRNA, culture with 0.6 mmol/L palmitate, 10 mmol/L glucose. Lower panel: After cells were incubated in medium containing 2 mmol/L glucose and 10 μmol/L CM-H_2DCFDA for 60 min, ROS production was measured (n = 5 in each bar). ‡P < 0.01 vs transfected with control siRNA, cultured without palmitate. §P < 0.01 vs control siRNA transfected, cultured with 0.6 mmol/L palmitate.

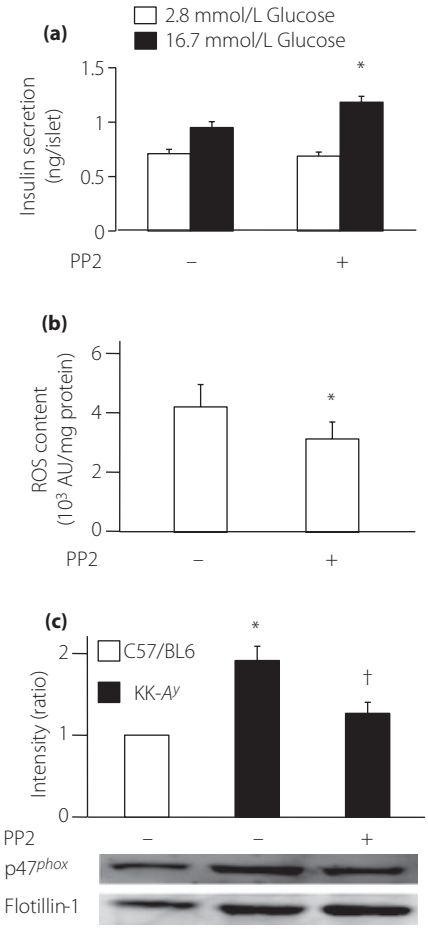


Figure 4 | Effect of Src inhibition on glucose-induced insulin secretion (GIIS) and reactive oxygen species (ROS) production, and the protein level of p47^{phox} in KK-A^{y} mouse islets. Values are mean ± standard error of the mean. (a) Effect of Src inhibition on GIIS. Freshly isolated islets of KK-A^{y} mice were batch-incubated in the presence of 2.8 mmol/L (white bar) and 16.7 mmol/L glucose (black bar) with or without 10 μmol/L 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d] pyrimidine (PP2) for 30 min, and released insulin in the medium was measured (n = 5 in each bar). *P < 0.01 vs without PP2, 16.7 mmol/L glucose. (b) Effect of Src inhibition on ROS production. Freshly isolated islets of KK-A^{y} mice were batch-incubated in the presence of 2.8 mmol/L with or without 10 μmol/L PP2 for 60 min, and ROS production was measured (n = 3 in each bar). *P < 0.05 vs without PP2. (c) Effect of cSrc inhibition on the protein level of p47^{phox}. Freshly isolated islets of control C57/BL6 mice (white bar) and KK-A^{y} mice (black bar) were batch-incubated in the presence of 2.8 mmol/L with or without 10 μmol/L PP2 for 30 min, and the protein level of p47^{phox} was measured using membrane fraction (n = 4 in each bar). Data are expressed relative to control values of C57/BL6 mice corrected by flotillin-1 level. *P < 0.01 vs C57/BL6. †P < 0.01 vs KK-A^{y} without PP2. Representative immunoblots are presented.

previously described[8,9]. We have previously described an important role of endogenous ROS production that involves Src activation in impaired GIIS in diabetic islets[13–15]. In the present study, we investigated the effects of PP2, a specific Src inhibitor, on impaired GIIS and augmented ROS production by lipotoxicity. Exposure to PP2 ameliorated impaired GIIS and decreased augmented ROS production by palmitate exposure (Figure 1c,d). In addition, Src downregulation ameliorated impairment of GIIS by palmitate exposure (Figure 1e). These results suggest that Src activation might be involved in impaired GIIS and augmented ROS production as a result of palmitate exposure.

Src is a non-receptor tyrosine kinase that is associated with the cell membrane and plays important roles in various signal transductions. Its activity is regulated by intramolecular interactions that depend on tyrosine phosphorylation and phosphorylation of Tyr^{418} at the kinase domain that results in Src activation[23]. Palmitate exposure caused Src activation indicated by an increased protein level of Tyr^{418}-phosphorylated Src (Figure 2a). In a recent study, it was proposed that palmitate alters membrane distribution of Src, causing it to partition into intracellular membrane subdomains, where it likely becomes autophosphorylated and activated[24]. NOX2 (gp91^{phox} and p22^{phox}), an isoform of NOX, and its related subunits (p47^{phox} and p67^{phox}) are expressed in β-cells[11,12,25–27]. Interestingly, exposure to palmitate affects expression level of p47^{phox} in islets[10]. In addition, impaired GIIS and an increase in apoptosis by chronic exposure to palmitate are restored by suppression of NOX2 with a decrease in ROS level in a β-cell line[9]. The effects of palmitate exposure on NOX2 and involvement of Src were therefore examined. NOX activity was prominently increased by palmitate exposure (Figure 2b). PP2 reduced augmented ROS production (Figure 2b), but did not affect protein level of NOX2 (Figure 2d). These results suggest that Src activation might affect regulatory factor(s) of NOX. An important role of Src in activation of NOX2 through translocation of p47^{phox} to plasma membrane has been reported in various cells[16,17]. In addition, cigarette smoke particle-phase extract induces Src activation, which causes NOX2, Src and p47^{phox} complex formation that increases NOX activity in smooth muscle cells[28]. Palmitate exposure was found to cause an increase in protein level of p47^{phox} in membrane fraction that was reduced by PP2 (Figure 2e). Taken together, these findings show that palmitate exposure increases NOX activity mainly by increasing translocation of p47^{phox} to plasma membrane through Src signaling.

To elucidate the role of p47^{phox} in impaired insulin secretion and ROS overproduction by palmitate exposure, the effect of *p47^{phox}* knockdown on GIIS and ROS production was examined. *p47^{phox}* Knockdown ameliorated impaired GIIS and decreased augmented ROS production by palmitate exposure (Figure 3B). These results show that p47^{phox} is involved in impaired GIIS and ROS overproduction by palmitate exposure.

Src inhibition by PP2 treatment completely recovered palmitate-induced GIIS impairment despite partial reduction of NOX activation and p47^{phox} expression in the membrane in INS-1D cells. Explanation of these findings is difficult using the data in the present study, but one possibility is that palmitate exposure

also decreases vulnerability to ROS in β-cell function. In addition, it is proposed that Src is constantly activated by forming an intracellular disulphide bond derived from ROS exposure[29], which could affect the concentration-dependent effect of Src inhibitors. Suppression of ROS content was partial despite complete suppression of the $p47^{phox}$ level by knockdown. These phenomena might be explained by the fact that palmitate exposure increases NOX2 expression in addition to the $p47^{phox}$ level (Figure 2c,d).

NOX2 expression is upregulated in islets from obese diabetic mice[11,12] and rats[11]. The pathophysiological significance of the results in the present study was examined using isolated islets of KK-A^y mice, an obese diabetic model with hyperlipidemia. Impairment of GIIS from islets of KK-A^y mice was ameliorated by PP2 (Figure 4a). In addition, ROS production and the level of $p47^{phox}$ protein in membrane fraction in the islets of KK-A^y mice was reduced by exposure to PP2 (Figure 4b,c). These results suggest that activation of NOX through Src signaling might be involved in impairment of GIIS from islets of KK-A^y mice.

The ameliorating effect of Src inhibition on GIIS is less in islets of obese KK-A^y mice than it is in islets of non-obese GK rats[13]. As shown in Figure S4, Src activation is similar in KK-A^y mice islets to that in GK rat islets[14]. This indicates that the differing effect of Src inhibition on GIIS is not derived from the different level of Src activation. Impairment of GIIS is more prominent in KK-A^y mice islets than in GK rat islets. Another mechanism of impaired GIIS independent of ROS overproduction might exist in obese mice islets. Severe impairment of GIIS is also observed in islets of *ob*/*ob* mice, another obese diabetic model, in which upregulation of UCP-2 derived from upregulation of TBP-2 plays an important role in impaired GIIS[19]. In contrast, we observed neither UCP-2 upregulation nor TBP-2 upregulation in GK rat islets (unpublished observation).

In conclusion, activation of NOX through Src signaling plays an important role in ROS overproduction and impaired GIIS caused by chronic exposure to palmitate, suggesting a lipotoxic mechanism of β-cell dysfunction in obese mice.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 | Effect of oleic acid (Ole) and arachidonic acid (Ara) exposure on reactive oxygen species (ROS). After INS-1D cells were cultured with 0.1 mmol/L palmitate, 0.6 mmol/L palmitate, 0.1 mmol/L Ole and 0.1 mmol/L Ara for 24 h, ROS production was measured. Values are mean ± standard error of the mean; $n = 4$ in each bar. $^*P < 0.01$ vs control without free fatty acid.
Figure S2 | Representative immunoblots of $p47^{phox}$ in the cytosol fraction from two independent experiments. After INS-1D cells were cultured with or without palmitate (Palm) for 24 h and incubated with Krebs–Ringer bicarbonate HEPES with or without 10 μmol/L 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) for 30 min, immunoblots of the cytosol fraction were carried out.
Figure S3 | Effect of *p47*^{phox} small interfering ribonucleic acid (siRNA) on basal $p47^{phox}$ level in membrane fraction without palmitate exposure. After INS-1D cells transfected with control, and *p47*^{phox} siRNA were cultured without palmitate for 24 h, protein levels were measured. Immunoblot was carried out using membrane fraction. Data are expressed relative to control values transfected with control siRNA corrected by flotillin-1 level. Values are mean ± standard error of the mean. $^*P < 0.01$ vs transfected with control siRNA; $n = 3$ in each bar.
Figure S4 | Representative immunoblots of Src in islets of KK-A^y mice and control C57/BL6 mice from two independent experiments. Src activation was detected by Tyr^{418}-phosphorylated Src. The numbers of the side of each panel express the ratio to value of control islets.