Regulation of insulin release by leucine and its implication in diabetes

Leucine stimulates insulin secretion via down-regulation of surface expression of adrenergic α2A receptor through the mTOR pathway and its implication in new-onset diabetes in renal transplantation.

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Background: Leucine can stimulate insulin release, but the mechanism has remained unclear.

Results: Leucine regulates adrenergic alpha2 receptor trafficking. Rapamycin and clonidine together increase the risk of diabetes.

Conclusion: mTOR activation by leucine elicits insulin release via adrenergic alpha2 receptors. Rapamycin and clonidine appear to synergistically facilitate new-onset diabetes.

Significance: Our findings may have relevance in the clinical management of renal transplant patients.

Summary

The amino acid leucine is a potent secretagogue, capable of inducing insulin secretion. It also plays an important role in the regulation of mTOR activity, therefore providing impetus to investigate if a leucine-sensing mechanism in the mTOR pathway is involved in insulin secretion. We found that leucine-induced insulin secretion was inhibited by both the mTOR inhibitor rapamycin as well as the adrenergic α2 receptor agonist clonidine. We also demonstrated that leucine down-regulated the surface expression of adrenergic α2A receptor via activation of the mTOR pathway. Leucine’s stimulatory effect on insulin secretion was attenuated in diabetic Goto-Kakizaki rats that overexpress adrenergic α2A receptors, confirming leucine’s role in insulin secretion. Thus, our data demonstrate that leucine regulates insulin secretion by modulating adrenergic α2 receptors through the mTOR pathway.
The role of the mTOR pathway in metabolic homeostasis led us to a second important finding in this study: retrospective analysis of clinical data showed that co-administration of rapamycin and clonidine was associated with an increased incidence of new-onset diabetes in renal transplantation patients over those receiving rapamycin alone.

We believe that inhibition of mTOR by rapamycin along with activation of adrenergic α2 receptors by clonidine represents a double-hit to pancreatic islets that synergistically disturbs glucose homeostasis. This new insight may have important implications for the clinical management of renal transplant patients.

Introduction

Protein restriction can negatively influence glucose homeostasis. Glucose tolerance was found to be compromised in animals fed a low-protein diet (1,2). Besides its possible effect on glucose uptake by skeletal muscle (2,3), a low-protein diet has been found to directly impair pancreatic secretion of insulin in response to glucose (1,4).

Conversely, ingestion of proteins or amino acids together with carbohydrates leads to strong insulin secretion in humans and animal models (2,3,5). Leucine is one of the most potent insulin secretagogues among the branched-chain amino acids that facilitate glucose-induced insulin release from pancreatic β-cells (6). The mechanisms by which leucine exerts its secretagogue effects vary (7). Leucine can either serve as a fuel source for ATP production or be converted to α-ketoisocaproate (KIC), a metabolic intermediate which in turn inhibits K_ATP channel activity, leading to membrane depolarization and triggering insulin secretion (8,9). Leucine also regulates insulin release by acting on glutamate dehydrogenase (6), a key enzyme that fuels amino acids into the tricarboxylic acid cycle (10). Additional routes of action include triggering calcium oscillations in pancreatic β-cells (7,11) and regulating the expression of some key genes that are critical for insulin secretion in pancreatic islets (12).

The mammalian target of rapamycin (mTOR) pathway has recently emerged as a critical regulator of cellular metabolism in response to growth factors and nutrients (13,14). Leucine is a potent stimulator of mTOR. Depletion of leucine results in inactivation of mTOR. Depletion of leucine results in inactivation of mTOR signaling and additional attenuation of growth factor signaling (14). In pancreatic β-cells, the mTOR pathway mediates nutrient sensing and regulates protein synthesis (15,16). Recent studies demonstrated that inhibition of mTOR by rapamycin impairs glucose-induced insulin secretion in pancreatic β-cells (17,18), and the use of rapamycin has been identified as a risk factor for new onset of diabetes in organ transplant patients (19). However, the downstream target of mTOR on insulin secretion in pancreatic islets remains enigmatic.

Pancreatic β-cells are heavily innervated by the sympathetic nervous system. Activation of α2 adrenergic receptors (α2ARs), particularly the α2A subunit, negatively influences glucose-stimulated insulin secretion (20,21). A recent study revealed that overexpression of adrenergic α2A receptor causes type 2 diabetes in a diabetic mouse model (22). In view of the implication of the mTOR pathway and adrenergic α2A receptor in regulating glucose homeostasis, we have undertaken a study of these pathways in leucine-induced acute insulin release in rats and evaluated their roles in new-onset diabetes in renal transplant patients.

Materials and Methods
Animals. Male rats, including strains of Sprague–Dawley (SD), Kyoto Wistar and Goto-Kakizaki (GK), were purchased from Taconic (Taconic, NY). Rats weighing from 100-150 g were used for glucose tolerance testing and for preparation of pancreatic islets. Rats were housed in a room with ad libitum access to food and water under a 12h light/dark cycle (lights on 0700 to 1900). All experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Albany Medical College.

Amino acid cocktail feeding, intraperitoneal glucose tolerance tests (IPGTT) and serum insulin measurement. The amino acid cocktail (in mg per 100 ml solution) consists of alanine 390, arginine 490, aspartic acid 900, cysteine 230, glutamic acid 2860, histidine 360, isoleucine 670, leucine 1210, lysine 1020, methionine 360, phenylalanine 670, proline 1650, serine 770, tryptophan 150, tyrosine 710, valine 800, glycine 270, and threonine 540, which was derived from a normal rat diet AIN-93M (TestDiet). Prior to glucose tolerance testing, rats were habituated to the experimental procedures by wrapping in a disposable plastic restraint for 60 min and orally gavaging with 3 ml distilled water daily for 6 days. After overnight fasting, rats were gavaged at 10.8 ml/kg body weight with an amino acid cocktail lacking leucine. Twenty min later, rats were given glucose (1.5 g/kg) either with or without leucine (13.1mg/kg) in 0.9% NaCl by intraperitoneal injection. In some experiments, rats were treated with rapamycin (Tecoland) at 3 mg/kg via i.p. 40 min before being fed the amino acid cocktail. Blood samples were collected via the tail vein before and 5, 15 and 30 min after glucose challenge. The serum samples were isolated by centrifugation and temporarily stored at -80°C. The serum insulin levels were determined by using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem).

Pancreatic islet preparation and in vitro insulin release assay. Pancreatic islets were isolated by collagenase digestion from Sprague–Dawley (SD), Kyoto Wistar and Goto-Kakizaki (GK) rats. Briefly, rats were anesthetized by pentobarbital and the bile duct was surgically exposed. Collagenase solution (0.5 mg/ml) was freshly made by dissolving collagenase XI (Sigma) in Hanks’ solution (Invitrogen) and then slowly injected into the pancreas via the bile duct. The pancreas was then removed and further digested in collagenase solution by incubation at 37 °C for an additional 15 min. The digested pancreas was filtered through a wet 70-μm cell strainer and washed in Hanks’ balanced salts solution without calcium chloride (Sigma). Individual islets were handpicked under a stereomicroscope and incubated in a 5% CO2 incubator at 37 °C in RPMI 1640 medium supplemented with 2.05 mM L-glutamine, 10% fetal bovine serum (FBS) and penicillin (100 U/ml)/ streptomycin (100 μg/ml). Purity of the islet preparations was verified by dithizone staining. The insulin release assay was performed using static incubations. Briefly, batches of 10 size-matched islets were first incubated at 37 °C for 1 h in a modified neural basal (NB) medium (23) containing all nutrients except glucose and leucine. Islets were then treated with clonidine (1 μM), yohimbine (10 μM), or rapamycin (0.2 μM) for 20 min, followed by adding leucine (0.8 mM) and glucose (16.7 mM). After 30 min of incubation, the medium was sampled for insulin assay. An aliquot of medium collected before the
addition of glucose served as a background sample.

Plasmid construction A human adrenergic alpha 2A receptor cDNA clone was purchased from Open Biosystems. The \( \alpha_{2A} \) AR-mCherry construct was generated by subcloning a full-length alpha 2A receptor coding region into pmCherry-N1 vector (Clontech) using the restriction enzymes NheI and BamHI. A DNA fragment encoding \( \alpha_{2A} \) AR was amplified by PCR using the forward primer: CTAGCTAGCCGCCACCATGTTCCGCCAGGAGCAGCC and the reverse primer: CGGGATCCTCACACGATCCGCTTC. Plasmids DsRed-Rab5 WT, eGFP-arrestin3 and clathrin-Lca-ECFP were purchased from Addgene. The Rab5-GFP plasmid was generated by inserting a Rab5 DNA fragment into the BglII and XhoI sites of peGFP-C1.

Co-immunoprecipitation The effect of leucine on complex formation between \( \alpha_{2A} \)AR and arrestin3 (\( \beta \)-arrestin2) was assessed by a co-immunoprecipitation strategy modified from a previously described method (24). Briefly, HEK 293T cells were transiently transfected with plasmids encoding eGFP-arrestin3 and \( \alpha_{2A} \)AR-mCherry (Addgene) using Lipofectamine 2000 (Invitrogen) and grown at 37°C for 24-36 h. The cells were first starved in leucine minus medium for 1 h and then replenished with 0.8 mM leucine for 5 or 10 min. Cells were lysed in buffer containing 20 mM HEPES, 0.5% Nonidet P-40, 10% glycerol, 2 mM EDTA, and protease inhibitors. The lysate preparations were then subjected to immunoprecipitation using anti-DsRed antibody (Clontech) at 1:200 dilution. This antibody specifically recognizes mCherry, but not eGFP. eGFP-tagged arrestin3 and \( \alpha_{2A} \)AR-mCherry were detected by anti-\( \beta \) arrestin (Cell Signaling) and anti- \( \alpha_{2A} \) AR (Novus Biologicals) antibodies.

Laser scanning confocal microscopy and total internal reflection fluorescence microscopy (TIR-FM) image acquisition Quantitative analysis of leucine-stimulated \( \alpha_{2A} \) AR trafficking was done with live cells imaged by confocal scanning microscopy. HEK-293T cells were grown on sterilized glass coverslips and transiently transfected with \( \alpha_{2A} \) AR-mcherry and Rab5-EGFP plasmids. Prior to living imaging, cells were starved in leucine-minus NB medium for at least 1 h. In some experiments, cells were also pretreated with rapamycin for 20 min before being loaded in an Attofluor cell chamber (Molecular Probes) for analysis. Z-series images of the cells were collected with a laser scanning confocal microscope (Zeiss Meta 510) using a PlanApo x60, 1.4 NA oil-immersion objective lens. Images were acquired immediately before and 2.5, 5 and 10 min after addition of leucine to a final concentration of 0.8 mM. Colocalization of \( \alpha_{2A} \)AR-mcherry and Rab5-EGFP was analyzed by using Zeiss LSM image software.

TIR-FM was performed at 37°C using a Zeiss TIRF system. HEK-293T cells were transiently transfected with \( \alpha_{2A} \)AR-mcherry and clathrin-Lca-ECFP a day before the experiment. The cells were incubated in leucine-minus NB medium for at least 1 h prior to being loaded into the recording chamber. Images were acquired immediately before and 1, 2, 5, 10 min after addition of leucine to a final concentration 0.8 mM. The depth of the TIRF field was set at 80 nm. Colocalization of clathrin and \( \alpha_{2A} \)AR was analyzed using the NIH Image J program.
**Surface biotinylation assay**  HEK293T or islet cells were first incubated at 37 °C in leucine-minus medium for 1 h. Cells were then treated with 0.2 μM rapamycin or vehicle for 5 min, followed by addition of leucine for 20 min. Afterwards, the cells were kept at 4°C for 30 min to stop membrane protein trafficking. After removal of the medium, the cells were rinsed with ice-cold PBS twice and then incubated in sulfo-NHS-SS-Biotin buffer (0.8 mM) at 4°C for 1 h. Biotinylation was stopped by adding a quenching solution (TBS: 25 mM Tris, 0.15M sodium chloride). The cells were then washed three times with cold PBS and lysed in ice-cold lysis buffer (25 mM Tris, pH 7.4; 150 mM NaCl; 1 mM EDTA; protease inhibitor cocktail; 1% Triton X-100) for 30 min, followed by a brief sonication. Insoluble cell debris was removed by centrifugation at 10,000 g for 5 min at 4°C. The supernatant was mixed with immobilized NeutrAvidin™ Gel at 4°C for 2 h. After washing 5 times, the samples were boiled in sample loading buffer and resolved by SDS PAGE. The surface biotinylation assay in islets was performed essentially as above. Briefly, islets were starved in leucine-minus neural basal medium for 1 h at 37°C, and harvested by dissociation from plates using non-enzymatic cell dissociation solution [0.48 mM EDTA and 0.5 mM phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) in the absence of leucine]. Afterwards, the cells were resuspended at a concentration of 4×10⁶ cells/ml and aliquoted in a volume of 10 μl into a 96-well plate. Cells were then treated with various concentrations of clonidine either in the presence or absence of 0.8 mM leucine for 30 min at room temperature. cAMP was measured using a LANCE cAMP kit (Perkin Elmer) and Victor 3V (Perkin Elmer) plate reader following the manufacturers’ instructions. cAMP concentrations were determined by using a standard curve.

**cAMP assay**  HEK293T cells were plated in 6-well plates, transiently transfected with α2AR, and allowed to grow overnight at 37°C. Cells were then starved in leucine-minus neural basal medium for 1 h at 37°C, and harvested by dissociation from plates using non-enzymatic cell dissociation solution [0.48 mM EDTA and 0.5 mM phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) in the absence of leucine]. Afterwards, the cells were resuspended at a concentration of 4×10⁶ cells/ml and aliquoted in a volume of 10 μl into a 96-well plate. Cells were then treated with various concentrations of clonidine either in the presence or absence of 0.8 mM leucine for 30 min at room temperature. cAMP was measured using a LANCE cAMP kit (Perkin Elmer) and Victor 3V (Perkin Elmer) plate reader following the manufacturers’ instructions. cAMP concentrations were determined by using a standard curve.

**Renal transplant patients chart review**  A retrospective chart review was approved by the Institutional Review Board (IRB) on Human Subjects Committee at Albany Medical College (AMC). Charts were systematically reviewed for all recipients who received renal transplantation at AMC during 2005-2010. Clinical data reviewed included entire history, laboratory tests and medications before and up to two years after transplantation. Those recipients with a prior history of diabetes were excluded. New-onset diabetes after transplantation (NODAT) was defined according to the criteria set by the American Diabetes Association/World Health Organization.
Specifically, the diagnosis was based on preprandial blood glucose levels exceeding 126 mg/dL at least in two separate measurements. We noticed that blood glucose levels were unstable during the perioperative period. Thus, NODAT was defined as sustained abnormal high glucose (exceeding 126 mg/dL) that occurred three months following renal transplantation.

**Statistical analysis**  Data were analyzed using Prism GraphPad software. NODAT incidence was determined by Chi-square testing, with a p value less than 0.05 considered statistically significant. Other tests, including student’s t-test and ANOVA, were also used as indicated either in the results section or in the figure legends.

**Results**

**Leucine stimulates insulin secretion and reduces blood glucose via the mTOR pathway**

Starved SD rats were first gavaged with an amino acid cocktail including all amino acids except leucine prior to a glucose tolerance test. Twenty minutes after gavage, they were challenged with 1.5 g/kg glucose either with or without leucine (131 mg/kg body weight) via i.p. injection as indicated in Fig 1A. We observed that blood glucose levels in the rats given both glucose and leucine were consistently lower at all time-points than control rats that received glucose only, with a statistically significant difference at 45 min (137.33 ± 8.63 for leucine-plus versus 200.22 ± 10.17 for leucine-minus in mg/dL) (Fig 1B). Conversely, blood insulin levels were higher in rats given leucine than that in control rats, with a statistically significant difference at 15 min (0.67 ± 0.11 for leucine-plus versus 0.38 ± 0.12 for leucine-minus in ng/dL) (Fig 1C). These data suggest that leucine stimulates insulin secretion and lowers blood glucose. To determine if leucine-induced insulin release involves the mTOR pathway, we treated rats with rapamycin (3 mg/kg) 40 min prior to the glucose tolerance test (Fig 1D). At all time points monitored, we found no difference in blood glucose levels between rats that received either leucine or vehicle. Therefore, rapamycin almost completely abolished the leucine-induced insulin release (Fig 1E and 1F). We also monitored blood glucose and insulin levels in rats that did not receive a glucose challenge (Fig 1G); both measurements remained steady and at low levels over the course of the experiment (Fig 1H and 1I) regardless of whether the rats were given leucine or vehicle, suggesting that the impact of animal handling on insulin release and thus blood glucose levels is minimal.

Besides its actions in pancreatic islet β-cells, leucine and the mTOR pathway could indirectly influence insulin secretion and glucose homeostasis by exerting influence on other organ systems such as the liver and muscles. To confirm that the effect we observed of leucine and rapamycin on insulin release in vivo involves a direct action on pancreatic β-cells, we performed an insulin release assay on pancreatic islets isolated from SD rats. Of note, in vitro insulin release assays reported in previous studies were carried out in Krebs buffer containing a high concentration of leucine (up to 20 mM) but lacking other amino acids (25,26). Conceivably, depletion of other amino acids could perturb the metabolic route in islets and force leucine to enter the TCA cycle. Thus, we performed all insulin release assays under physiological conditions in the presence of all other amino acids along with leucine at 0.8 mM, a standard concentration in regular cell culture media. Under such conditions, we found that leucine alone was able to stimulate insulin release by 1.7-fold (Fig 2).
Similarly, glucose itself stimulated insulin release by 2.4-fold. However, leucine and glucose together elicited a much larger increase in insulin release, by approximately 4.5-fold, suggesting an additive effect on insulin release by leucine and glucose. To determine if leucine regulates insulin release through the mTOR pathway, we included 0.2 μM rapamycin in the assay 20 min prior to performing the in vitro insulin release assay (Fig 2). Rapamycin almost completely suppressed insulin release under all assay conditions, including the presence of leucine or glucose or both. These data suggest that the mTOR pathway is involved in leucine-induced insulin release in addition to its role in glucose-induced insulin release (17).

**α2AR activation attenuates the response of pancreatic islets to leucine**

It is well documented that inhibition of α2ARs stimulates insulin secretion (20,21). To test if the secretagogue effect of leucine involves α2ARs, we treated islets with an α2AR antagonist, yohimbine, at 10 μM. We found that yohimbine stimulated insulin release to a level comparable to that induced by either glucose or leucine (Fig 2), suggesting that α2AR regulates insulin secretion of pancreatic islets. Yohimbine also slightly enhanced (26%) glucose-induced insulin release. However, it had little, if any, further effect on insulin release elicited by leucine or by a combination of leucine and glucose (Fig 2). To ascertain that leucine-induced insulin release involves α2ARs, we treated the islets with the α2AR agonist clonidine. We found that clonidine markedly suppressed insulin release triggered by either glucose or leucine, or both (Fig 2).

**Leucine regulates α2AR trafficking via the mTOR pathway**

Having demonstrated that leucine-induced insulin release is blocked by the α2AR agonist clonidine, we asked how α2ARs could influence the leucine-induced release of insulin. As receptor trafficking between the plasma surface and intracellular compartments is an important point of regulation of α2ARs, we hypothesized that leucine could exert its stimulatory effect on insulin release by regulating α2AR trafficking. Previous studies revealed that α2AR internalization involves clathrin-coated pits (24). We transiently transfected α2AR, the major subtype in regulating insulin release (22,27), into HEK293T cells in which its expression is low, along with clathrin-CFP. We monitored α2AR internalization using total internal reflection fluorescent microscopy (TIRF). Under the condition of leucine depletion, about 40% α2AR-mCherry puncta were colocalized with clathrin-CFP (green) (Fig 3A). Within 1-2 min following the addition of leucine, we observed a noticeable increase in colocalization of α2AR-mCherry and clathrin, indicating that leucine stimulates the formation of new clathrin-coated pits (Fig 3A). The effect peaked within 5-10 min after addition of leucine (Fig 3A and 3B). Furthermore, the number of α2AR-mCherry (red) puncta was reduced in the TIRF field (Fig 3A), suggesting that α2AR was internalized. A recent study reported that α2AR internalization is regulated by arrestin-dependent clathrin-mediated endocytosis (24). To determine if arrestin is involved in the internalization of α2AR, we performed a co-immunoprecipitation assay in HEK293T cells coexpressing eGFP-arrestin3 and α2AR-mCherry (red). We employed an mCherry-specific antibody to pull down α2AR complex and then monitored the level of associated arrestin3 using anti-arrestin3 antibody. We observed
an increased amount of arrestin3 associated with α2AR in the presence of leucine.

Replenishing leucine activates mTOR. To determine if the effect of leucine involves activation of mTOR, we treated HEK293T cells with 200 nM rapamycin 20 min before leucine stimulation. We found that the effect of leucine on colocalization of α2AR and clathrin was largely inhibited (Fig 3D and 3E). To further confirm if the α2AR puncta that disappeared from the TIRF field following leucine stimulation were internalized, we utilized confocal microscopy to trace α2AR subcellular localization in HEK293T cells transiently transfected with α2AR-mCherry along with Rab5-GFP, an early endosome marker. We observed an increased amount of colocalization of α2AR-mCherry and Rab5-GFP (Fig 4A and 4B), indicating that α2AR is internalized and concentrated in the endosomal compartment. Again, the effect of leucine was blocked by rapamycin (Fig 4C and 4D), suggesting that mTOR is involved in α2AR internalization.

To further confirm that leucine reduces the surface expression of α2ARs, we performed a surface biotinylation assay in HEK293T cells transiently transfected with α2AAR. We found a marked decrease in surface expression of α2AAR by 45.1% (Fig 5A and 5B) 20 min after leucine stimulation. However, it had little effect on the surface expression of glycosylphosphatidyl-inositol (GPI) -anchored GFP, a membrane-bound target protein that was co-expressed in HEK293T cells and served as a control for the surface biotinylation assay (Fig 5A). To test if leucine regulates α2AAR trafficking through the mTOR pathway, we treated HEK293T cells with rapamycin 5 min prior to the assay. We observed that rapamycin led to a marked increase in surface expression of α2AAR and averted the down-regulation of surface α2AAR induced by leucine (Fig 5C and 5D). Apparently, the effect of leucine on α2AAR surface expression inversely correlates with mTOR activity, as indicated in Fig 5E-5G wherein the phosphorylation of p70 and S6 induced by leucine was largely inhibited by rapamycin. To confirm if the down-regulation of surface α2AAR by leucine observed in HEK293T cells reflects its action in pancreatic islets, we repeated the assay using pancreatic islets prepared from SD rats. As anticipated, we observed a similar effect from leucine treatment, which was prevented by rapamycin (Fig 5H and 5I). This effect was also accompanied by a change in phosphorylation of p70 and S6 (Fig 5J-5L). We also performed the surface biotinylation assay in islets at 5 and 15 min after leucine addition. A noticeable effect on the surface level of α2AAR occurred as early as 5 min after leucine stimulation (Fig 5M). Taken together, these data strongly suggest that leucine down-regulates the surface expression of α2AAR in pancreatic islets through activation of mTOR.

α2ARs are coupled to G_i protein, with their activation reducing cAMP production. Because leucine reduces the surface expression of α2AAR, we asked if leucine regulates cAMP production in HEK293T cells expressing α2AAR. In the absence of leucine, the level of cAMP was low and was not affected by clonidine (Fig 5N). This suggests that α2AARs are tonically activated in HEK293T cells. The addition of leucine to the medium markedly augmented the level of cAMP. Clonidine at 10 μM and 200 μM was able to suppress cAMP production induced by leucine by 29.0% and 31.4%, respectively (P< 0.05) (Fig 5N).

Impaired pancreatic response to leucine in GK rats
A recent study revealed that overexpression of α2AAR in GK rats is responsible for impaired insulin release and glucose intolerance (22). Accordingly, we employed this diabetic model to test the involvement of α2AAR in leucine-induced insulin release. We compared the leucine-stimulated insulin release in GK to that in the genetically matched Wistar control rats. While leucine effectively stimulated insulin secretion in Wistar rats by 155.3%, 93.6%, and 87.8% at 5, 15, and 30 min, respectively, after challenge with glucose (Fig 6B) it had a modest effect in GK rats, with a 59.3% increase seen only at 30 min (Fig 6D). Blood glucose levels were also significantly lower in the presence of leucine compared to those in the absence of leucine in Wistar control rats at 30 min (181.00 + 7.92 in leucine-plus versus 212.73 + 12.66 in leucine-minus, in mg/dL) (Fig 6C) and at 45 min (151.62 + 8.06 in leucine-plus versus 179.10 + 13.14 in leucine-minus, in mg/dL). However, we observed only a transient decrease in blood glucose in GK rats at 30 min (345.13 + 42.11 in leucine-plus versus 484.00 + 35.05 in leucine-minus, in mg/dL) (Fig 6E). These data suggest that the stimulatory effect of leucine on insulin release is compromised in GK rats. Western blot confirmed that islets from GK rats express more α2AAR concurrent with an increased amount of surface α2AAR (Fig 6F). To confirm if this observation indicates a poor response of pancreatic β-cells to leucine in GK rats, we performed an in vitro insulin release assay in pancreatic islets isolated from both GK and Wistar rats. We observed that glucose effectively stimulated insulin release from pancreatic islets prepared from Wistar rats (2.05 ± 0.10 fold increase), but it had a very modest effect on pancreatic islets from GK rats (1.02 ± 0.06) (Fig 4G), which is consistent with previous studies (22). Likewise, leucine- and leucine/glucose-induced insulin release were significantly less in GK islets than in Wistar islets (1.2±0.08 and 2.22±0.11; 2.37 ± 0.08 and 2.98 ± 0.24, respectively). Yohimbine also became less potent in the stimulation of insulin release elicited by leucine and glucose in GK islets than in Wistar islets (2. 1.82+0.15 and 2.82+0.15; 2. 27 ± 0.09 and 2.66 ± 0.08, respectively).

New-onset diabetes in renal transplant patients treated with rapamycin and clonidine

The findings just described suggested to us the need to evaluate their clinical significance, as both rapamycin and clonidine are frequently used in the management of renal transplant patients. The remainder of this paper deals with the potential clinical application of these findings. New-onset diabetes mellitus is frequently observed in patients following solid organ transplantation and is known to be associated with the administration of immunosuppressants, including rapamycin (also called sirolimus) and FK506 (also called tacrolimus) (19,28-31). Intriguingly, most transplant patients also develop high blood pressure (32) and require additional anti-hypertensive therapy. Clonidine is sometimes used in an antihypertensive drug regimen as it has less impact on blood flow to the kidney. We therefore expected that at least a portion of renal transplant patients could have been treated by both rapamycin and clonidine, which would allow an opportunity to determine if concomitant exposure to rapamycin and clonidine has a synergistic impact on the development of new-onset diabetes after transplantation (NODAT) in humans. We performed a retrospective chart review of the incidence of NODAT in renal transplant recipients who were treated with rapamycin. Of the 287 recipients reviewed, 207 had a previous
history of glucose abnormalities prior to renal transplantation and were excluded from further analysis. In the remaining 80 patients who received a standard immunosuppressive regimen including rapamycin, tacrolimus and mycophenolate mofetil, we found that 31 developed diabetes (fasting blood glucose level of ≥ 126 mg/dL). Thus in this population the overall incidence of NODAT is about 38.75%, which is comparable to the incidence levels reported in recent studies (19,30,31). We also found that 21 patients within this group also received clonidine for hypertension treatment following renal transplantation (rapamycin/tacrolimus/mycophenolate mofetil/clonidine), in which 15 patients (71.4%) developed NODAT. In contrast, among 59 patients (rapamycin/tacrolimus/mycophenolate mofetil) without exposure to clonidine, only 16 (27.1%) developed NODAT (Fig 7 A). Therefore the incidence of NODAT in renal transplant patients who received rapamycin and clonidine (rapamycin/tacrolimus/mycophenolate mofetil/clonidine) was significantly higher than in those who did not (rapamycin/tacrolimus/mycophenolate mofetil) (P=0.025, Chi-square test, two-sided). These data suggest that the combination of rapamycin and clonidine significantly increases the risk of diabetes. We also analyzed 45 renal transplant patients who were not treated with rapamycin. Eighteen patients had a history of diabetes and were excluded from this study. In the remaining 27 patients, we found only 4 patients who received clonidine treatment (tacrolimus/mycophenolate mofetil/clonidine), but none of them developed new-onset diabetes. However, in the 23 patients who were not treated with clonidine (tacrolimus/mycophenolate mofetil), 7 developed NODAT (Fig 7B). As hypertension in this setting is generally thought to be associated with the use of rapamycin (32), the less frequent use of clonidine we observed in the non-rapamycin-treated group perhaps reflects the low incidence of hypertension.

Methylprednisolone was also prescribed in some patients to reduce rejection reactions. As it is a corticosteroid, conceivably, it could be a confounding factor of NODAT. We found that only a very small portion of patients in the rapamycin group (rapamycin/tacrolimus/mycophenolate mofetil/methylprednisolone) were additionally treated with methylprednisolone. Apparently, methylprednisolone does not significantly contribute to the incidence of NODAT caused by the combination of rapamycin and clonidine in the present study (Fig 7C and 7D).

Discussion

Most well-documented mechanisms by which leucine stimulates insulin release involve metabolic pathways, including those producing the metabolic intermediate KIC or facilitating ATP production (6,8-10). In the present study, we found that leucine also regulates pancreatic insulin release through the mTOR pathway and α2AR. This finding has additionally led us to identify co-administration of rapamycin and clonidine as a risk factor for new-onset diabetes in renal transplant patients.

Pancreatic β-cells are innervated by the peripheral sympathetic nervous system, which mediates acute regulation of blood glucose under stress conditions. Although α2BAR is also expressed in the pancreatic islets, α2AAR is the major subtype in regulating insulin release (27). Previous studies revealed that activation of α2AAR by either electric stimulation or norepinephrine inhibits insulin secretion, which is likely
through inhibition of cAMP production (33). The role of the $\alpha_{2A}$AR in regulating pancreatic function and glucose homeostasis was further cemented by a recent genetic study revealing that overexpression of $\alpha_{2A}$AR causes impaired insulin granule membrane docking and secretion in $\beta$-cells in the diabetic GK rat (22). In the present study, we found that leucine down-regulates membrane surface $\alpha_{2A}$AR and increases cAMP production, thus revealing a novel route regulating insulin release by leucine-sensing mTOR signaling. Additional lines of evidence that support the involvement of $\alpha_{2A}$AR in leucine-induced insulin release include compromised insulin release in vivo as well as in vitro in pancreatic islets prepared from diabetic GK rats overexpressing $\alpha_{2A}$AR. These observations are also consistent with a previous study showing poor response of insulin release to leucine in pancreatic islets prepared from the GK rat (34). We also observed that, in the absence of leucine, the $\alpha_{2A}$AR agonist clonidine has little, if any, effect on cAMP production. This suggests that $\alpha_{2A}$AR is already tonically activated under a basal condition. Consistent with this notion, we also observed that the $\alpha_{2A}$AR antagonist yohimbine stimulates insulin release in pancreatic islets. This was also reported by previous studies (22). We observed that rapamycin possesses an inhibitory effect on glucose-stimulated insulin release even in the absence of leucine. Similarly, the $\alpha_{2A}$AR inhibitor clonidine also inhibits glucose-induced insulin release in the absence of leucine. A possible interpretation would be that rapamycin may have a stronger effect on $\alpha_{2A}$AR than does leucine depletion. Alternatively, besides attenuating leucine-induced internalization of $\alpha_{2A}$AR, rapamycin may regulate glucose-induced insulin release involving an additional mechanism.

The membrane trafficking of $\alpha_{2A}$ARs is one of principal mechanisms of regulating receptor activity (35,36). We found that leucine reduces the surface expression of $\alpha_{2A}$AR via activation of mTOR, suggesting a new pathway for nutrient sensing in pancreatic islets. However, the detailed cellular processes by which leucine regulates $\alpha_{2A}$AR membrane trafficking remain to be elucidated. A recent study revealed that leucine can elicit a transient rise in intracellular calcium, resulting in activation of the class III lipid kinase Vps34 and the mTOR complex 1 (37). Interestingly, leucine also induces calcium oscillation in pancreatic islets as well, which is thought to be a critical step in regulating insulin granule docking and membrane fusion (7). Because Vps34 is known to regulate receptor vesicle recycling (38 56), conceivably it could be a candidate that regulates $\alpha_{2A}$AR trafficking. Of particular note, glucose stimulates insulin release in part by depolarizing beta cells and thus resulting in Ca$^{2+}$ influx (7,11). It will be interesting to know if the Ca$^{2+}$ transient elicited by leucine involves a similar mechanism, for example via electrogenic amino acid transporters (39). Because leucine and glucose together have a synergistic effect on insulin release, they likely act through different mechanisms.

New-onset diabetes has been increasingly recognized as a complication associated with organ transplantation, which creates additional challenges for patient care and likely generates significant negative impacts on long-term patient survival following organ transplantation (40). Recent clinical studies revealed that NODAT is strongly associated with immunosuppressive medications (40), in which rapamycin is one of the major risk factors (19). We found that inhibition of mTOR by rapamycin increases the surface level of $\alpha_{2A}$AR. This result led
us to hypothesize that exposure to clonidine in addition to rapamycin could lead to stronger activation of α2AAR, consequently generating a greater negative impact on blood glucose homeostasis. Indeed, the incidence of NODAT is significantly higher in patients treated with both rapamycin and clonidine than those just receiving rapamycin alone. Therefore, our data suggest that administration of both rapamycin and clonidine could represent a double-hit that contributes to the high incidence of NODAT in renal transplant patients.

In the 27 renal transplant patients who were not treated with rapamycin, only 4 patients were treated with clonidine, but none of them developed NODAT. While we acknowledge that the interpretability is constrained by the small number of cases, this observation tends to suggest that clonidine itself, without rapamycin, has a minimal effect on NODAT. Moreover, it is also in line with previous studies reporting that α2AR agonists such as clonidine have very limited effect on blood glucose in humans (41), which is perhaps due to a general attenuation of adrenergic input into the pancreas because of its additional effect on sympathetic output (42). Nevertheless, the overall high incidence of NODAT we observed in the renal transplant recipients more likely reflects a joint effect from rapamycin and clonidine. Methylprednisolone was also prescribed in a small portion of renal transplant patients; apparently it has no significant effect on NODAT in the present study.

In summary, our study suggests that amino acids influence pancreatic function and glucose homeostasis in part through α2AR. Additionally, we found a significant high risk of NODAT in transplant patients undergoing treatment with both rapamycin and clonidine. This finding is of particular importance because hypertension has been a pressing issue in renal transplant patients (32) and clonidine has been considered as a favorable option for hypertension control. Our data suggest that clonidine should be prescribed with precaution in renal transplant patients who are undergoing treatment with rapamycin.

References Cited

31. Sharif, A., and Baboolal, K. Nat Rev Nephrol 6, 415-423
Acknowledgement: This work was funded by grants from the National Institute of Neurological Disorders and Strokes NS062068 (Y.H.) and the National Institute of Diabetes and Digestive and Kidney Diseases K08DK088950 (X. Z.).

Figure legends:

Figure 1 Leucine enhances insulin release and lowers blood glucose. Schematic diagrams showing the order of drug treatment, feeding of amino acid cocktail, glucose challenge and blood sampling in three experimental paradigms (A,D,G). (B) Intraperitoneal glucose tolerance test (IPGTT). Blood glucose levels in the presence (Leu (+)) or absence (Leu (-)) of leucine (131 mg/kg) via i.p. injection. (C) Insulin measurements. Blood samples were collected as indicated for measurements of glucose (E) and insulin (F). Blood glucose (H) and insulin (I) without glucose challenge. Data are presented as means ± SEM (N=4-7). * and ** indicate p<0.05 or 0.01, respectively (ANOVA test).

Figure 2 Stimulatory effect of leucine on insulin release from pancreatic islets. Insulin release from pancreatic islets in the presence of leucine (0.8 mM), glucose (16.7 mM), rapamycin (RAPA) (0.2 μM), yohimbine (Yoh) (10 μM), and clonidine (Clo) (1 μM) either individually or as a combination as indicated. Data are presented as means ± SEM (N=4-6). * and ** indicate p<0.05 or 0.01, respectively, between the test group and the control (ANOVA test).

Figure 3 Leucine stimulates colocalization of α2AAR and clathrin. Surface α2AAR-mCherry and clathrin-ECFP were monitored by multichannel TIRF immediately before and 1, 2, 5, 10 min after addition of leucine in HEK293T cells (A) or in cells pretreated with 200 nM rapamycin (E). Quantification of colocalization of α2AAR-mCherry and clathrin-ECFP (B, D). Data are presented as means ± SE (n=8-11). * indicates p<0.05 (ANOVA). Co-immunoprecipitation assay monitoring complex formation between α2AAR and arrestin 3 in HEK293 cells 5 and 10 min after stimulation with 800μM (C). α2A AR-mChery and GFP-arrestin3 were detected by anti-DsRed and anti-arrestin antibodies respectively. The experiment was repeated once with similar results.

Figure 4 Leucine stimulates α2AAR endocytosis in HEK293T cells. HEK293T cells were transiently transfected with α2AAR-mCherry and Rab5-EGFP. Time-lapses of confocal images were acquired immediately before and 2.5, 5, 10 min after leucine addition in the absence (A) or presence of rapamycin (C). Quantification of overlap correlation between Rab5 and α2AAR in the
absence (B) or presence of rapamycin (D). Data are presented as means ± SE (n=5-7). * indicates $p<0.05$ (ANOVA).

**Figure 5** Leucine reduces membrane $\alpha_{2A}\text{AR}$ and increases cAMP production. HEK293T cells were transiently transfected with $\alpha_{2A}\text{AR}$ and GPI-GFP and incubated in a medium with leucine (Leu(+)) or without leucine (Leu(-)). Surface $\alpha_{2A}\text{AR}$ was evaluated by surface biotinylation assay (A) and quantified (B). ** indicates $p<0.01$ (t test). Rapamycin blocks leucine-induced reduction in surface expression of $\alpha_{2A}\text{AR}$ in HEK293 cells (C) and quantification (D). ** indicates $p<0.01$ (ANOVA). Leucine reduces surface expression of $\alpha_{2A}\text{AR}$ in pancreatic islets, which was blocked by rapamycin (H), and quantification (I). Leucine stimulates phosphorylation of p70 and S6, which was inhibited by rapamycin in HEK293T cells (E, F, & G) and in pancreatic islets (J, K, & L). Surface biotinylation assay on surface expression of $\alpha_{2A}\text{AR}$ 5 and 15 min after addition of leucine (M). Leucine stimulates accumulation of cAMP in HEK293 cells overexpressing $\alpha_{2A}\text{AR}$ (N) in the absence or presence of clonidine at a concentration of 10 or 200 $\mu$M. Data are presented as means ± SEM.** and * indicate $p<0.01$ and $p<0.05$ respectively (N=12) (ANOVA) between leucine-plus and their leucine-minus control. The symbol # indicates that clonidine significantly reduces cAMP production at $p<0.05$ (ANOVA).

**Figure 6** Schematic diagram showing the order of treatments in Wistar and GK rats (A). Insulin (B & D) and glucose (C & E) measurements from blood samples collected from Wistar control or GK rats immediately before and 5, 15, and 30 min after glucose challenge. Data are presented as means ± SEM. The symbol * indicates $p<0.05$ between leucine-plus and -minus groups (N=4-8, ANOVA). (F) Total and surface levels of $\alpha_{2A}\text{AR}$ in islets prepared from Wistar and GK rats. (G) Pancreatic islets isolated from Wistar or GK rats were incubated in media in the presence of glucose (16.7 mM), leucine (0.8 mM), yohimbine (10 $\mu$M), or rapamycin (0.2 $\mu$M) either individually or in a combination as indicated. The symbol ** indicates $p<0.01$ between the test and control groups. The symbol ## indicates $p<0.01$ between pancreatic islet preparations from Wistar and GK rats (N=3-7).

**Figure 7** (A) Significant increase in NODAT in renal transplant patients treated with both rapamycin and clonidine. After excluding those with a prior history of diabetes, the remaining renal transplant patients who received immunosuppressive regimens [rapamycin (RAPA), tacrolimus (TRL) and mycophenolate mofetil (MMF)] were assigned to two groups, either with or without clonidine treatment. The table indicates gender ratio, average age, and the total number of patients who had fasting blood glucose levels over 126 mg/dL in each group. The symbol * indicates $p<0.05$ between two groups (Chi-square test). (B) Renal transplantation recipients who received immunosuppressive regimens [tacrolimus (TRL) and mycophenolate mofetil (MMF)] and with or without clonidine. (C) Renal transplantation recipients who received immunosuppressive regimens [rapamycin (RAPA), tacrolimus (TRL), mycophenolate (MP) and mycophenolate mofetil (MMF)] and with or without clonidine. (D) Renal transplantation recipients who received immunosuppressive regimens [tacrolimus (TRL) and mycophenolate mofetil (MMF)] and with or without mycophenolate. (E) A proposed model showing how rapamycin and clonidine synergistically increase the incidence of NODAT. Inhibition of mTOR by rapamycin increases surface expression of $\alpha_{2A}\text{AR}$, which leads to stronger activation of $\alpha_{2A}\text{AR}$ by its agonist clonidine, thereby interfering with glucose homeostasis.
Fig 1

A

Starvation 16hs  AA cocktail without leu  Glucose or glucose with leu
20'

B

Plasma Glucose (mg/dl)

0 20 40 60

Time (min)

0 60 120 180 240

Leu +  Leu -

C

Serum Insulin (ng/ml)

0 0.2 0.4 0.6 0.8

Time (min)

0 10 20 30

Leu +  Leu -

D

Starvation 16hs  RAPA  AA cocktail without leu  Glucose or glucose with leu
40' 20'

E

Plasma Glucose (mg/dl)

0 20 40 60

Time (min)

0 60 120 180 240

RAPA & Leu +  RAPA & Leu -

F

Serum Insulin (ng/ml)

0 0.25 0.50 0.75 1.00

Time (min)

0 10 20 30

RAPA & Leu +  RAPA & Leu -

G

Starvation 16hs  AA cocktail without leu  Leu/Veh

H

Plasma Glucose (mg/dl)

0 20 40 60

Time (min)

0 60 120 180 240

Leu +  Leu -

I

Serum Insulin (ng/ml)

0 0.2 0.4 0.6 0.8

Time (min)

0 10 20 30

Leu +  Leu -
Fold increase of insulin release

Fig 2
Fig 3

A

Leu 0.8 mM  0'  1'  2’  5’  10’

\[\begin{array}{cccccc}
\alpha_2\text{AR-mCherry} & \text{Clathrin-GFP} & \text{Overlay} & \text{Enlarged} \\
\end{array}\]

B

% of colocalization of \(\alpha_2\text{AR}\) and clathrin

C

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<tr>
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<td>EGFP-arrestin</td>
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Fig 3

D

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Clathrin-GFP

$\alpha_2$AR-mCherry

Overlay

Enlarged

E

% of colocalization of $\alpha_2$AR and clathrin

0 10 20 30 40

Leu 0  Leu 1  Leu 2  Leu 5  Leu 10

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Fig 4

A

\(\alpha_2\)AR-mCherry  Enlarged  Rab5-GFP  Enlarged  Overlay  Enlarged

Leu (-) 0

Leu (+) 2.5'

Leu (+) 5'

Leu (+) 10'

B

Overlap Correlation

Leu0  Leu2.5  Leu5  Leu10

0.00  0.03  0.06  0.09  0.12

* * * *
Fig 5

A

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\( \alpha_2A \) AR

GPI-GFP

GAPDH

B

Surface \( \alpha_2A \) AR

(Arbitrary unit)

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C

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<td>RAPA (0.2 ( \mu )M)</td>
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\( \alpha_2A \) AR

GAPDH

D

Surface \( \alpha_2A \) AR

(Arbitrary unit)

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P-p70

P-S6

p70

S6

GAPDH

F

\% of P-p70\(^{T389}\)

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G

\% of P-S6\(^{235/236}\)

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Fig 5

H

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<td>RAPA (0.2 µM) - + + - + + +</td>
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<tr>
<td>α2A AR</td>
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I


J

| Leu (0.8 mM) | + + - |
| RAPA (0.2 µM) | - + - |
| P-p70 | GAPDH |
| P-S6 | GAPDH |
| p70 | GAPDH |
| S6 | GAPDH |

K

Leu+ Leu- RAPA & Leu+

L

% of P-p70(T389)

Leu+ Leu- RAPA & Leu+

M

| Leu(min) | 0 | 5 | 15 |
| sympathec | |
| α2A AR | GAPDH |

N

GluMP(nM)

Leu- Leu+

log[clonidine]M
Fig 6

A

Starvation 16hs

AA cocktail without leu

Glucose or glucose with leu

20'

B

Wistar

Leu +

Leu -

C

Wistar

Leu +

Leu -

D

GK

Leu +

Leu -

E

GK

Leu +

Leu -

F

Lysate

Surface

Anti- α2a AR

Anti-GAPDH

G

Fold increase of insulin release

Wistar

GK

Cont

Glu

Leu

Glu & Yoh

Leu & Yoh

Glu & Leu

Glu & Rapa

Wistar

GK

**

#

**

**

**

**
### A

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### Table D

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Leucine stimulates insulin secretion via down-regulation of surface expression of adrenergic alpha 2A receptor through the mTOR pathway and its implication in new-onset diabetes in renal transplantation

Jun Yang, Micheal Dolinger, Gabrielle Ritaccio, Joseph Mazurkiewicz, David Conti, Xinjun Zhu and Yunfei Huang

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