

# ARA290 Improves Insulin Release and Glucose Tolerance in Type 2 Diabetic Goto-Kakizaki Rats

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Effects of ARA290 on glucose homeostasis were studied in type 2 diabetic Goto-Kakizaki (GK) rats. In GK rats receiving ARA290 daily for up to 4 wks, plasma glucose concentrations were lower after 3 and 4 wks, and hemoglobin A<sub>1c</sub> (Hb A<sub>1c</sub>) was reduced by ~20% without changes in whole body and hepatic insulin sensitivity. Glucose-stimulated insulin secretion was increased in islets from ARA290-treated rats. Additionally, in response to glucose, carbachol and KCl, islet cytoplasmic free Ca<sup>2+</sup> concentrations, (Ca<sup>2+</sup>)<sub>i</sub>, were higher and the frequency of (Ca<sup>2+</sup>)<sub>i</sub> oscillations enhanced compared with placebo. ARA290 also improved stimulus–secretion coupling for glucose in GK rat islets, as shown by an improved glucose oxidation rate, ATP production and acutely enhanced glucose-stimulated insulin secretion. ARA290 also exerted an effect distal to the ATP-sensitive potassium (K<sub>ATP</sub>) channel on the insulin exocytotic pathway, since the insulin response was improved following islet depolarization by KCl when K<sub>ATP</sub> channels were kept open by diazoxide. Finally, inhibition of protein kinase A completely abolished effects of ARA290 on insulin secretion. In conclusion, ARA290 improved glucose tolerance without affecting hematocrit in diabetic GK rats. This effect appears to be due to improved  $\beta$ -cell glucose metabolism and (Ca<sup>2+</sup>)<sub>i</sub> handling, and thereby enhanced glucose-induced insulin release.

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## INTRODUCTION

Impaired  $\beta$ -cell function and insulin secretion play a primary role in type 2 diabetes (1,2). Although the mechanisms behind impaired insulin secretion may reside in inherited defects related to  $\beta$ -cell development and metabolism, immunological events such as low-grade inflammation and apoptosis may also contribute to  $\beta$ -cell dysfunction. Indeed, increased expression of cytokines and chemokines has been demonstrated in pancreatic islets of patients with type 2 diabetes as well as in animal models of the disease (3,4).

Erythropoietin (EPO) is a cytokine that regulates hematopoiesis mediated by its binding to the erythropoietin receptor (EPOR), that is present also in nonerythroid tissues, including pancreatic islets (5). In addition to its hematopoietic action, EPO has been shown to exert antiinflammatory, antiapoptotic and cytoprotective effects in a wide variety of cell types by binding to the innate repair receptor (IRR) which is a heteromer of EPOR and CD131, the  $\beta$  common receptor (6). EPO treatment has been shown to protect against diabetes

development in streptozotocin-induced and db/db mouse models of type 1 and type 2 diabetes, respectively, while exerting antiapoptotic, antiinflammatory, proliferative and angiogenic effects within the islets (7).

Since prolonged treatment with EPO can increase the hematocrit and provoke thrombosis, we have studied an EPO analogue, ARA290 (8). This 11 amino acid peptide lacks hematopoietic action, binds to the IRR and protects a number of tissues in response to injury (9). A recent phase 2 clinical trial evaluating ARA290 in patients with type 2 diabetes and painful neuropathy showed that ARA290 significantly reduced hemoglobin A<sub>1c</sub> (Hb A<sub>1c</sub>) levels as well as neuropathic symptoms (10). In an effort to explore the mechanism of action of ARA290 in diabetes, we now report effects of ARA290 on different aspects of glucose homeostasis in spontaneously diabetic Goto-Kakizaki (GK) rats (2) compared with nondiabetic controls.

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## MATERIALS AND METHODS

### ARA290

The nonhematopoietic erythropoietin analogue ARA290 consists of 11 amino acids (MW 1258 daltons) (8), and was supplied by Araim Pharmaceuticals. It was dissolved in phosphate buffered saline (PBS) at a concentration of 2 mg/mL and kept at 4°C for up to 4 wks.

### Animals

Diabetic Goto-Kakizaki (GK) rats, originating from Wistar rats, were bred in our department (2). Normal Wistar (W) rats were purchased from a commercial breeder (B&K Universal) and used as nondiabetic controls. All animals were about six weeks old and with body weights 100 to 150 g when treatment was initiated. They were kept at 22°C on a reversed 12-h light-dark cycle with free access to food, except when fasted overnight as noted below. The study was approved by the Laboratory Animal Ethics Committee of Karolinska Institutet (N333/09). All *in vivo* experiments were performed in a blinded manner. Rats were treated over 4 wks with ARA290 by a once daily subcutaneous (s.c.) injection at a dose of 30 µg/kg bodyweight or PBS. Blood samples for determination of glucose were taken after a small tail incision and analyzed every week before morning s.c. injection of either ARA290 or placebo (Accu-Check Aviva, Roche Diagnostics). During the experimental period, body weights were measured weekly.

### Tolerance Testing

Prior to treatment and after 2 and 4 wks, intraperitoneal (IP) glucose tolerance tests (IPGTT; 2 mg glucose/g BW) were performed in overnight fasted rats. Additionally, plasma samples were collected at 0 and 30 min for insulin analyses using radioimmunoassay (11). IP pyruvate tolerance (IPPTT; 2 mg sodium pyruvate/g BW) and s.c. insulin tolerance (SCITT; 0.5 mU insulin/g BW) tests were carried out in overnight fasted GK rats.

### Islet Experiments

Islets were isolated from W and GK rats by collagenase digestion of the exocrine pancreas as previously described (12). Islets were separated using a Histopaque gradient and picked up under a stereomicroscope and cultured overnight in RPMI 1640 medium supplemented with 11 mmol/L glucose, 2 mmol/L glutamine, 10% heat inactivated FCS, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin at 37°C, under a 95% O<sub>2</sub>, 5% CO<sub>2</sub> atmosphere.

### Batch-Incubation Experiments

After the overnight culture, the islets were preincubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer solution (KRB), supplemented with 2 mg/mL bovine albumin, 10 mmol/L HEPES and 3.3 mmol/L glucose, pH 7.4. After preincubation, batches of three islets were incubated for 60 min in a shaking water bath at 37°C in 300 µL of KRB with either 3.3 or 16.7 mmol/L glucose. In both conditions, islets were treated with or without addition of 1, 5 or 10 ng/mL ARA290. When effects of ARA290 on the insulin secretion pathway was analyzed, KRB with either 3.3 mmol/L or 16.7 mmol/L glucose was supplemented with 0.25 mmol/L diazoxide ± 30 mmol/L KCl to analyze proximal or distal effect of ARA290 on the ATP-sensitive potassium (K<sub>ATP</sub>) channels, or with 10 µmol/L PKA inhibitor H89, or 5 µmol/L nimodipine to block the L-type of Ca<sup>2+</sup> channels. After incubations, aliquots of the media were taken for radioimmunoassay of insulin (11).

### Islets Perfusion

After overnight culture in RPMI, GK rat islets were preincubated for 30 min in KRB buffer supplemented with 3.3 mmol/L glucose. Batches of 50 islets were layered between two layers of bio-gel (Bio-Rad) in a perfusion chamber and perfused at a flow rate of 200 µL/min, at 37°C, with KRB and 3.3 mmol/L glucose for 20 min prior to the start of collecting samples. To determine the dynamic response of insulin secretion, the islets were first perfused for 20 min

with 3.3 mmol/L glucose followed by 10 min with 3.3 mmol/L glucose plus 10 ng/mL ARA290, then for 30 min with 16.7 mmol/L glucose in absence or presence of 10 ng/mL ARA290, then for 15 min KCl 30 mmol/L in absence or presence of 10 ng/mL ARA290, followed by 30 min 3.3 mmol/L glucose only. Samples were collected every 2 min and stored at -20°C until insulin was analyzed by radioimmunoassay.

### Glucose Oxidation

After overnight culture in RPMI, GK rat islets were preincubated for 30 min in KRB buffer supplemented with 3.3 mmol/L glucose. Ten islets were placed in glass incubation vials with either 3.3 mmol/L or 16.7 mmol/L glucose in 100 µL KRB (pH 7.4), with or without 10 ng/mL ARA290 and also containing 1 µCi D-[U-<sup>14</sup>C]glucose (PerkinElmer). Incubation vials were then placed in 20 mL scintillation bottles containing 1.5 mL water and sealed with a rubber-membrane equipped cap under 95% O<sub>2</sub>, 5% CO<sub>2</sub> and incubated for 2 h in 37°C in a water bath. The incubation was terminated by injecting through the rubber membrane 100 µL of 0.05 mmol/L antimycin in 70% ethanol, followed by 250 µL of hyamine in the scintillation bottles and 100 µL of 0.4 mmol/L sodium phosphate buffer, pH 6.0, in the incubation vials. The <sup>14</sup>CO<sub>2</sub> was allowed to absorb overnight into the hyamine. Incubation vials were discarded and 5 mL of scintillation liquid (Ultima Gold, Perkin Elmer) were added in the scintillation bottles. Radioactivity in <sup>14</sup>CO<sub>2</sub> was measured with a Liquid Scintillator Analyzer (Tricarb 1900-TR, Packard), and results were expressed as pmoles glucose oxidized/islet per 2 h.

### ATP Determination

After preincubation as above, batches of 20 islets were incubated for 1 h in 300 µL KRB with either 3.3 mmol/L or 16.7 mmol/L glucose, and with or without 10 ng/mL ARA290, at 37°C in a shaking water bath. ATP levels were measured using the ATP Bioluminescence Assay Kit

HS II (Roche Diagnostics) according to the manufacturer's instructions. Bioluminescence was measured after 1-s delay and with 1-s integration time, using a GloMax 96 Microplate Luminometer (Promega). Protein concentration in the lysis suspension was determined by the Bradford protein assay (Bio-Rad), and results were expressed as pmol ATP/mg protein.

### Measurements of Cytoplasmic Free $\text{Ca}^{2+}$ Concentration, $(\text{Ca}^{2+})_i$

Islets were incubated with 2  $\mu\text{mol/L}$  fura-2 AM and changes in  $(\text{Ca}^{2+})_i$ , that is, fluorescence ratio 355/380 nm, were analyzed on a microscope consisting of a Zeiss Axiovert 200M with a fluorescence imaging system using Andor iQ software with an Andor iXon DV887DCS-BV camera and a Cairn monochromator for excitation (Andor Technology plc) (13,14).

### Power Spectral Analysis of $(\text{Ca}^{2+})_i$ Oscillations

All  $(\text{Ca}^{2+})_i$  data were subjected to visual inspection and  $(\text{Ca}^{2+})_i$  oscillations were analyzed using power spectral analysis using Matlab (The Mathworks Inc.) for analysis of the oscillation patterns commonly observed in pancreatic islets, partly based on SpectralAnalysis (16). The amplitudes of fast and slow oscillations were calculated as the square root of the total power of periods from 6 to 60 s (fast oscillation) and 60 to 600 s (slow oscillation) respectively (17). Power spectral density for fast oscillations was calculated by the method of Welsh (18), and standard fast Fourier transform power spectrum was used for slow oscillations. The dominant fast and slow periods were obtained from peaks in respective power spectrum.

### Statistical Analysis

Statistical analyses were carried out with Sigmaplot (2001). The results have been calculated as mean  $\pm$  SEM and comparisons of the means have been done by unpaired Student *t* test.  $P < 0.05$  was regarded as statistically significant.

All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).

## RESULTS

### ARA290 Prevents Progressive Worsening of Glucose Control without Affecting Body Weight

Neither GK nor Wistar rats showed significant weight differences after 4 wks treatment with ARA290 compared with placebo (Supplementary Figures 1A–B). In W rats, the morning PG levels did not differ between the ARA290-treated and placebo groups (Supplementary Figure 1C). By contrast, morning PG levels in GK rats treated with ARA290 were significantly lower after wk 2 compared with placebo (Supplementary Figure 1D). Importantly, ARA290 had no effect on hematocrit (Supplementary Figure 1E).

### ARA290 Improves Glucose Tolerance

In GK rats, the total area under the glucose curve (AUCs) in IPGTT performed at baseline before starting treatment (d 0) was similar in both ARA290-treated and placebo groups (Figures 1A, D). However, glucose AUCs in IPGTT were significantly lower in ARA290 treatment compared with placebo treatment after 2 wks (Figures 1B, D) and after 4 wks (Figures 1C, D) of treatment. Additionally, hemoglobin  $\text{A}_{1c}$  as determined by mass spectroscopy (see Supplementary Materials) was significantly lower in the ARA290-treated rats (Figure 1E). Furthermore, the insulin response during IPGTT after 4 wks (at min 0 and 30 during IPGTT) was augmented in ARA290-treated compared with placebo GK rats (from  $14.2 \pm 1.0$  to  $22.8 \pm 2.9$  mU/L,  $n = 7$ ,  $p < 0.05$ , and from  $15.5 \pm 3.4$  to  $18.1 \pm 2.5$  mU/L,  $n = 7$ ,  $p = 0.2$ , respectively). In nondiabetic W rats, AUCs during IPGTT were similar in ARA290-treated and placebo groups both at baseline and after 2 and 4 wks of treatment (Supplementary Figures 2A–C).

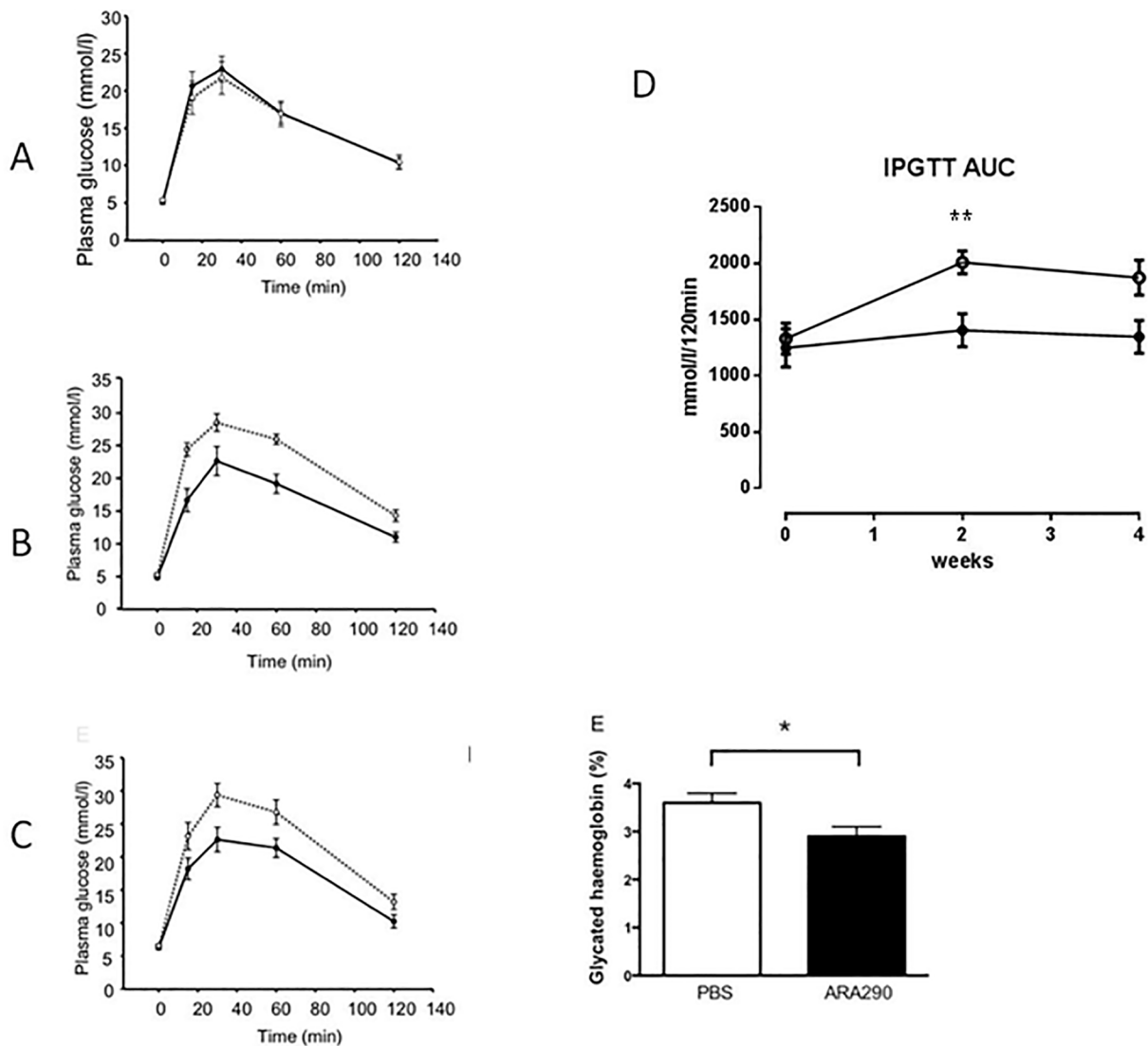
ARA290 treatment had no effect on fasting plasma glucose levels in GK rats (Figures 1A–C). Moreover, in the insulin sensitivity test (SCITT; Supplementary Figure 3A) and the IP pyruvate tolerance test, (IPPTT; Supplementary Figure 3B) performed after 4 wks, results were similar in ARA290-treated and placebo groups, suggesting that altered extrahepatic and hepatic insulin sensitivity do not contribute to the improvement in plasma glucose levels.

### ARA290 Improves $\beta$ Cell Secretory Function in GK Islets

To assess whether ARA290 exerts a direct effect on  $\beta$ -cell secretory function, which may account for the improved glucose homeostasis in GK rat, we assessed effects of ARA290 on insulin secretion by performing GSIS experiments on islets isolated after 4 wks of ARA290 treatment in W and GK rats as well as batch incubations and islets perfusion experiments. In islets from ARA290-treated GK rats, insulin responses to 16.7 mmol/L glucose, in relation to basal (3.3 mmol/L) glucose, were significantly increased about two-fold compared with responses in islets from the placebo-treated rats, the fold increase in response being  $3.8 \pm 0.5$  versus  $2.0 \pm 0.4$ , respectively ( $p < 0.05$ ).

Islets isolated from untreated W and GK rats were exposed to ARA290 in the range of 1 to 10 ng/mL at both 3.3 and 16.7 mmol/L glucose. In W rat islets, ARA290 did not change insulin secretion during either basal or high glucose stimulation (Figure 2A). In GK rat islets, exposure to ARA290 at basal glucose conditions did not enhance insulin secretion, although in the presence of the high glucose concentration, 1 ng/mL ARA290 significantly improved insulin secretion 2.7-fold compared with 16.7 mmol/L glucose alone (Figure 2B). Higher concentrations of ARA290 further increased insulin secretion.

Islet perfusion experiments showed that ARA290 greatly augmented the first phase insulin response (control  $0.076 \pm 0.017$  versus ARA290  $0.303 \pm 0.184$   $\mu\text{U}/\text{islet}/\text{min}$  at 34 min,  $p < 0.01$ ) (Figure 2C). By contrast,



**Figure 1.** Effect of ARA290 on IPGTT in GK rats. (A) Baseline, (B) wk 2, and (C) 4 wks IPGTT in GK rats treated with ARA290 or PBS. (D) Area under the curve expressed as mmol/120 min in the IPGTT in GK rats. (E) Hemoglobin A<sub>1c</sub> percent in ARA290 versus PBS treated rats at 4 wks. Plasma glucose levels (mmol/L) are represented as means ± SEM. (● ARA290-treated, ○ placebo-treated). \**P* < 0.05, \*\**P* < 0.01.

ARA290 did not exert a significant effect on the second phase insulin secretion. However, addition of KCl and ARA290 increased insulin secretion further (Figure 2C), suggesting that ARA290 affects not only the glucose stimulatory pathway but also the amplification pathway.

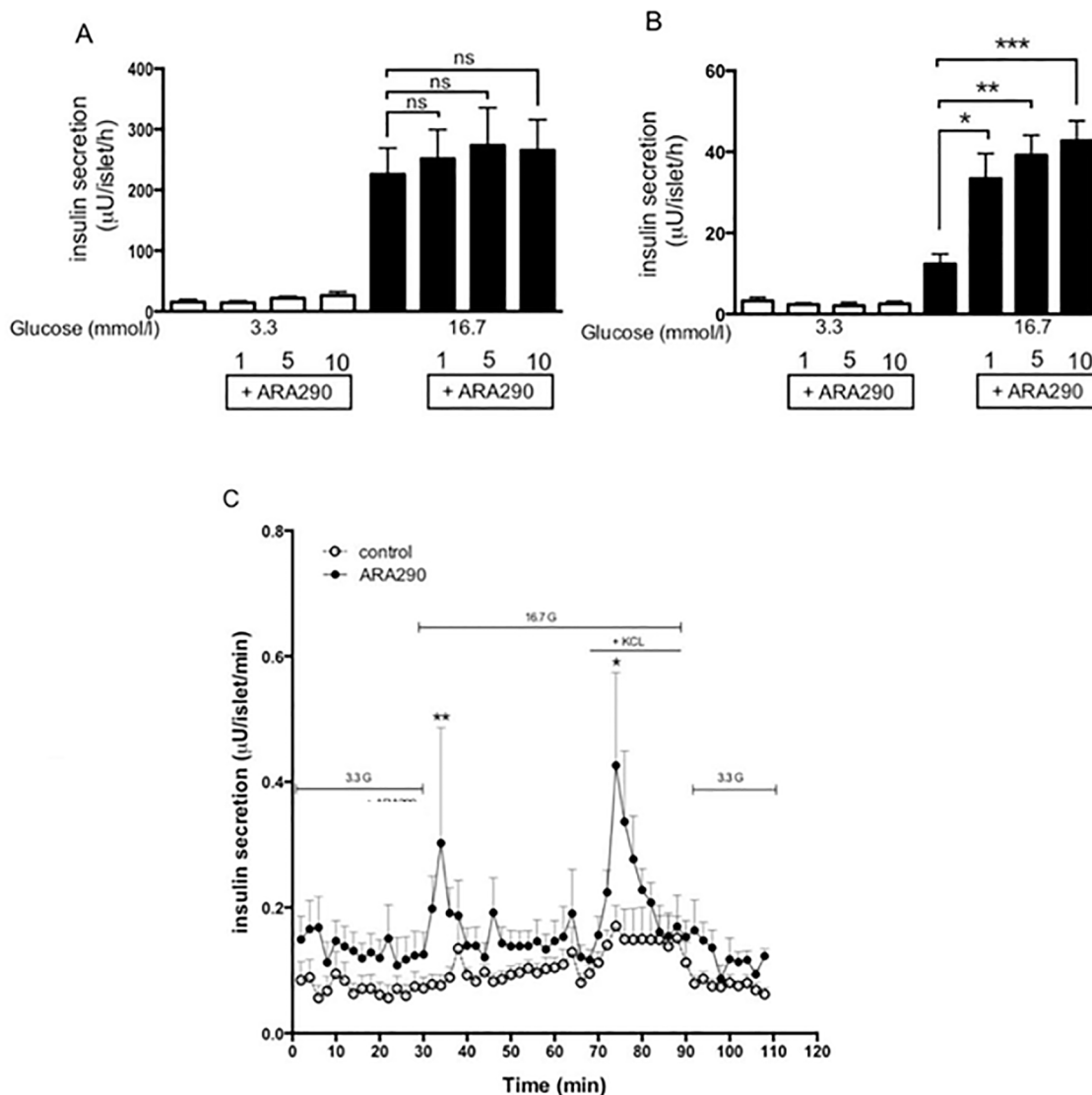
**ARA290 Improves Islet Glucose Metabolism**

To address whether improved first phase insulin secretion could result from

a beneficial effect of A290 on glucose metabolism, we assessed effects of ARA290 on islet glucose oxidation and ATP production. Under hyperglycemic conditions, ARA290 significantly increased glucose oxidation (Figure 3A) in GK islets. Treatment by ARA290 for 1 h also improved ATP production in GK islets (Figure 3B). These results suggest that ARA290 has a direct effect on glucose metabolism by increasing Krebs cycle activity and ATP production by the mitochondria.

**ARA290 Augments the Insulin Secretion Pathway**

To investigate whether ARA290 has effects on the islet K<sub>ATP</sub> channel in the insulin secretion pathway, we used the K<sub>ATP</sub> channel opener diazoxide. At 3.3 mmol/L glucose, diazoxide did not suppress basal insulin secretion, and the stimulation of insulin secretion by addition of diazoxide and KCl was not significantly modulated by ARA290 (Figure 4A). However, in the presence of 16.7 mmol/L glucose, coincubation of

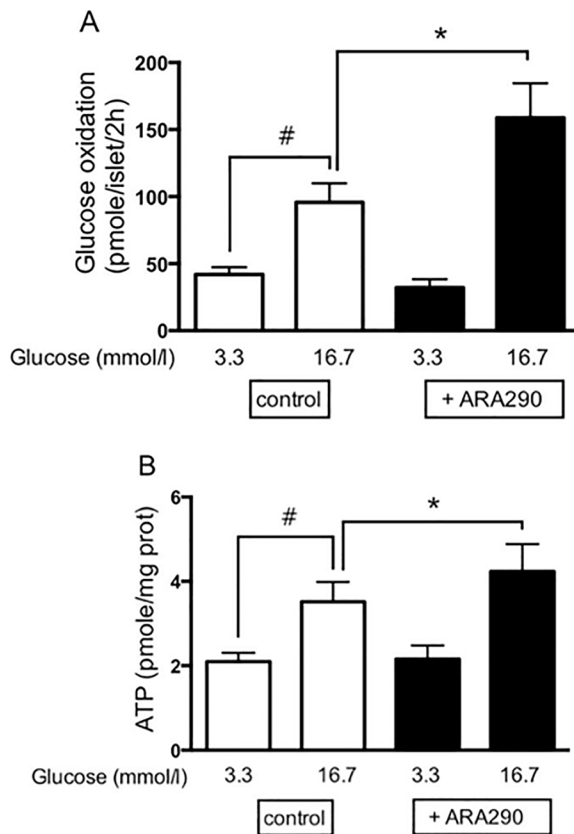


**Figure 2.** ARA290 increases insulin secretion from GK rat islets. (A) Insulin secretion expressed as  $\mu$ U/islet/h is measured in batch incubations of 3 W rat islets. Islets were treated with 1, 5 or 10 ng/mL of ARA290 as indicated with either 3.3 mmol/L G ( $\square$ ) or 16.7 mmol/L G ( $\blacksquare$ ). (B) Insulin secretion expressed as  $\mu$ U/islet/h is measured in batch incubations of 3 GK rat islets. Islets were treated with 1, 5 or 10 ng/mL of ARA290 as indicated in the graph in either 3.3 mmol/L G ( $\square$ ) or 16.7 mmol/L G ( $\blacksquare$ ). Results represent means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus 16.7 mmol/L G, ns indicates no statistical difference. (C) Dynamic glucose-stimulated insulin secretion is assessed by islets perfusion. Batches of 50 islets were exposed to 3.3 mmol/L G for 15 min, and then for an additional 15 min to 3.3 mmol/L G alone (O curve) or plus 10 ng/mL ARA290 ( $\bullet$  curve), followed by stimulation with 16.7 mmol/L G alone (O curve) or plus 10 ng/mL ARA290 ( $\bullet$  curve) for 40 min, islets were then subjected to an additional stimulation with 30 mmol/L KCl for 20 min, finally perfusion medium was changed to 3.3 mmol/L G for an additional 20 min. Results represent means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$  versus control.

GK rat islets with 10 ng/mL ARA290 and 0.25 mmol/L diazoxide neutralized the stimulatory effect of ARA290 on GSIS (Figure 4B). This suggests that the stimulatory effect on GSIS by ARA290 is mediated through  $K_{ATP}$  channels.

However, at high glucose concentrations when the  $K_{ATP}$  channels were kept open by diazoxide and were depolarized by 30 mmol/L KCl, ARA290 potentiated the GSIS of GK islets (Figure 4B). These results show that

ARA290 has an additional effect in the insulin secretion pathway that is distal of the  $K_{ATP}$  channels. Indeed, when GK islets were coincubated with 10 ng/mL ARA290 and 10  $\mu$ mol/L of the protein kinase A (PKA) inhibitor



**Figure 3.** Effect of ARA290 on glucose metabolism in GK rat islets. (A) After 30 min preincubation of islets in KRB supplemented with 3.3 mmol/L G, glucose oxidation was measured in batches of 10 GK islets during incubation for 2 h with 3.3 or 16.7 mmol/L G alone (□) or in the presence of 10 ng/mL ARA290 (■). Results represent means ± SEM of n = 6 experiments. #P < 0.05 versus 3.3 mmol/L G control, \*P < 0.02 versus 16.7 mmol/L G control. (B) Islets were preincubated for 30 min in KRB supplemented with 3.3 mmol/L G, batches of 20 islets were incubated for 1 h with 3.3 or 16.7 mmol/L G alone (□) or in the presence of 10 ng/mL ARA290 (■) and ATP content was measured. Results are expressed as pmole of ATP normalized by the total protein content of islets. Results represent means ± SEM of n = 6 experiments. #P < 0.05 versus 3.3 mmol/L G control, \*P < 0.05 versus 16.7 mmol/L G control.

H89, the effect of ARA290 on GSIS was abolished (Figure 4C). Altogether these results suggest that ARA290 exerts its effect on GSIS both on the  $K_{ATP}$  channels and on the insulin amplification pathway through PKA. Finally, we examined whether the effect of ARA290 was dependent on  $Ca^{2+}$  signaling. The effect of ARA290 on GSIS was blocked by the addition of the L-type Ca channel blocker nimodipine (5  $\mu$ mol/L) (Figure 4D). This suggests that improvement of insulin secretion by ARA290 involves an entry of  $Ca^{2+}$  through the L-type  $Ca^{2+}$  channels.

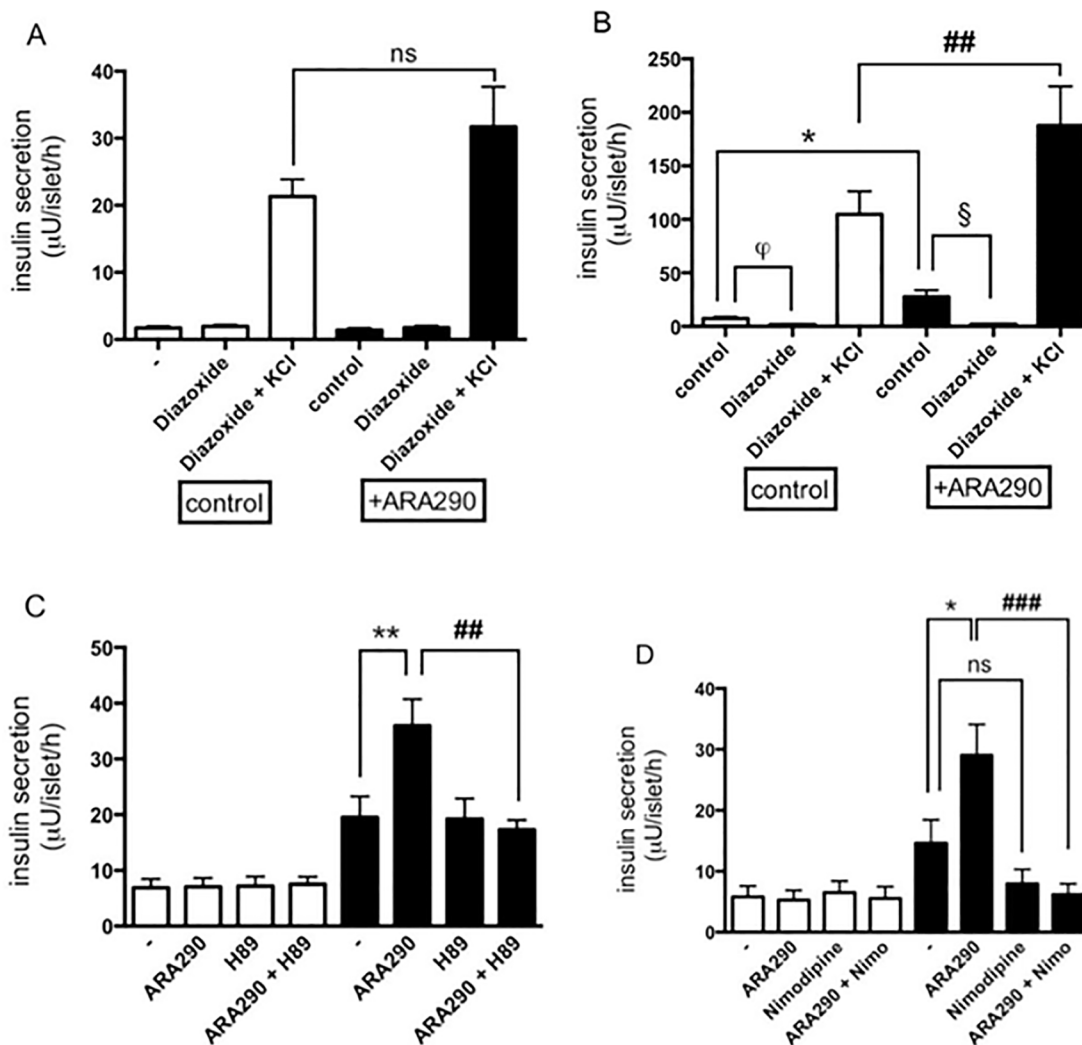
### ARA290 Improves Islet $(Ca^{2+})_i$ Oscillations in the GK Rat

Figures 5A and 5B show representative traces of glucose-stimulated  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_i$  oscillations in isolated islets from GK rats treated with the vehicle (PBS) and ARA290, respectively. There was no statistically significant difference between the two groups in basal  $[Ca^{2+}]_i$  in islets perfused with 3 mmol/L glucose (Figure 5C). We observed an increase in peak  $[Ca^{2+}]_i$  values, that is, fura-2 ratio, after 16.7 mmol/L glucose stimulation in ARA290-treated islets

as compared with vehicle-treated islets (Figure 5D). We also found increases in carbamylcholine- and KCl-induced  $[Ca^{2+}]_i$  in ARA290-treated islets (Figures 5E, F). Furthermore, we performed power spectrum analysis for the  $[Ca^{2+}]_i$  oscillation data and observed that islets from ARA290-treated GK rats displayed an increased frequency in slow  $[Ca^{2+}]_i$  oscillations compared with controls (Figures 5A, B, G). The average period for slow  $[Ca^{2+}]_i$  oscillation (slow oscillations in Figure 5G) frequency in islets from vehicle-treated GK rats was 178.5 s, whereas the average period in islets from ARA290-treated GK rats was 101.5 s, which corresponded to a 43% increase in  $[Ca^{2+}]_i$  oscillation frequency versus the control islets. However, there was no difference in amplitude of slow  $[Ca^{2+}]_i$  oscillations between the groups (Figure 5H). Also, there was no difference in frequency and amplitude of fast  $[Ca^{2+}]_i$  oscillations (Figures 5I, J).

### ARA290 Treatment Increases Intracellular $Ca^{2+}$ Mobilization after Cholinergic Stimulation

To investigate effects of ARA290 treatment on  $Ca^{2+}$  mobilization from intracellular stores, we studied the direct effects of carbamylcholine, a cholinergic agonist that activates the acetylcholine receptor, on PLC/InsP<sub>3</sub>-mediated  $Ca^{2+}$  release in islets from ARA290-treated GK rats as compared with vehicle-treated GK rats. We observed an increase in peak  $[Ca^{2+}]_i$  values in ARA290-treated islets during stimulation by 200  $\mu$ mol/L carbamylcholine, in the presence or in the absence of extracellular  $Ca^{2+}$ , as compared with the vehicle-treated islets (Phase I and Phase III in Figure 6). These results were from islets perfused with basal glucose (3 mmol/L) and were compatible to the increase in  $[Ca^{2+}]_i$  stimulated by 100  $\mu$ mol/L carbamylcholine in the presence of 16.7 mmol/L glucose in ARA290-treated islets (Figure 5E). There was no statistically significant difference in  $Ca^{2+}$  entry over the plasma membranes between the two groups (Phase II in Figure 6).



**Figure 4.** Effect of ARA290 on insulin secretion pathway in GK rat islets. (A) Insulin secretion ( $\mu\text{U}/\text{islet}/\text{h}$ ) was assessed in batches of three islets incubated in 3.3 mmol/L G without ARA290 ( $\square$ ) or with 10 ng/mL ARA290 ( $\blacksquare$ ) and supplemented with 0.25 mmol/L diazoxide or 30 mmol/L KCl as indicated in the graph. (B) Insulin secretion ( $\mu\text{U}/\text{islet}/\text{h}$ ) was assessed in batches of three islets incubated in 16.7 mmol/L G without ARA290 ( $\square$ ) or with 10 ng/mL ARA290 ( $\blacksquare$ ) and supplemented with 0.25 mmol/L diazoxide or 30 mmol/L KCl as indicated in the graph. Results represent means  $\pm$  SEM of  $n = 7$  experiments.  $^{\circ}P < 0.02$  versus 16.7 mmol/L G control,  $^*P < 0.02$  versus 16.7 mmol/L G control,  $^{\S}P < 0.02$  versus ARA290,  $^{\#\#}P < 0.01$  versus diazoxide + KCl, ns indicates no statistical difference. (C) Insulin secretion ( $\mu\text{U}/\text{islet}/\text{h}$ ) was assessed in batches of three islets incubated for 1 h with 3.3 mmol/L G ( $\square$ ) or 16.7 mmol/L G ( $\blacksquare$ ) and supplemented with 10 ng/mL ARA290 or PKA inhibitor H89 (10  $\mu\text{mol}/\text{L}$ ). Results represent means  $\pm$  SEM of  $n = 5$  experiments.  $^{**}P < 0.01$  versus 16.7 mmol/L G,  $^{\#\#}P < 0.01$  versus ARA290. (D) Insulin secretion ( $\mu\text{U}/\text{islet}/\text{h}$ ) was assessed in batches of three islets incubated for 1 h with 3.3 mmol/L G ( $\square$ ) or 16.7 mmol/L G ( $\blacksquare$ ) and supplemented with 10 ng/mL ARA290 or nimodipine (5  $\mu\text{mol}/\text{L}$ ). Results represent means  $\pm$  SEM of  $n = 4$  experiments.  $^*P < 0.05$  versus 16.7 mmol/L G,  $^{\#\#\#}P < 0.001$  versus ARA290.

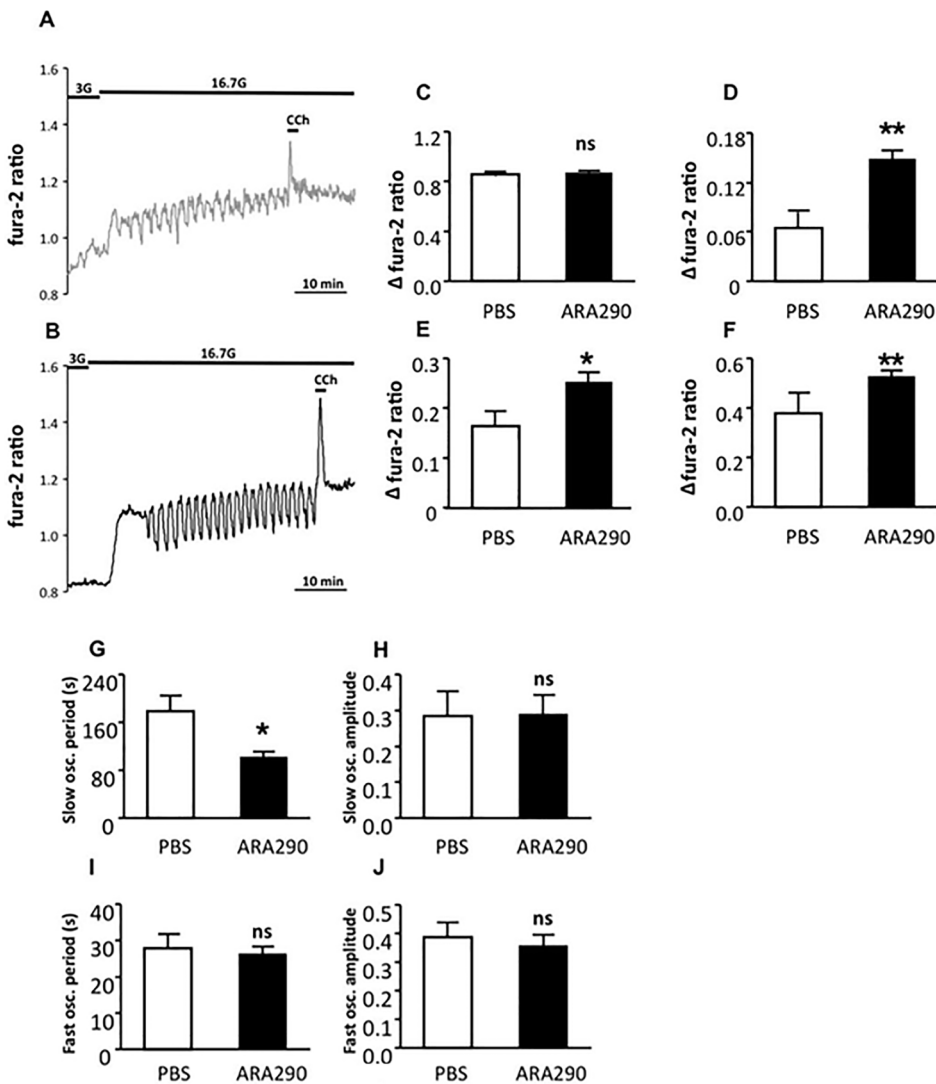
## DISCUSSION

The results of these experiments show that ARA290 prevents the progressive worsening of glucose tolerance that occurs in the GK rat, a nonobese model of type 2 diabetes characterized by impaired insulin secretion (2). Notably,

although fasting PG concentrations remained normal in both treatment groups, IPGTT showed that the glucose responses were significantly lower after 2 and 4 wks of treatment in the ARA290 group. This was supported by a significantly reduced HbA<sub>1c</sub> concentration after

4 wks of treatment in ARA290-treated GK rats compared with control.

The improved glucose tolerance observed in ARA290-treated GK rats could be due to enhancement of insulin sensitivity and/or increased insulin secretion. Since there were no significant



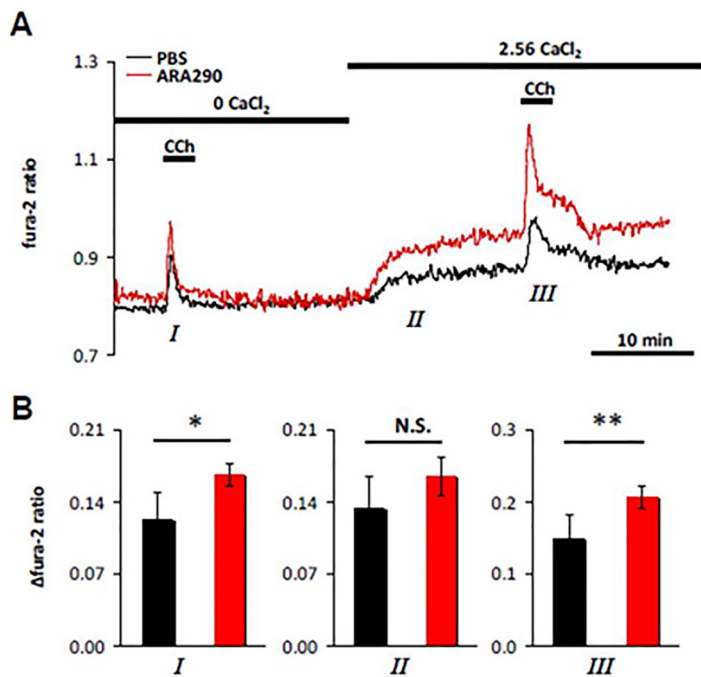
**Figure 5.** Effect of ARA290 on cytoplasmic free calcium concentrations ( $Ca^{2+}_i$ ) in pancreatic islets isolated from GK rats. (A) Traces represent dynamics in  $Ca^{2+}_i$  changes in islets from placebo-treated rats. (B) Traces represent dynamics in  $Ca^{2+}_i$  changes in islets from rats treated with ARA290. Bars above the traces indicate the duration of stimulation. 3G and 16.7G indicate 3 mmol/L and 16.7 mmol/L glucose, respectively. CCh indicates 0.1 mmol/L carbamylcholine. The displayed traces are representative of 13 traces from the placebo-treated rats ( $n = 4$ ) and 17 traces from ARA290-treated rats ( $n = 4$ ). (C–F) Analysis of changes in  $Ca^{2+}_i$  from (A) and (B). (C) Basal  $Ca^{2+}_i$  changes in peak  $Ca^{2+}_i$  values after (D) 16.7 mmol/L G, or (E) in response to 0.1 mmol/L CCh in the presence of 16.7 mmol/L G and (F) in response to 30 mmol/L KCl are represented as mean  $\Delta$  fura-2 ratio  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , ns indicates no statistical significance. (G–J) Analysis of glucose-stimulated  $Ca^{2+}_i$  oscillation frequency from (A) and (B). (G) Period of  $Ca^{2+}_i$  oscillations  $>60$  s are considered as slow oscillations. Glucose-stimulated slow  $Ca^{2+}_i$  oscillation frequency in islets are shown as average slow  $Ca^{2+}_i$  oscillation period  $\pm$  SEM, \* $P < 0.05$ . (H) Average slow  $Ca^{2+}_i$  oscillation amplitude  $\pm$  SEM. (I) Period of  $Ca^{2+}_i$  oscillation between 6 and 60 s are considered as fast oscillations. Glucose-stimulated fast  $Ca^{2+}_i$  oscillation frequency in islets are shown as average fast  $Ca^{2+}_i$  oscillation period  $\pm$  SEM. (J) Average fast  $Ca^{2+}_i$  oscillation amplitude  $\pm$  SEM.

differences between ARA290-treated and placebo-treated rats regarding their responses in the pyruvate tolerance and the insulin sensitivity tests, it is not plausible that ARA290 exerts its primary action on insulin sensitivity in this model. This is further supported by similar fasting plasma insulin levels in treated and control rats, suggesting that the analogue does not improve glucose homeostasis by acting on the liver. Thus, ARA290-induced improvement of glucose tolerance in the GK rat can be accounted for by a direct effect on the pancreatic  $\beta$  cells. This conclusion is supported by the observation that islets isolated from GK rats exhibit an increased glucose-induced insulin secretion when compared with control.

In pancreatic  $\beta$  cells, glucose stimulates insulin secretion by virtue of its metabolism. Following rapid transport through the  $\beta$ -cell plasma membrane, the hexose is phosphorylated to glucose-6-phosphate, and then further metabolized through glycolysis and citric acid cycle to yield ATP (15). The subsequent increase in the cytosolic ATP/ADP ratio leads to closure of the ATP-regulated  $K^+$  channels, plasma membrane depolarization and opening of voltage-dependent L-type  $Ca^{2+}$  channels (13,14). The resulting increase in  $[Ca^{2+}]_i$  stimulates the exocytosis of insulin granules (16). Our results show that the effect of ARA290 on  $\beta$  cells occurs via an improvement of glucose metabolism as evidenced by increased glucose oxidation and ATP production. Additional data show that the increase in insulin secretion also depends on the activation of PKA-dependent pathways, that is, the effects of ARA290 occur via known insulin secretory pathways.

Interestingly, ARA290 did not affect glucose concentrations in W rats or stimulate glucose-induced insulin release in W islets. This may imply that ARA290 specifically improves the mechanisms responsible for defective insulin secretion in the GK rat. In this animal model of type 2 diabetes, reduced  $\beta$ -cell mass associated with defective insulin secretion is a hallmark of its phenotype and can be attributed to several abnormalities in the  $\beta$  cell function, similar to what is found in human type 2 diabetes (2).





**Figure 6.** Measurements of  $\text{Ca}^{2+}$  mobilization from intracellular stores and  $\text{Ca}^{2+}$  influx over the plasma membrane. (A) Effects of 0.2 mmol/L CCh on changes in  $(\text{Ca}^{2+})_i$  in islets from GK rats treated with ARA290 compared with placebo treatment. Bars above the traces indicate the duration of stimulation. The values 0  $\text{CaCl}_2$  and 2.56  $\text{CaCl}_2$  indicate 0 mmol/L and 2.56 mmol/L  $\text{CaCl}_2$ , respectively. The concentration of CCh is 0.2 mmol/L. Phase I shows changes in  $(\text{Ca}^{2+})_i$  in response to CCh in the absence of extracellular  $\text{Ca}^{2+}$ . Phase II shows effects of adding 2.56 mmol/L  $\text{CaCl}_2$  in the perfusion chamber on changes in  $(\text{Ca}^{2+})_i$ , indicating  $\text{Ca}^{2+}$  influx over the plasma membrane. Phase III shows changes in  $(\text{Ca}^{2+})_i$  in response to CCh in the presence of extracellular  $\text{Ca}^{2+}$ . The displayed traces are representatives of 13 traces from the placebo-treated rats ( $n = 4$ ) and 19 traces from ARA290-treated rats ( $n = 4$ ). (B) Phase I: Average  $\Delta$  peak  $(\text{Ca}^{2+})_i$  values, that is, fura-2 ratio, in islets stimulated with CCh in the  $\text{Ca}^{2+}$  free buffer; Phase II: Average basal  $\text{Ca}^{2+}$  influx over the plasma membrane; Phase III: Average  $\Delta$  peak fura-2 ratio in islets stimulated with CCh in the presence of extracellular  $\text{Ca}^{2+}$ . Data are shown as means  $\pm$  SEM.  $P$  values between the two groups (PBS placebo, black bars, versus ARA290, red bars) are shown. \* $P < 0.05$ , \*\* $P < 0.01$ , N.S. indicates no statistical significance.

After an initial transient rise in  $[\text{Ca}^{2+}]_i$  stimulated by glucose,  $[\text{Ca}^{2+}]_i$  in pancreatic islets normally oscillates due to a sophisticated interplay between  $\text{Ca}^{2+}$  entry through voltage-activated  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  mobilization from intracellular stores. In the present study, we aimed at determining whether changes in  $[\text{Ca}^{2+}]_i$  dynamics, that is,  $[\text{Ca}^{2+}]_i$  oscillations, in pancreatic  $\beta$  cells contribute to the significant improvement on insulin secretion and glucose homeostasis after ARA290 treatments. Although 16.7 mmol/L glucose-induced  $[\text{Ca}^{2+}]_i$  oscillations occurred in both placebo- and ARA290-treated islets in

GK rats, power spectrum analysis found that ARA290 treatment increased  $[\text{Ca}^{2+}]_i$  oscillation frequency in GK rat  $\beta$  cells. Results from the  $\text{Ca}^{2+}$  experiments suggest that the enhanced  $[\text{Ca}^{2+}]_i$  dynamics in pancreatic  $\beta$  cells, that is, increased initial peak  $[\text{Ca}^{2+}]_i$  values and faster  $[\text{Ca}^{2+}]_i$  oscillation frequency in response to glucose, is a key feature of the increased insulin secretion (17,18) and therefore improved glucose tolerance after ARA290 treatments in GK rats. The improved  $[\text{Ca}^{2+}]_i$  oscillation frequency observed in the isolated islets were mirrored by increased glucose-induced insulin secretion *in vivo*.

To determine how ARA290 treatment increases  $[\text{Ca}^{2+}]_i$  oscillation frequency in GK rat  $\beta$  cells, we measured changes in CCh-stimulated  $\text{Ca}^{2+}$  mobilization from intracellular stores and  $\text{Ca}^{2+}$  influx over the plasma membrane. We first observed increased amounts of PLC/InsP<sub>3</sub>-generated signals in ARA290-treated islets induced by 0.1 mmol/L CCh, a cholinergic agonist, in the presence of 16.7 mmol/L glucose. We thereafter found increased CCh-stimulated  $\text{Ca}^{2+}$  mobilization from intracellular stores. These findings are compatible with more releasable  $\text{Ca}^{2+}$  in the endoplasmic reticulum (ER) pool in  $\beta$  cells after ARA290 exposure and may be explained by an increased basal  $\text{Ca}^{2+}$  influx over the plasma membrane in ARA290-treated islets (Phase II in Figures 6A, B). These results suggest that, in ARA290-treated islets, more  $\text{Ca}^{2+}$  was available to fill the ER pool, and, there was also an increase in the ER  $\text{Ca}^{2+}$  uptake, which leads to a significant increase in the peak  $[\text{Ca}^{2+}]_i$  values and an increase in  $[\text{Ca}^{2+}]_i$  oscillation frequency in response to glucose. Moreover, to determine whether ARA290 enhanced depolarization-induced  $\text{Ca}^{2+}$  influx over plasma membrane, we found that ARA290-treated islets displayed a 39% increase in peak  $[\text{Ca}^{2+}]_i$  values after 30 mmol/L KCl treatment versus control islets (Figure 5D). The latter finding is compatible with an increased activity of the voltage-activated  $\text{Ca}^{2+}$  channels in plasma membrane and that, in turn, contributes to the enhancement in  $\beta$  cell  $[\text{Ca}^{2+}]_i$  dynamics. Overall, these experiments demonstrate for the first time that the enhancement in  $[\text{Ca}^{2+}]_i$  oscillations in pancreatic  $\beta$  cells of GK rats treated with the non-hematopoietic erythropoietin analogue, ARA290, is associated with increased insulin secretion and improved glucose tolerance. ARA290-induced fine tuning of the  $[\text{Ca}^{2+}]_i$  signal, that is, increased  $[\text{Ca}^{2+}]_i$  oscillation frequency, in GK islets may be further explained by activation of PLC/InsP<sub>3</sub>-mediated  $\text{Ca}^{2+}$  mobilization from intracellular stores and enhancement of membrane depolarization-induced  $\text{Ca}^{2+}$  influx.

Altogether, these observations mean that ARA290 could act on insulin secretion by a mechanism involving both  $Ca^{2+}$ -independent mechanisms through the PKA activation and  $Ca^{2+}$ -dependent mechanisms, the latter supported by the fact that the  $Ca^{2+}$  channel blocker nimodipine suppressed the effect by ARA290 on insulin release.

Our findings are in agreement with the recently published study of db/db mice treated with rhEPO, resulting in improvement of insulin secretion (7). However, in this study the authors failed to show a direct effect of EPO on the  $\beta$  cell secretory function, and rather showed that EPO rescued diabetes in the db/db mouse by an effect on the proliferation and antiapoptotic mechanisms and therefore on  $\beta$  cell mass. The discrepancy in the results may be explained by the specific pathophysiologic mechanisms of diabetes in the GK rats, that is, that the defect in these rats is not primarily due to an increased rate of apoptosis in the pancreatic  $\beta$  cells but rather to molecular defects in these cells (2).

## CONCLUSION

Treatment with ARA290 significantly improved glucose tolerance in diabetic GK rats but had no effects on nondiabetic Wistar rats. The beneficial effect in glucose tolerance in GK rats results from an increase in insulin release. ARA290 increases GSIS by a sequence of events focused on the  $\beta$  cell that culminates in improved glucose oxidation and ATP production, reflecting enhanced mitochondrial oxidation. This in turn leads to  $K_{ATP}$  channel closure and an increase in  $Ca^{2+}$  entry through voltage-dependent  $Ca^{2+}$  channels. Further, the mechanism behind the increased insulin secretion also appears to be mediated by improved  $\beta$  cell  $[Ca^{2+}]_i$  handling and thus of the insulin exocytotic machinery.

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## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

## REFERENCES

1. Frayling TM. (2007) Genome-wide association studies provide new insights into type 2 diabetes aetiology. *Nat. Rev. Genet.* 8:657–62.
2. Ostenson CG, Efendic S. (2007) Islet gene expression and function in type 2 diabetes; studies in the Goto-Kakizaki rat and humans. *Diabetes Obes. Metab.* 9 Suppl 2:180–6.
3. Homo-Delarche F, et al. (2006) Islet inflammation and fibrosis in a spontaneous model of type 2 diabetes, the GK rat. *Diabetes.* 55:1625–33.
4. Donath MY, Storling J, Maedler K, Mandrup-Poulsen T. (2003) Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. *J. Mol. Med. (Berl)* 81:455–70.
5. Fenjves ES, et al. (2003) Human, nonhuman primate, and rat pancreatic islets express erythropoietin receptors. *Transplantation.* 75:1356–60.
6. Brines M, Cerami A. (2006) Discovering erythropoietin's extra-hematopoietic functions: biology and clinical promise. *Kidney Int.* 70:246–50.
7. Choi D, et al. (2010) Erythropoietin protects against diabetes through direct effects on pancreatic beta cells. *J. Exp. Med.* 207:2831–42.
8. Brines M, et al. (2008) Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. *Proc. Natl. Acad. Sci. U. S. A.* 105:10925–30.
9. Brines M, Cerami A. (2012) The receptor that tames the innate immune response. *Mol. Med.* 18:486–96.
10. Brines M, et al. (2015) ARA 290, a nonerythropoietic peptide engineered from erythropoietin, improves metabolic control and neuropathic symptoms in patients with type 2 diabetes. *Mol. Med.* 20:658–66.
11. Herbert V, Lau KS, Gottlieb CW, Bleicher SJ. (1965) Coated charcoal immunoassay of insulin. *J. Clin. Endocrinol. Metab.* 25:1375–84.
12. Quynh NT, Islam MS, Floren A, Bartfai T, Langel U, Ostenson CG. (2005) Effects of galnon, a nonpeptide galanin-receptor agonist, on insulin release from rat pancreatic islets. *Biochem. Biophys. Res. Commun.* 328:213–20.
13. Yang Y, Gillis KD. (2004) A highly  $Ca^{2+}$ -sensitive pool of granules is regulated by glucose and protein kinases in insulin-secreting INS-1 cells. *J. Gen. Physiol.* 124:641–51.
14. Healy JA, et al. (2010) Cholinergic augmentation of insulin release requires ankyrin-B. *Sci. Signal.* 3:ra19.
15. Wiederkehr A, Wollheim CB. (2006) Minireview: implication of mitochondria in insulin secretion and action. *Endocrinology.* 147:2643–9.
16. Uhlén P. (2004) Spectral analysis of calcium oscillations. *Sci. STKE.* 258:p115.
17. Stein KM, et al. (1993) Prognostic value and physiological correlates of heart rate variability in chronic severe mitral regurgitation. *Circulation.* 88:127–35.
18. Welsh PD. (1967) The use of fast Fourier transform for the estimation of power spectra: a method based on time averaging over short, modified periodograms. *IEEE Trans. Audio. Electroacoust.* 15:70–3.

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