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Dimethyl amiloride improves glucose homeostasis in mouse models of type 2 diabetes

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Gunawardana SC, Head WS, Piston DW. Dimethyl amiloride improves glucose homeostasis in mouse models of type 2 diabetes. *Am J Physiol Endocrinol Metab* 294: E1097–E1108, 2008. First published April 15, 2008; doi:10.1152/ajpendo.00748.2007.—Dimethyl amiloride (DMA) enhances insulin secretion in the pancreatic β-cell. DMA also enhances time-dependent potentiation (TDP) and enables TDP to occur in situations where it is normally absent. As we have demonstrated before, these effects are mediated in part through inhibition of neuronal nitric oxide synthase (nNOS), resulting in increased availability of arginine. Thus both DMA and arginine have the potential to correct the secretory defect in diabetes by enabling or enhancing TDP. In the current study we have demonstrated the ability of these agents to improve blood glucose homeostasis in three mouse models of type 2 diabetes. The pattern of TDP under different conditions indicates that inhibition of NOS is not the only mechanism through which DMA exerts its positive effects. Thus we also have explored another possible mechanism through which DMA enables/enhances TDP, via the activation of mitochondrial α-ketoglutarate dehydrogenase.

pancreatic islets; β-cell memory; insulin secretion

AMILORIDE DERIVATIVES INHIBIT Na^+/H^+ exchangers (NHE) in cellular membranes, resulting in retention of H^+ ions and decrease of intracellular pH. In pancreatic β-cells, studies have reported strong positive effects of amiloride derivatives on insulin release and on other related functions such as cell membrane electrical activity, increase of intracellular Ca^{2+}, and retention of K^+ (7, 8, 15, 41, 46, 54, 55). Our previous studies demonstrated that dimethyl amiloride (DMA) strongly enhances nutrient-stimulated insulin release in islets from both healthy and diabetic mice (29, 31).

We also found remarkable effects of DMA on time-dependent potentiation (TDP) (29, 31–33), which is defined as the enhancement of the insulin response resulting from a memory induced by a previous exposure to certain secretagogues. Such secretagogues include glucose and other compounds such as glyceraldehydes, leucine, methyl pyruvate, α-ketoisocaproate (KIC), and 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH), all of which enhance mitochondrial metabolism (22, 25, 30, 43, 72–74). These compounds induce a memory, which remains after the compound is removed, and enhances subsequent secretory responses of the β-cell. Once induced, TDP enhances insulin secretion in response to all secretagogues (both metabolic and nonmetabolic) in a nonspecific manner, bringing about a general strengthening of the secretory capacity of the β-cell (12, 26, 28). Only certain secretagogues can induce TDP, and a common feature among them is the ability to enhance mitochondrial metabolism and anaplerotic input, i.e., to provide intermediates into the TCA cycle (30). Although DMA is not an inducer of TDP, DMA strongly enhances TDP induced by other compounds and enables TDP to occur even under Ca^{2+}-free conditions (33). Whereas it was generally believed that Ca^{2+} was necessary for TDP, our previous studies showed that TDP can occur under stringent Ca^{2+}-free conditions as long as proper pH is maintained, through conditions such as addition of DMA (30, 33). TDP is a species-specific function, reported to exist in species such as humans, rats, and rabbits (10–13, 16, 22, 24, 26, 71), whereas it is absent in mice (5, 6, 71). We previously demonstrated that DMA can enable TDP to occur in mouse islets, where this function is normally absent (29, 31). We also found that the positive effect of DMA on TDP is mediated, at least in part, through inhibition of neuronal nitric oxide synthase (nNOS), since this inhibition increases the amount of arginine available for other cellular functions (32). Therefore, DMA, nNOS inhibitors, and arginine, all have the potential to correct the secretory defect in type 2 diabetes (T2D) by enabling and/or enhancing TDP. Although direct insulin release is usually impaired in T2D, TDP can remain intact in some patients, and the impaired insulin response can be restored to normal by inducing TDP (11, 13, 50). In other cases, both the direct insulin response and TDP are impaired (23, 27, 49), and restoring the TDP response may automatically repair the defect in direct insulin secretion. Thus enabling TDP without raising blood glucose would be a useful therapeutic approach in T2D, and agents such as DMA and arginine have the potential to do so. In the current study, we tested the potential therapeutic value of DMA and arginine for diabetes. In mouse models of T2D, both compounds produced considerable improvement of glucose tolerance, glucose-stimulated insulin response, and basal glucose and insulin levels.

Whereas increasing the availability of arginine via nNOS inhibition seems to be a major mechanism of action of DMA (32), the pattern and magnitude of TDP by different secretagogues indicates the presence of other mechanisms. Since TDP is dependent on mitochondrial metabolism (30) but completely independent of Ca^{2+} and K^+ (33, 67, 68), these mechanisms are likely to be enzymatic rather than inotropic. In addition to inhibiting cytosolic nNOS, it is likely that DMA enables TDP by activating key metabolic enzymes in the mitochondria. DMA is widely known to decrease cellular pH (7, 8, 41, 46, 54, 55), presumably through its inhibitory action on the membrane NHE. Previously, we found that DMA causes a large and sustained decrease in mouse islet cytosolic pH (31). The

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current study demonstrates that DMA causes a smaller but significant decrease in organelle pH, reaching the mitochondria, where many of the metabolic enzymes for TDP-inducing agents are located. Previous studies suggested that TDP signals originate largely in the mitochondria. All TDP-generating compounds are involved in mitochondrial metabolism and are dependent on anaplerotic input, i.e., provision of intermediates to the TCA cycle (30). Therefore, it is likely that DMA enhances TDP by activating specific pH-sensitive mitochondrial enzymes related to the TCA cycle. A common feature of all compounds that induce TDP is to supply α-ketoglutarate (KG) to the TCA cycle to be metabolized via the α-ketoglutarate dehydrogenase (KGDH) reaction (28, 30). Although different TDP-inducing compounds produce KG through different enzymatic reactions, they all increase the levels of cellular KG and share the common step of utilizing KG via KGDH, a strongly pH-sensitive enzyme. Although there are several pH-sensitive enzymes involved in mitochondrial metabolism, KGDH is a more likely candidate, because *1*) it is a common step for all TDP-inducing compounds (see Fig. 9), and *2*) unlike other enzymes, it is consistently activated by low pH (28, 39, 45, 47, 48, 51, 52). Thus we hypothesized that KGDH is another enzyme involved in the signaling for TDP and that DMA enables TDP through low-pH mediated activation of KGDH. We tested the involvement of KGDH in TDP with in vitro studies on isolated mouse islets, using acetyl salicylate, a reasonably specific inhibitor of KGDH.

Inhibition of KGDH partially inhibits glucose-induced TDP and completely inhibits TDP by mitochondrial secretagogues such as α-KIC. (To ensure that this inhibition was not mediated by a general inhibition of the TCA cycle, we added the subsequent TCA intermediates to the reaction mixture and monitored direct insulin secretion as well as TDP). These data suggest that activation of KGDH is another possible component in the mechanism whereby DMA enhances TDP.

EXPERIMENTAL PROCEDURES

Animals

In vitro studies with isolated islets. nNOS knockout mice (NOS KO mice) were strain B6; 129S4-*Nos1*^{tm1Plh}/J (stock no. 002633), obtained from Jackson Laboratories (Bar Harbor, ME) (36). C57BL6 mice (Harlan Laboratories, Indianapolis, IN) were used as wild-type (WT) controls. All mice used were males ages 6–10 wk.

In vivo studies for blood metabolic parameters. Two of the T2D models, i.e., strains NON/LtJ (NON mice; stock no. 002423) and BKS.Cg-*m*$^{+/+}$Lepr^{db}/J (Ldb mice; stock no. 0006423), were purchased from Jackson Laboratories (37, 38). *gk*$^{lox/w}$+*Rip-cre* mice (GK mice) (57) were donated by Dr. Mark Magnuson's laboratory at Vanderbilt University. The diabetic Ldb and NON mice were used at 2–4 and 5–9 mo of age, respectively, to allow time for T2D to develop. All mice were males except for GK mice, in which both males and females had to be used due to difficulty in breeding. GK mice were used at 2–6 mo of age. The animals were fed standard laboratory chow and were cared for guidelines approved by the Vanderbilt Institutional Animal Care and Use Committee.

In Vivo Studies

Effect of oral treatment with DMA and arginine on glucose homeostasis was monitored in diabetic NON mice, GK mice, and Ldb mice. Each strain was treated with DMA or arginine administered in drinking water to each test groups for 3 wk. The estimated dosage of DMA was 1 mg·kg^{-1}·day^{-1}, and that of arginine was 2.5 g·kg^{-1}·day^{-1}, calculated by daily water intake. Each corresponding control group was given regular drinking water. Glucose homeostasis was assessed by intraperitoneal glucose tolerance test (IPGTT), insulin response to glucose bolus, and average basal blood glucose and insulin levels before and after treatment. For IPGTT, after a 12-h fast, mice were anesthetized with isoflurane/oxygen and injected with 2 g/kg sterile glucose solution intraperitoneally. Blood samples were taken from the tail before and after glucose injection at denoted intervals.

Media

Pancreatic islets were isolated in Hanks' balanced salt solution, and HEPES-buffered Krebs-Ringer bicarbonate solution (KRBH) was used for the static incubations in secretion experiments. The components of KRBH are as follows: 128.8 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, 5 mmol/l $NaHCO_3$, 10 mmol/l HEPES, and 0.1% bovine serum albumin. The medium pH was maintained at 7.4. In the Ca^{2+}-free KRBH medium used during the first exposure to glucose (priming period) of certain indicated experiments, Ca^{2+} was omitted from the medium and the following Ca^{2+} chelators were added to ensure stringent Ca^{2+}-free conditions: the extracellular Ca^{2+} chelator EGTA (5 mmol/l) and the intracellular Ca^{2+} chelator BAPTA-AM (20 μmol/l), purchased from Sigma-Aldrich. Except where specifically indicated for Ca^{2+}-free priming conditions, all incubations were carried out in regular Ca^{2+}-containing media. Basal KRBH used for preincubation, and nonstimulated controls contained 2.8 mmol/l glucose, whereas the stimulating media contained either 16.7 mmol/l glucose or other secretagogues as indicated in the presence of 2.8 mmol/l glucose. The nonglucose secretagogues included 20 mmol/l KIC or 20 mmol/l BCH. KIC, a mitochondrially metabolized secretagogue, is a metabolic product of the amino acid leucine. BCH is a nonmetabolizable analog of leucine and stimulates insulin secretion presumably through allosteric activation of mitochondrial dehydrogenases. For forced decrease of islet intracellular pH (pH_i), 40 μmol/l DMA (Alexis Biochemicals) was added to the medium to produce intracellular acidification. Methyl acetyl salicylate (5 mmol/l; Sigma-Aldrich) was added to inhibit the activity of KGDH where indicated, and methyl fumarate (20 mmol/l; Sigma-Aldrich) and diethyl malate (20 mmol/l; Sigma-Aldrich) were added to the reaction mixture to prevent blockage of the TCA cycle. In preparation for imaging experiments, islets were cultured in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 0.1 g/l streptomycin, and 11 mmol/l glucose.

Isolation of Islets

A modified version of the collagenase digestion method described by Lacy and Kostianovsky (40) was used. Mice were terminally anesthetized with intraperitoneal injection of ketamine (80 mg/kg)-xylazine (20 mg/kg). The pancreas was removed, placed in ice-cold Hanks' solution, and minced with scissors. Collagenase (3 mg/ml) was added, and the mixture was shaken in a 37°C water bath until the tissue was adequately digested. The mixture was then centrifuged, the supernatant was removed, and the pellet was resuspended in Hanks' solution. Centrifugation and resuspension were repeated several times to remove exocrine tissue. The final pellet was resuspended in either basal KRBH medium for secretion experiments or RPMI medium for islet culture. Islets were handpicked under a stereomicroscope.

Culture of Islets

The method described by Arkhammar et al. (1) was used with minor modifications (31, 56, 58). Culture dishes (35 mm) with glass-bottomed wells (Mat-Tek) were used. The dishes were preprepared by coating the wells with human extracellular matrix (BD Biosciences). Freshly isolated islets were placed carefully in each

well, covered with RPMI medium containing 11 mmol/l glucose, and cultured at 37°C in 95% O_2 and 5% CO_2. Under these conditions, the cells in the islet spread out within 14 days, greatly reducing the islet thickness and making it particularly suitable for imaging with confocal microscopy. Islets cultured under these conditions exhibit normal responses of Ca^{2+}, NAD(P)H, and insulin release to glucose stimulation, as described in previous studies (1, 56, 58) and confirmed in our preliminary experiments.

Mitochondrial pH Measurements

Our established method for pH measurements in pancreatic islets (31) was used in combination with the method described in a previous study for mitochondrion-specific loading of indicator (69). Carboxy-seminaphthorhodofluor-5 (SNARF-5) (44), a pH-sensitive fluorescent indicator, was used for pH measurements with ratiometric confocal microscopy as described earlier (31). pH calibration and measurements were performed as described previously, except with the loading conditions modified for the dye to leave the cytosol and accumulate in the mitochondria (69). Cultured islets were loaded with SNARF-5-AM (5 μmol/l) for 30 min and then incubated in SNARF-free medium for 4+ h in room temperature. Islets were excited at 514 nm, and emission fluorescence from loaded regions was recorded at 570 and 630 nm. Emission fluorescence was recorded before and after DMA treatment as described previously (31), and pH was calculated from the emission ratio in the nonnuclear regions using LSM software.

Secretion Measurements

All incubations were done with freshly isolated islets in a 37°C water bath. Except in Figs. 2 and 7*B*, where direct insulin secretion was measured, the following protocol was followed for quantitation of TDP. Groups of islets were exposed to four different incubation periods, i.e., preincubation, priming or memory-inducing period, rest in basal glucose, and final stimulation with high glucose, or memory-manifesting period. Fresh islets were first preincubated for 40 min in basal KRBH containing 2.8 mmol/l glucose. Islets were then divided into groups and exposed to different conditions for 40 min, as indicated in RESULTS, for possible induction of TDP. Control groups were maintained in basal glucose. Subsequently, all groups were washed and rested in basal KRBH for 20 min and then stimulated with 16.7 mmol/l glucose for 40 min. At the end of this final stimulation period, samples were collected for insulin measurement by radioimmunoassay (performed by the DRTC Core facility at Vanderbilt University). Islet insulin content was measured after freezing islets overnight in 1% Triton-X. Insulin secretion is expressed as fractional release, i.e., the percentage of total insulin content released over the period of stimulation, and *n* denotes the number of times each experiment was repeated with islets from a different mouse.

Statistical Analysis

Values are means ± SE. Groups were compared using paired Student's *t*-test. In secretion studies, *n* denotes the number of times each experiment was repeated with islets from different mice. In metabolic studies, *n* denotes the number of mice in each group. In imaging experiments for pH_i, *n* denotes the number of islets imaged for each condition.

RESULTS

As previous data indicate (29–33), DMA is a powerful agent that can dramatically enhance both direct insulin release and TDP. Our previous in vitro data showed that DMA can correct the secretory defect in islets isolated from diabetic mice (29). Because amiloride derivatives are already on the market as diuretics and antihypertensive agents, we wanted to investigate their potential therapeutic value in T2D. Arginine, one of the messenger molecules whereby DMA exerts its effect, is also a valuable alternative therapeutic agent with potentially fewer side effects. We tested the effect of both DMA and arginine on blood glucose homeostasis in diabetic mouse models. Oral administration of each compound in drinking water resulted in considerable improvement of basal glucose and insulin levels, glucose tolerance, and insulin response to glucose bolus.

Three mouse models of T2D were used in these experiments. First, diabetic NON mice (37) are an inbred strain, originally developed as a control strain for the well-known insulin-dependent diabetes mellitus model of NOD/LtJ. NON mice are genetically closely related to NOD mice but have a diabetes-resistant MHC haplotype *H2*^{nb1} (K^b, A^{nb1}, E^k, D^b) and are homozygous for the retinal degeneration allele *Pde6b*^{rd1}. NON mice have genes predisposing to T2D, as evidenced by early impaired glucose tolerance and development of maturity onset obesity and hypoinsulinemia. At 20 wk of age, NON males exceed 40 g in body weight, with blood glucose and insulin levels reported around 200 mg/dl and 1 ng/ml, respectively (37). Our previous data showed that oral treatment of DMA results in marked improvement of insulin release in islets isolated from these mice (29), and the current study shows similar positive effects on blood glucose homeostasis in vivo. Second, *gk*$^{lox/w}$+*Rip-cre* mice are a β-cell-specific glucokinase heterozygous knockout strain developed by the Magnuson laboratory (57). These mice exhibit considerable hyperglycemia and severe hypoinsulinemia from birth (57), providing a suitable phenotype to test the ability of DMA and arginine to enhance insulin release at the β-cell level. Finally, Ldb mice are a severely diabetic model available from Jackson Laboratory (38). Mice homozygous for the diabetes spontaneous mutation (*Lepr*^{db}) become identifiably obese around 3–4 wk of age. Elevations of plasma insulin begin at 10–14 days, and elevations of blood glucose begin at 4–8 wk. Homozygous mutant mice are polyphagic, polydipsic, and polyuric. The course of the disease is influenced by genetic background, and a number of features are observed on the C57BLKS background, including an uncontrolled rise in blood glucose, β-cell depletion, and death by 10 mo of age (38).

Oral treatment with DMA or arginine resulted in a decrease in basal blood glucose and an increase in plasma insulin levels in all three strains to varying degrees (Table 1), although these

Table 1. *Changes in basal blood glucose and insulin levels in response to oral treatment with DMA or arginine for 3 wk*

		DMA Treatment		Arginine Treatment	
Mice	*n*	Before	After	Before	After
		Blood glucose, mg/dl			
NON	8	140±8.1	102±8.3*	142±11.5	125±8.5
GK	3	190±9.33	140±16.2	188±10.8	148±15.8
Ldb	4	503±20.6	351±32.5*	431.3±23.9	319±38
		Plasma insulin, ng/ml			
NON	8	0.97±0.14	1.46±0.14*	1.14±0.26	1.37±0.25
GK	3	0.28±0.05	0.51±0.11	0.19±0.03	0.32±0.05*
Ldb	4	3.77±.0.7	6.25±1.66	4.13±1.1	11.5±2.68

Values are means ± SE of changes in basal blood glucose and insulin levels in response to oral treatment with dimethyl amiloride (DMA; 1 $mg \cdot kg^{-1} \cdot day^{-1}$) or arginine (2.5 $g \cdot kg^{-1} \cdot day^{-1}$) for 3 wk in NON, GK, and Ldb mice. *$P < 0.05$.

changes were not always significant. Both treatments produced significant improvement in glucose tolerance in NON mice and Ldb mice (Fig. 1, *A* and *C*), but not in GK mice (Fig. 1*B*). In NON mice, the insulin response to glucose challenge was enhanced by both treatments, and glucose tolerance showed a corresponding improvement. Although both DMA and arginine improved basal and fasting blood glucose levels and glucose tolerance, arginine produced a more effective response on glucose homeostasis, particularly at the 30-min time point, before the insulin levels went up significantly. In GK mice, neither compound produced significant changes in basal or fasting glucose levels or in glucose tolerance. Interestingly,

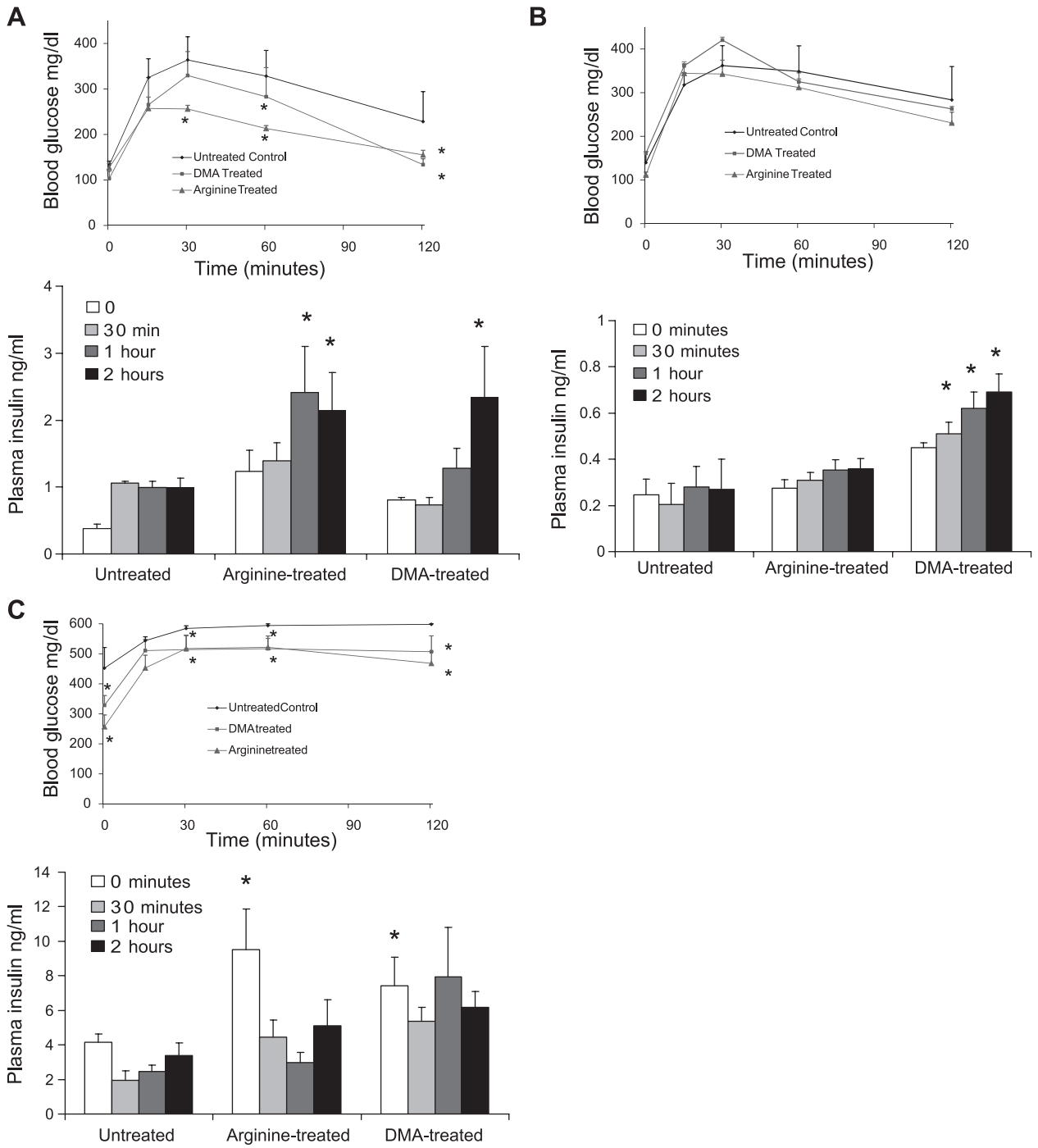


Fig. 1. Prior oral treatment with dimethyl amiloride (DMA) and arginine improve blood glucose homeostasis in three mouse models of type 2 diabetes. Intraperitoneal glucose tolerance test (IPGTT; *top*) and insulin response to glucose bolus (*bottom*) were determined in diabetic mice treated with each compound compared with untreated controls. Diabetic NON/Ltj mice (*A*; $n = 8$ per group), GK mice (*B*; $n = 3$ per group), and Ldb mice (*C*; $n = 4$ per group) were given DMA (1 mg·kg^{-1}·day^{-1}) or arginine (2.5 g·kg^{-1}·day^{-1}) in drinking water for 3 wk. Control groups were given regular drinking water. IPGTT was performed by sampling blood glucose at designated intervals before and after intraperitoneal injection of 2 g/kg sterile glucose solution. Plasma insulin levels in response to the same glucose challenge were measured at the time points indicated. *$P < 0.05$ compared with each corresponding untreated control group.

DMA did improve insulin response to glucose bolus, but this had little effect on glucose tolerance, suggesting that this strain also has additional defects on insulin sensitivity. Isolated islets from these mice showed an impaired insulin response to glucose in vitro, which was restored to some extent by DMA (Fig. 2). In Ldb mice, where the initial hyperglycemia is very severe, both treatments increased fasting plasma insulin levels, but there was no further enhancement of the insulin secretion in response to a glucose challenge. Despite this relative lack of insulin response, fasting glucose levels and glucose tolerance were significantly improved, although they were still far from normal. Such improvement suggests that in addition to enhancing insulin release at the β-cell level, DMA and arginine may have an action of directly decreasing insulin resistance at the peripheral tissue level. Previous studies have also reported insulin sensitivity-enhancing actions of arginine associated with other drugs such as sildenaphil (4). DMA can have a similar effect by making more arginine available through inhibition of nNOS.

As shown in our previous findings, one mechanism whereby DMA unmasks TDP is through inhibition of nNOS, which causes an increase the amount of arginine available for cellular functions. Glucose does not induce TDP in normal mouse islets, but it does so in the presence of DMA, nNOS inhibitors, or added arginine (32). However, this may not be the only mechanism, since the magnitude of glucose-induced TDP in the presence of DMA is much greater than that in the presence of nNOS inhibitors or arginine (32). To further explore any additional mechanisms, we tested the ability of selected nonglucose secretagogues to induce TDP in the presence of DMA or (L-NAME), an inhibitor of nNOS. The secretagogues included KIC and BCH. KIC is a metabolite of the amino acid leucine and is a mitochondrially metabolized nutrient that stimulates insulin release better than leucine, albeit to a lesser extent than glucose (29, 31). BCH, a nonmetabolizable analog of leucine, produces TDP to the same extent as glucose, although it is not metabolized (30).

As expected, neither KIC nor BCH could induce TDP in WT mouse islets. Whereas DMA enabled both KIC and BCH to induce significant TDP, L-NAME did not (Fig. 3). Thus, unlike with glucose, inhibition of nNOS is not sufficient to enable

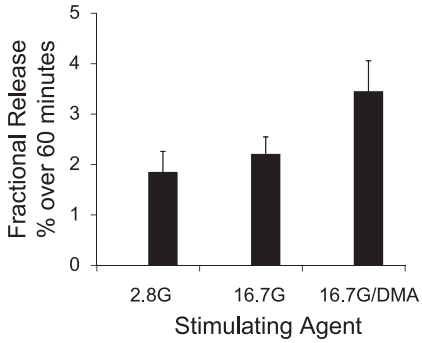


Fig. 2. DMA restores the impaired insulin response to glucose in islets from diabetic GK mice. Freshly isolated islets were exposed to different conditions as denoted, and insulin secretion was measured over a period of 60 min. Values are means ± SE, expressed as release percentage over 60 min ($n = 6$). Insulin secretion in response to high glucose (G, glucose in mmol/l) was impaired and was restored to some extent by the presence of DMA (40 μmol/l). Corresponding absolute insulin release values for each condition from *left* to *right* are 3.26 ± 1.31, 3.12 ± 0.99, and 5.17 ± 1.0 ng/ml.

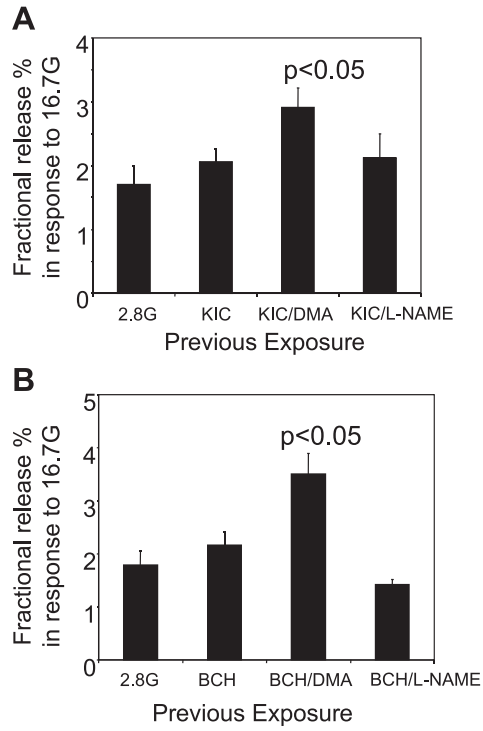


Fig. 3. Time-dependent potentiation (TDP) by nonglucose secretagogues in wild-type (WT) mice. Insulin secretion was measured in response to 16.7 mmol/l glucose in freshly isolated islets from WT mice. As indicated, islets were previously exposed to α-ketoisocaproate (KIC; *A*) or 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH; *B*) (20 mmol/l) in the presence or absence of DMA (40 μmol/l) or N^G-nitro-L-arginine methyl ester (L-NAME; 5 mmol/l). Control islets were maintained in basal glucose. TDP was observed only when the previous exposure to KIC or BCH was done in the presence of DMA. Values are means ± SE, expressed as fractional release percentage over 40 min ($n = 5$ for KIC and 4 for BCH). Corresponding absolute insulin release values for each condition from *left* to *right* are A 1.79 ± 0.3, 1.84 ± 0.23, 6.58 ± 2.6, and 1.32 ± 0.24 ng/ml in *A* and 3.36 ± 0.54, 2.93 ± 0.35, 6.6 ± 0.7, and 2.1 ± 0.63 ng/ml in *B*.

TDP by these nonglucose compounds. This is further confirmed by the pattern of TDP in NOS KO islets, where glucose normally induces TDP to a large magnitude (32). In NOS KO islets, KIC induced a low degree of TDP that was significantly enhanced by DMA (Fig. 4*A*), whereas BCH did not induce TDP except in the presence of DMA (Fig. 4*B*). These results and previous data (32) on the magnitude of glucose-induced TDP indicate that nNOS inhibition is not the only mechanism whereby DMA enables TDP. The activation/inhibition of another pH-sensitive factor(s) appears to be essential for these mitochondrial secretagogues to exert their memory effect. Although DMA increases K^+ retention and intracellular Ca^{2+}, this cannot be the other pH-sensitive mechanism, because *1*) TDP is a function independent of K^+ and Ca^{2+} (33, 67, 68), and *2*) DMA enables and/or enhances TDP under strict Ca^{2+}-free conditions to the same extent it does in the presence of Ca^{2+} (Fig. 5) (28, 33).

All compounds that induce TDP require mitochondrial metabolism and are dependent on anaplerotic input (30). Glucose is the only agent that is partially metabolized in the cytosol first, whereas all others directly affect the mitochondria. Although BCH is not metabolized itself, BCH is believed to act by activating glutamate dehydrogenase (GDH) and enhancing entry of glutamate into the TCA cycle (43, 59). A major effect

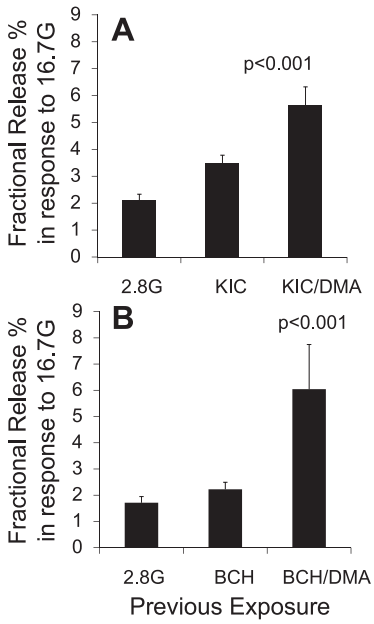


Fig. 4. TDP by nonglucose secretagogues in NOS knockout (KO) mice: Insulin secretion was measured in response to 16.7 mmol/l glucose in islets freshly isolated from NOS KO mice. As indicated, islets were previously exposed to KIC (*A*) or BCH (*B*) (20 mmol/l) in the presence or absence of DMA (40 μmol/l). Controls were maintained in basal glucose. Significant TDP was observed only when the previous exposure to KIC or BCH was done in the presence of DMA. Values are means ± SE, expressed as fractional release percentage over 40 min (n = 8 for KIC and 6 for BCH). Corresponding absolute insulin release values for each condition from *left* to *right* are 4.48 ± 0.6, 6.85 ± 0.83, and 9.45 ± 0.87 ng/ml in *A* and 5.59 ± 1.6, 7.56 ± 1.3, and 12.4 ± 2.5 ng/ml in *B*.

of DMA is to inhibit cell membrane NHE, which in turn decreases intracellular pH, and may influence metabolic enzymes particularly sensitive to pH. Although there is some controversy on the mechanism whereby DMA decreases cellular pH (65), there is no doubt that DMA causes a strong and sustained decrease of cellular pH in a HEPES-buffered medium (33, 62, 63). Thus it is possible that the low pH created by DMA activates a specific mitochondrial enzyme(s) essential for generating the signals for TDP. Our previous studies showed that DMA decreases cytosolic pH in islets from both healthy and diabetic mice (29, 31). To verify whether DMA also decreases mitochondrial pH, we measured organelle pH in islet cells in the presence and absence of DMA, using our established pH-measurement techniques for pancreatic islets (31) with modifications for mitochondrion-specific dye loading (69). Mitochondria constitute a major part of the organelle mass in the β-cells (34, 70). Addition of DMA to islets caused an average drop of 0.33 pH units in perinuclear organelles (Fig. 6), a small but significant decrease adequate to affect the activity of pH-sensitive metabolic enzymes.

A common feature among all compounds that induce TDP is anaplerotic input, with particular emphasis on the reactions that generate and utilize KG in the TCA cycle (30). These include isocitrate dehydrogenase (ICDH), which generates KG from isocitrate; GDH, which generates KG from glutamate; and KGDH, which converts KG to succinyl-CoA (30) (see Fig. 9). TDP is a pH-dependent function, and all these enzymes are also pH dependent. GDH and ICDH, although pH sensitive, are not always activated by low pH. Their pH optimum sometimes falls in the alkaline range (39, 51, 52), whereas KGDH is consistently activated by low pH in all tissues tested (45, 47, 48). Work in rat pancreatic islets has demonstrated that KGDH is specifically activated by low pH in both the presence and absence of Ca^{2+}, whereas GDH does not show the same sensitivity to pH (28). Furthermore, TDP-inducing compounds such as BCH and leucine also activate KGDH (28), whereas GDH is indirectly inhibited by glucose, the major physiological priming agent, through the action of GTP (30, 61, 64). In addition, KGDH is also known to retain a "memory" to a prior exposure, a feature similar to that of TDP (60). Therefore, KGDH is a more likely candidate than the other enzymes, and it is possible that part of the mechanism whereby DMA enables TDP is simply by increasing the activity of mitochondrial KGDH.

To determine whether KGDH is a necessary factor for TDP, we tested whether inhibition of KGDH would inhibit TDP. Acetyl salicylate, an inhibitor of KGDH that is not toxic to cellular metabolism (53), was used to inhibit KGDH. Acetyl salicylate was administered in its methylated form to facilitate entry into intact cells. Since KGDH is a key enzyme in the TCA cycle, its inhibition would halt a major part of cellular metabolism. To prevent this problem, we added two subsequent intermediates of the TCA cycle, fumarate and malate, to the reaction mix to keep the TCA cycle going and verified the functionality of the system by measuring direct insulin secretion in addition to TDP. These results show that TDP was inhibited by the KGDH-inhibitor (see Figs. 7 and 8).

Although glucose cannot induce TDP in WT mouse islets, glucose normally does induce TDP in NOS KO mouse islets (32). Inhibition of KGDH by acetyl salicylate partially inhibited glucose-induced TDP in NOS KO islets (Fig. 7*A*), regardless of the presence of fumarate and malate added to compensate for blockage of overall TCA cycle metabolism. Acetyl salicylate did not block direct insulin secretion by glucose during the priming (memory inducing) period, as long as the TCA cycle was kept going with the added fumarate and malate (Fig. 7*B*). Hence, acetyl salicylate is a reasonably appropriate

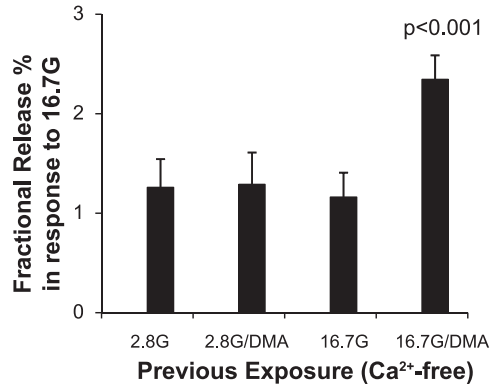
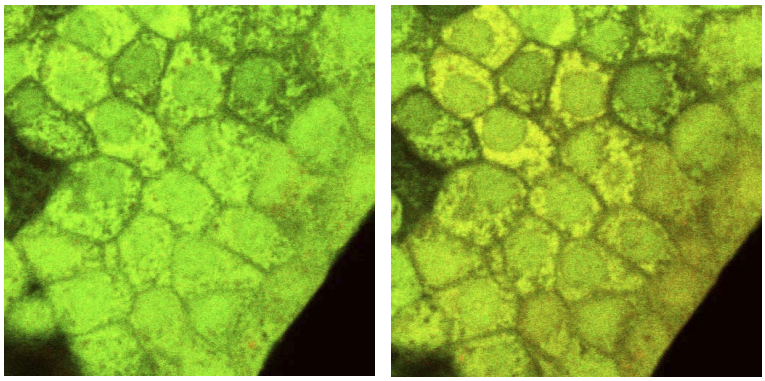


Fig. 5. DMA enables TDP in mouse islets even under stringent Ca^{2+}-free conditions. Insulin secretion was measured in response to 16.7 mmol/l glucose in freshly isolated islets from WT mice. As indicated, islets were previously exposed to basal or high glucose in the presence or absence of DMA (40 μmol/l) under stringent Ca^{2+}-free conditions ensured by omitting Ca^{2+} from the medium and addition of EGTA (5 mmol/l) and BAPTA-AM (20 μmol/l) for extracellular and intracellular Ca^{2+} chelation, respectively. DMA enabled glucose to induce TDP independently of Ca^{2+}. Values are means ± SE, expressed as fractional release percentage over 40 min (n = 5). Corresponding absolute insulin release values for each condition from *left* to *right* are 3.85 ± 1, 1.91 ± 0.45, 3.01 ± 0.68, and 6.53 ± 1.12 ng/ml.



Islet	1	2	3	4	5	6	7	8	Mean
Average pH: Basal	7.06051	7.04342	7.03755	7.03726	7.04469	7.13379	6.36155	7.03779	**6.96957**
Average pH: After DMA	6.71139	6.69744	6.72456	6.70355	6.68319	6.89406	6.34085	6.36089	**6.63949**
pH Change	0.34919	0.34598	0.31299	0.33371	0.36149	0.23973	0.02070	0.67689	**0.33007 ***

Fig. 6. DMA causes a significant decrease in islet mitochondrial pH. Changes in emission fluorescence of carboxy-seminaphthorhodofluor-5 acetoxymethyl ester (SNARF-5-AM) were measured in response to changes in organelle pH. Cultured islets were loaded with SNARF-5-AM (5 μmol/l) for 30 min. Islets were then washed and incubated in SNARF-5-free medium for 4+ h to allow SNARF-5 to accumulate in mitochondria and remaining cytosolic SNARF-5 to diffuse out. Islets were excited at 514 nm, and emission fluorescence was recorded at 580 and 630 nm. Emission fluorescence at 580 and 630 nm are denoted by red and green, respectively (pseudocolors). Recordings were taken before and after treatment with DMA to a final concentration of 40 μmol/l. Addition of DMA causes a marked decrease in the green fluorescence and an increase in the red fluorescence that can be quantitated by LSM software and is sometimes visible to the eye. One representative image is shown at *top*, part of one islet before and after treatment with DMA. pH was calculated using the emission ratio in specific regions of interest before and 20 min after treatment with DMA. pH in 6–10 selected perinuclear regions was calculated and averaged for each islet. The table at *bottom* shows such averaged values for 8 islets. Average change of pH in nonnuclear regions of all islets after DMA treatment = 0.33 units. $*P < 0.002$.

inhibitor to determine the role of KGDH in TDP. Although acetyl salicylate has another nonspecific effect of inhibiting cyclooxygenase, this is not likely to adversely affect interpretation of the current data. Inhibition of cyclooxygenase by acetyl salicylate and other NSAIDs is reported to increase rather than inhibit insulin secretion in pancreatic islets (19–21). In addition, acetyl salicylate may close membrane ATP-dependent K^+ (K_{ATP}) channels, further increasing direct insulin release (42). Despite such favorable effects of acetyl salicylate on direct insulin release, the current data show that acetyl salicylate selectively inhibits TDP (but not direct insulin release), which further confirms an important role for its other target, KGDH, in TDP. Unlike glucose, BCH and KIC can induce significant TDP only in the presence of DMA. Such TDP was also completely blocked by acetyl salicylate (Fig. 8), confirming that KGDH plays a role in TDP.

DISCUSSION

The major findings of this study are the positive systemic effects of DMA upon in vivo treatment of diabetic mice. DMA consistently produced significant improvement of basal and fasting blood glucose levels, glucose tolerance, and insulin response to glucose challenge in two of three diabetic models tested. The GK mice, which did not show an improvement of glucose tolerance, still responded to DMA with an increased insulin response in vivo and an enhanced insulin response to glucose in isolated islets in vitro. Thus the ability of DMA to improve nutrient-stimulated insulin response at the β-cell level is consistent and may be utilized as a potential therapeutic approach for T2D.

The mechanisms by which DMA improves systemic glucose homeostasis are not fully understood. The well-known systemic effects of DMA include its diuretic and antihypertensive actions, K^+ sparing, and decrease of intracellular pH through inhibition of membrane NHEs. Although we cannot rule out the influence of the other systemic effects, evidence from this and previous studies point more toward the influence of DMA on intracellular pH and consequent enzymatic changes leading to TDP as mediators of the improvement of glucose homeostasis. Although diuresis and increased fluid intake occurred in the DMA-treated groups, the net positive effects on blood glucose homeostasis were not significantly different from those in the arginine-treated groups, which did not have these side effects. As demonstrated before, DMA can enable TDP to occur in situations where it is absent and improve insulin release where it is impaired (29, 31, 33). The pH-lowering effect of DMA is widely documented, and our previous studies indicated that this acidifying effect inhibits cellular nNOS, increasing the availability of arginine as a messenger for cellular functions such as TDP (32). Current data confirm a mechanism of action through arginine availability, since oral treatment with arginine produced positive effects on glucose homeostasis similar to those of DMA. In NON/LtJ mice, arginine treatment improved glucose tolerance better than DMA did (Fig. 1*A*). In hyperinsulinemic Ldb mice, although arginine failed to increase plasma insulin levels in response to a glucose challenge, both DMA and arginine improved glucose tolerance significantly. Such improvement suggests that in addition to enhancing insulin release at the β-cell level, DMA and arginine may have an action of directly decreasing insulin resistance at the peripheral tissue level. Previous studies have also reported insulin sensitivity-enhancing actions of arginine associated with other drugs such as sildenaphil (4). DMA can have a similar effect by making more arginine available through inhibition of nNOS. Both DMA and arginine treatment resulted in significant improvement of glucose homeostasis, and neither compound produced serious adverse effects. Considering that arginine produced slightly better glucose tolerance than DMA (despite greater insulin response with DMA treatment), it

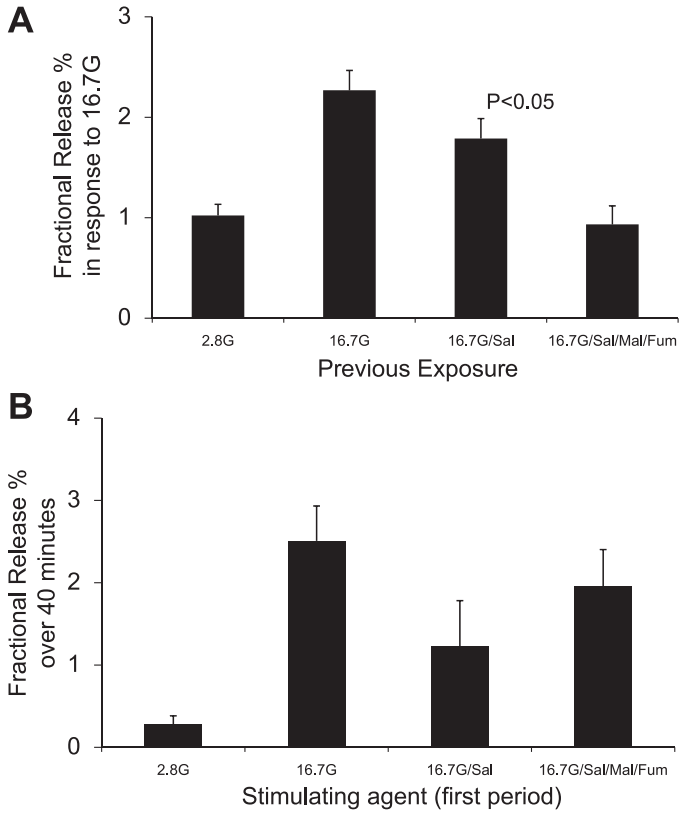


Fig. 7. Effect of inhibition of α-ketoglutarate dehydrogenase (KGDH) in islets from NOS KO mice: *A*: acetyl salicylate inhibits glucose-induced TDP. Insulin secretion was measured in response to 16.7 mmol/l glucose in islets freshly isolated from NOS KO mice. As indicated, islets were previously exposed to 16.7 mmol/l glucose in the presence or absence of acetyl salicylate (5 mmol/l), with or without the subsequent intermediates of the TCA cycle, fumarate (20 mmol/l) and malate (20 mmol/l). All compounds (acetyl salicylate, malate, and fumarate) were added in methylated from to facilitate entry into intact cells. Control groups were maintained in basal glucose. TDP normally observed with 16.7 mmol/l glucose was significantly inhibited in the presence of acetyl salicylate. Sal, acetyl salicylate; Mal, malate; Fum, fumarate. Values are means ± SE, expressed as fractional release percentage over 40 min (n = 8). Corresponding absolute insulin release values for each condition from *left* to *right* are 2.03 ± 0.3, 7.15 ± 0.83, 4.7 ± 0.54, and 2.93 ± 0.98 ng/ml. *B*: acetyl salicylate does not inhibit direct insulin secretion by glucose, provided the TCA cycle is not blocked. Direct insulin secretion during the first stimulation period (memory-inducing period) of the experiment in *A* was measured. Direct insulin secretion over the first period (40 min) in response to each condition is shown. The presence of acetyl salicylate did not significantly inhibit direct insulin secretion in response to 16.7 mmol/l glucose, particularly when fumarate and malate were added to keep the TCA cycle functioning.

would be worthwhile to further explore dietary supplementation of arginine as a therapeutic option for T2D.

The observed improvement of glucose homeostasis could also be influenced by other systemic effects of DMA. K^+ retention, a well-documented effect of DMA, would be expected to elevate basal insulin levels through stimulation of first-phase insulin release. All three strains showed elevated basal insulin levels upon DMA treatment, albeit not significant in NON mice. Such increase in basal insulin could also be due to an increased supply of arginine (a cationic compound that directly stimulates insulin release in addition to its effect on TDP) secondary to DMA-induced decrease of pH, since arginine-treated groups also showed elevated basal insulin levels (Fig. 1). Although it is possible that the action of DMA is partly due to direct stimulation of insulin release through cations such as K^+ or arginine, this would only contribute a small percentage to its glucose homeostatic effect, since cation-stimulated insulin release only accounts for the first-phase insulin release, a relatively small portion of the total insulin response of in the β-cell (66). The major part of nutrient-stimulated insulin secretion occurs over time through the second phase (augmentation pathways) and TDP via metabolic signals, whereas <1% of the insulin content of the β-cell is released during the cation-stimulated first phase (9, 66). Because DMA is known to exert strongly positive effects on TDP (31–33), it appears that the most likely mechanism whereby DMA improves glucose homeostasis in diabetes is by helping the induction of TDP. Previous in vivo studies in rats, a species that normally has TDP, showed that certain amiloride derivatives consistently improve glucose homeostasis and related functions in T2D (2, 3, 17, 18). Previous in vitro studies in rats showed that DMA consistently enhances insulin release and TDP in response to all metabolic secretagogues tested (28, 30, 33).

As demonstrated before, DMA can enable TDP to occur in situations where it is absent and improve insulin release where it is impaired. Although nNOS inhibition through lowered pH is one mechanism for this (32), the presence of additional mechanisms is suggested by the pattern and magnitude of TDP

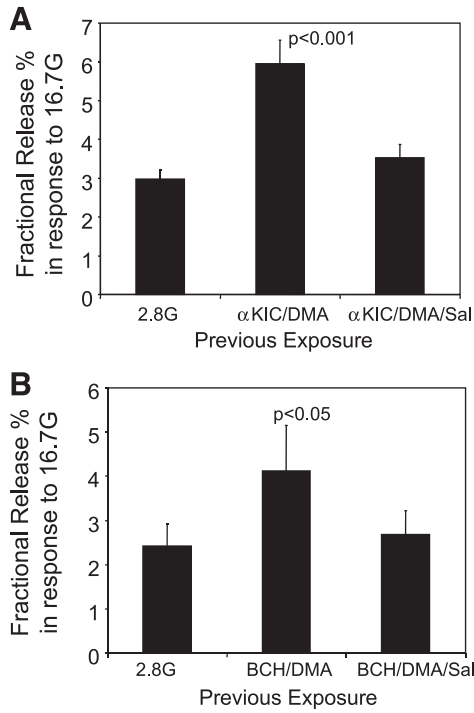


Fig. 8. Acetyl salicylate inhibits TDP induced by nonglucose secretagogues. Insulin secretion was measured in response to 16.7 mmol/l glucose in islets freshly isolated from NOS KO mice. As indicated, islets were previously exposed to KIC (*A*) or BCH (*B*) (20 mmol/l) with DMA (40 μmol/l), in the presence or absence of acetyl salicylate (5 mmol/l), fumarate (20 mmol/l), and malate (20 mmol/l). Controls were maintained in basal glucose. TDP normally observed with KIC or BCH in the presence of DMA was significantly inhibited in the presence of acetyl salicylate. Sal, acetyl salicylate + fumarate + malate. Values are means ± SE, expressed as fractional release percentage over 40 min (n = 5). Corresponding absolute insulin release values for each condition from *left* to *right* are 6.17 ± 0.92, 12.4 ± 1.87, and 8.03 ± 1.54 ng/ml in *A* and 3.87 ± 0.69, 11.38 ± 2.44, and 4.97 ± 1.2 ng/ml in *B*.

induced by different compounds. Glucose can induce TDP in the presence of either DMA or nNOS inhibitors, but the magnitude is much greater in the presence of DMA (32). Unlike with glucose, nNOS inhibition cannot enable TDP by nonglucose compounds such as KIC or BCH. These compounds can induce TDP only in the presence of DMA (Figs. 3 and 4). This indicates the presence of additional pH-sensitive factors involved in the generation of TDP. A common feature among all compounds that induce TDP is anaplerotic input, with particular emphasis on the reactions that provide KG into the TCA cycle (30). These include ICDH, which generates KG from isocitrate, GDH, which generates KG from glutamate, and KGDH, which converts KG to succinyl-CoA (30) (Fig. 9). TDP is a pH-dependent function, and all these enzymes are also pH dependent. GDH and ICDH, although pH sensitive, are not always activated by low pH. Their pH optimum sometimes falls in the alkaline range (39, 51, 52), whereas KGDH is consistently activated by low pH in all tissues tested (45, 47, 48). Work in rat pancreatic islets has demonstrated that KGDH is specifically activated by low pH in both the presence and absence of Ca^{2+}, whereas GDH does not show the same pH sensitivity (28). Furthermore, TDP-inducing compounds such as BCH and leucine also activate KGDH (28), whereas GDH is indirectly inhibited by glucose, the major physiological TDP-inducing agent, through the action of GTP (30, 61, 64). In addition, KGDH is also known to retain a memory to a prior exposure, a feature similar to TDP (60). All this indirect evidence points to KGDH as the most likely candidate to be another pH-sensitive factor involved in the generation of TDP. If this hypothesis were true, i.e., if KGDH contributes to the signaling in TDP, inhibition of KGDH should inhibit TDP to some extent.

Limitations in experimental inhibition of KGDH include the critical role KGDH plays in cellular functions and the limited specificity of available inhibitors. With KGDH being a central enzyme in the TCA cycle, inhibition of KGDH would result in cessation of a large part of cellular metabolism. We circumvented this problem by providing the subsequent TCA cycle intermediates into the reaction mixture to keep the TCA cycle going (Figs. 7 and 9). Acetyl methyl salicylate is a reasonably specific inhibitor of KGDH, although it also inhibits cyclooxygenase. However, this inhibition is not expected to adversely

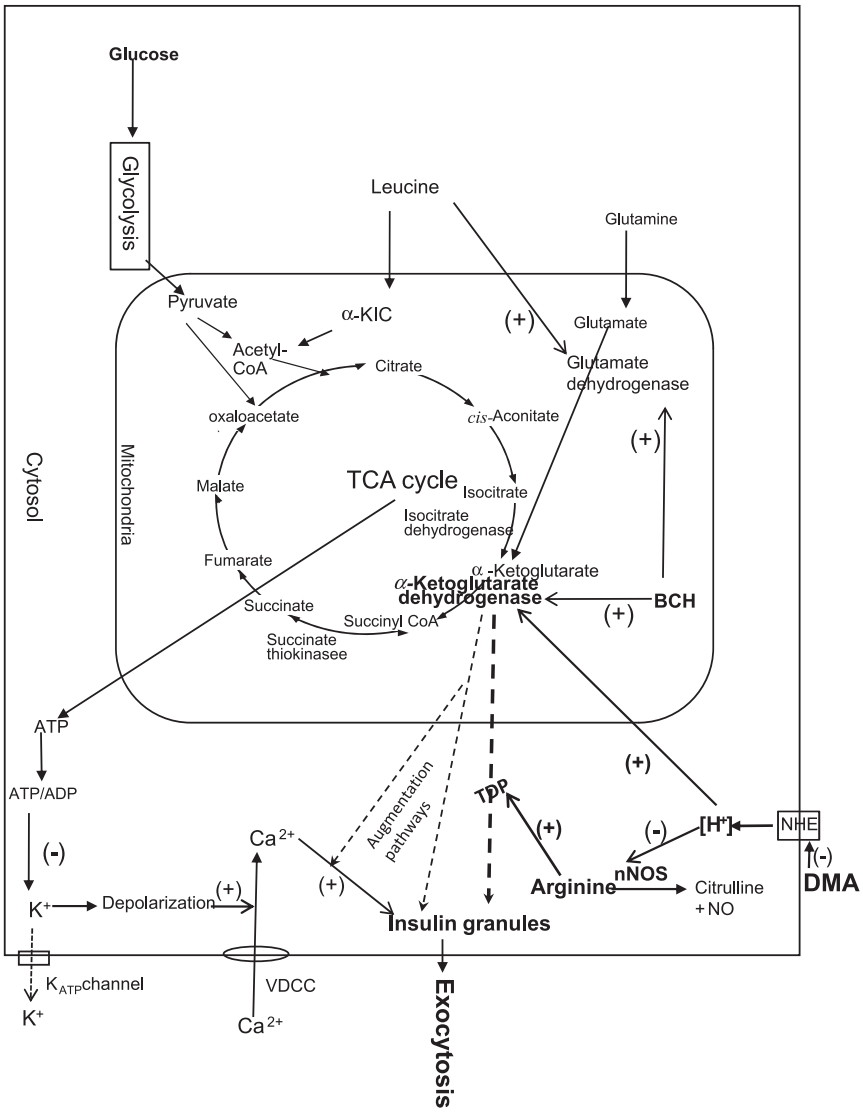


Fig. 9. Illustration of the effects of DMA on different pathways of nutrient-stimulated insulin response in the β-cell. All nutrients enter the mitochondria, where they enhance anaplerotic input by being metabolized in the TCA cycle (*top*). BCH, although not metabolizable, enhances anaplerotic input by activating GDH and KGDH activity (*middle right*). Metabolism of nutrients in the TCA cycle generates reducing equivalents (NADH and $FADH_2$), which then go through the respiratory chain to generate ATP. (This well-known step is not shown due to space limitations.) Increased ATP production leads to Ca^{2+} influx via the steps illustrated at *bottom left*, triggering the first-phase insulin release. Once the first phase is exhausted, nutrient-stimulated insulin release continues via the prolonged second phase or augmentation pathways, which include a Ca^{2+}-dependent component and a Ca^{2+}-independent component (broken lines). In addition, mitochondrial secretagogues also impart a "memory" into the cell, which enhance subsequent insulin responses of the cell. This function is the third component of the insulin response, also referred to as TDP (bold broken line). The underlying mechanisms of the second and third components of the insulin response are not fully known, and the signals for these components are believed to originate in the mitochondria. The positive effects of DMA on TDP are most likely mediated through inhibition of cytosolic nNOS and activation of mitochondrial KGDH (*bottom right*). K_{ATP}, ATP-dependent K^+ channel; VDCC, voltage-dependent Ca^{2+} channel; NHE, Na^+/H^+ exchanger.

affect interpretation of the current data, because several studies have reported that the inhibition of cyclooxygenase by acetyl salicylate and other NSAIDs would increase rather than inhibit direct insulin secretion in pancreatic islets (19–21). In addition, acetyl salicylate may close membrane K_{ATP} channels, further increasing direct insulin release (42). Despite such favorable effects of acetyl salicylate on direct insulin release, our data show that acetyl salicylate selectively inhibits TDP (Figs. 7 and 8) (but not direct insulin release), which further confirms a role for its other target, KGDH, in TDP. Several studies have reported severe morphological disruption of mitochondria in T2D (14, 35). Such alterations may be associated with decreased activity of KGDH, resulting in impaired insulin secretion.

Figure 9 illustrates a model of the different components of nutrient-stimulated insulin release and the possible effects of DMA on these pathways. All nutrients ultimately enter the mitochondria, where they enhance anaplerotic input. Glucose is first metabolized into pyruvate, and leucine into KIC. They both enter the TCA cycle as acetyl-CoA. Glutamine is deaminated into glutamate and is converted to KG via GDH. In addition to being metabolized to produce energy, leucine also enhances anaplerotic input by activating GDH activity. BCH, although not metabolizable, enhances anaplerotic input by activating GDH and KGDH activity. Enhanced TCA cycle activity produces reducing equivalents (NADH and $FADH_2$), which then go through the respiratory chain to generate ATP. (This well-known step is not shown in Fig. 9 due to space limitations.) Increased ATP production elevates the cellular ATP/ADP ratio, which leads to closure of K_{ATP} channels, causing accumulation of K^+ inside the cell. The resulting membrane depolarization opens voltage-dependent Ca^{2+} channels, leading to Ca^{2+} influx. This Ca^{2+} signal triggers the release of the readily releasable pool of insulin granules, bringing about the initiation or first phase of insulin release (9, 66). Once the first phase is exhausted, nutrient-stimulated insulin release continues via the prolonged second phase or augmentation pathways, which include a Ca^{2+}-dependent component and a Ca^{2+}-independent component (9, 66). Continuation of insulin secretion through these pathways requires movement of insulin granules from the reserve pool to the readily releasable pool (9, 66). The specific steps responsible for such movement are not known. In addition to the augmentation pathways, mitochondrial secretagogues also impart a memory into the cell, which enhance subsequent insulin responses of the cell. This function is the third component of the insulin response, also referred to as TDP. TDP is distinct from the augmentation pathways in several respects: *1*) all compounds that induce TDP do not stimulate the augmentation pathways, and vice versa (30); *2*) augmentation pathways require the presence of the inducing compound in the system, whereas TDP can occur after the compound is taken away (22, 26, 28); *3*) TDP is completely independent of Ca^{2+} (33), whereas augmentation pathways are partially dependent on Ca^{2+} (9, 66); *4*) anaplerotic input alone is sufficient for the induction of TDP, whereas augmentation pathways require both anaplerotic input and increased levels of acetyl CoA (30); and *5*) signaling molecules such as cAMP, long-chain acyl-CoA, and protein kinase A are involved in augmentation pathways (9, 66), whereas TDP occurs independently of these signals (22, 28, 30). Nevertheless, there are several important similarities between TDP and the Ca^{2+}-independent component of the augmentation pathway, such as the need for mitochondrial metabolism and independence of Ca^{2+}, K_{ATP} channels, cAMP, and insulin biosynthesis. Since the specific steps involved in either of these pathways are not yet known, at this point it is difficult to determine the exact relationship between augmentation pathways and TDP. Although we could not rule out the possibility of shared steps, we chose to treat TDP and the Ca^{2+}-independent augmentation pathway as two potentially separate components (Fig. 9), since the known differences between the two pathways are greater than their similarities. Our studies have documented that TDP is critically dependent on islet intracellular pH (30–33). pH-lowering drugs such as DMA have remarkable positive effects on TDP. These effects are most likely mediated through two particular pH-sensitive metabolic enzymes: cytosolic nNOS, with pH optimum in the alkaline range (32); and mitochondrial KGDH, with pH optimum in the acidic range (28, 45, 47, 48). nNOS normally keeps TDP inhibited in mouse islets by removing arginine, its substrate. DMA-induced decrease of intracellular pH inhibits nNOS, making more arginine available as a messenger for TDP as well as for direct insulin release through its cationic action. As described earlier, mitochondrial KGDH is another enzyme important in the generation of TDP signals. DMA activates KGDH, thereby enhancing anaplerotic input, increasing signals for both TDP and augmentation of direct insulin release (Fig. 9). Although the current and previous data (30) suggest a link between activation of KGDH and exocytosis of insulin granules, the specific steps involved in these processes remain to be discovered. One likely mechanism is enhanced movement of granules from the reserve pool to the readily releasable pool. It has been established that the Ca^{2+}-triggered first-phase insulin release results in the exocytosis of most of the insulin granules in the "immediately releasable pool" (9). Since the percentage of insulin granules in a releasable state are relatively small and limited, it is important to keep granules moving from the reserve pool into the releasable pool (9, 66). KGDH reaction may aid this process by providing energy through both reducing equivalents and ATP, whereas nNOS may inhibit this process through depletion of NADPH. Another possible mechanism of KGDH in TDP is increased production of succinyl-CoA and succinate, compounds that stimulate proinsulin biosynthesis, thereby maintaining enhanced insulin release in response to subsequent stimuli.

In conclusion, this study demonstrates the potential therapeutic value of oral treatment of DMA and arginine for type 2 diabetes and suggests a role for mitochondrial KGDH as a messenger in the mechanisms of TDP. Our future directions include studying the long-term effects of these compounds as therapeutic agents for diabetes in larger species and further exploration into their mechanisms of action in the peripheral tissues as well as the β-cell.

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