

Genipin inhibits UCP2-mediated proton leak and acutely reverses obesity- and high glucose-induced β cell dysfunction in isolated pancreatic islets

Chen-Yu Zhang,^{1,2,5,*} Laura E. Parton,^{1,5} Chian Ping Ye,^{1,5} Stefan Krauss,^{1,4} Ruichao Shen,³ Cheng-Ting Lin,³ John A. Porco Jr.,³ and Bradford B. Lowell^{1,*}

¹Department of Medicine, Division of Endocrinology, Beth Israel Deaconess Medical Center and Harvard Medical School, 99 Brookline Avenue, Boston, Massachusetts 02215

²State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, 22 Hankou Road, Nanjing, Jiangsu, China

³Department of Chemistry and Center for Chemical Methodology and Library Development, Boston University, 590 Commonwealth Avenue, Boston, Massachusetts 02215

⁴Present address: Department of Cancer Biology and Therapeutics, Merck Research Laboratories, 33 Avenue Louis Pasteur, Boston, Massachusetts 02215

⁵These authors contributed equally to this work.

*Correspondence: cyzhang@nju.edu.cn (C.-Y.Z.); blowell@bidmc.harvard.edu (B.B.L.)

Summary

Uncoupling protein 2 (UCP2) negatively regulates insulin secretion. UCP2 deficiency (by means of gene knockout) improves obesity- and high glucose-induced β cell dysfunction and consequently improves type 2 diabetes in mice. In the present study, we have discovered that the small molecule, genipin, rapidly inhibits UCP2-mediated proton leak. In isolated mitochondria, genipin inhibits UCP2-mediated proton leak. In pancreatic islet cells, genipin increases mitochondrial membrane potential, increases ATP levels, closes K_{ATP} channels, and stimulates insulin secretion. These actions of genipin occur in a UCP2-dependent manner. Importantly, acute addition of genipin to isolated islets reverses high glucose- and obesity-induced β cell dysfunction. Thus, genipin and/or chemically modified variants of genipin are useful research tools for studying biological processes thought to be controlled by UCP2. In addition, these agents represent lead compounds that comprise a starting point for the development of therapies aimed at treating β cell dysfunction.

Introduction

Uncoupling protein 2 (UCP2) is a mitochondrial carrier protein with homology to other uncoupling proteins like UCP1 and UCP3 (Fleury et al., 1997). UCP2 is expressed in a number of tissues, including pancreatic islets (Krauss et al., 2003; Zhang et al., 2001). In previous studies, it has been demonstrated that UCP2 is a negative regulator of insulin secretion (Chan et al., 2001; Zhang et al., 2001). UCP2 mediates proton leak across the inner mitochondrial membrane. This has been demonstrated in studies using proteoliposomes (Echtay et al., 2001; Jaburek et al., 1999), isolated mitochondria (Echtay et al., 2002), and in intact cells (Krauss et al., 2002). In pancreatic β cells, UCP2-mediated proton leak decreases the yield of ATP from glucose (Chan et al., 2001; Zhang et al., 2001). Consequently, UCP2 negatively regulates glucose-stimulated insulin secretion (Chan et al., 2001; Zhang et al., 2001).

As UCP2 is a negative regulator of insulin secretion, it has been proposed that increased UCP2 expression in β cells could result in β cell dysfunction and the development of type 2 diabetes. Consistent with this idea, UCP2 mRNA and protein were found to be markedly increased in pancreatic islets of animal models of type 2 diabetes (Kassis et al., 2000; Laybutt et al., 2002; Winzell et al., 2003; Zhang et al., 2001). There is also evidence that increased amounts of UCP2 expression in humans can downregulate insulin secretion and increase the risk of type 2 diabetes (Krempler et al., 2002; Sasahara et al., 2004; Sesti et al., 2003).

It has been shown that UCP2 levels in islets are increased by high glucose and high lipids in vitro (Laybutt et al., 2002; Patane et al., 2002). Of note, gene knockout of UCP2 restores first-phase insulin secretion, increases serum insulin levels, and greatly decreases levels of glycemia in *ob/ob* mice (Zhang et al., 2001). Furthermore, deficiency of UCP2 prevents obesity-, high glucose-, and high lipid-induced β cell dysfunction (Joseph et al., 2002, 2004; Krauss et al., 2003). Taken together, these findings indicate that UCP2 is an important mediator of β cell dysfunction. Consequently, a UCP2 inhibitor lacking adverse side effects could be a potentially useful drug for treatment of β cell dysfunction and type 2 diabetes. It is important to note that, at present, no therapy exists for type 2 diabetes that is specifically aimed at a pathogenic cause of β cell dysfunction.

The "classical" regulators of UCPs are fatty acids and purine nucleotides. Purine nucleotides (ATP, ADP, GTP, and GDP) inhibit and fatty acids activate UCP2 and 3 when these are expressed in *E. coli* and reconstituted into liposomes (Echtay et al., 2001; Jaburek et al., 1999; Zackova et al., 2003). Recently, using mitochondria isolated from yeast and various rodent tissues, Echtay et al. (2002) showed that UCP2 and 3 catalyze proton leak when activated by exogenously added superoxide. In isolated mitochondria, purine nucleotides have been shown to inhibit superoxide-induced, UCP2- or 3-dependent proton leak (Echtay et al., 2002). However, purine nucleotides are not cell permeable and therefore are unable to inhibit UCPs when added to intact cells. The goal of the present study was to

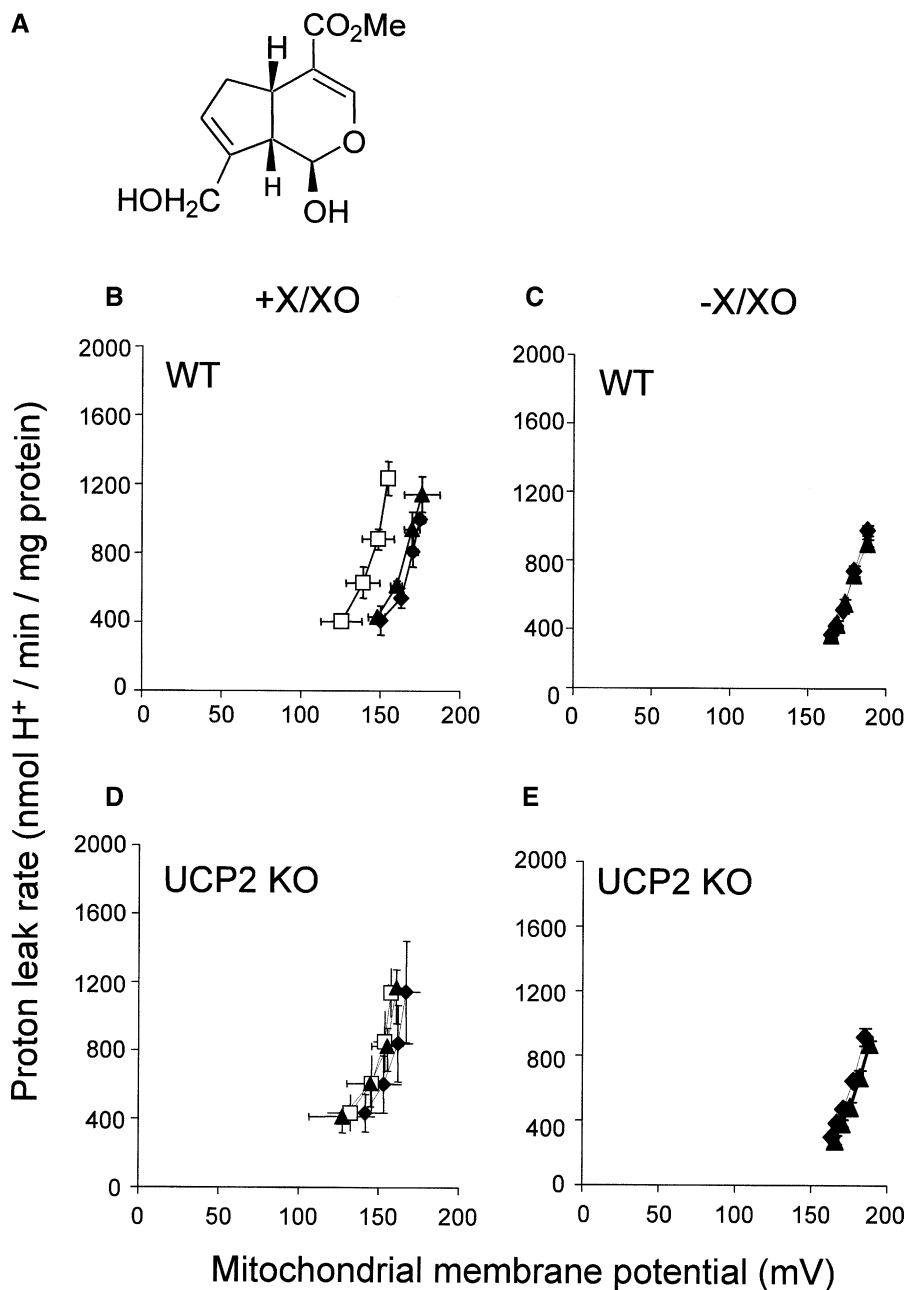


Figure 1. Chemical structure of genipin and effect of genipin on superoxide-dependent proton leak of kidney mitochondria isolated from wild-type and UCP2-deficient mice

Chemical structure of genipin (**A**) and effect of genipin on superoxide-dependent proton leak of kidney mitochondria isolated from WT (**B** and **C**) and UCP2-deficient (**D** and **E**) mice. Proton leak titrations were performed in the presence (**B** and **D**) and absence (**C** and **E**) of a superoxide-generating system (xanthine plus xanthine oxidase, +X/XO), essentially as described previously (Krauss et al., 2003), and with or without 50 μ M genipin. Graphs show the rate of proton leak as a function of its driving force, mitochondrial membrane potential. Left panels (**B** and **D**): filled triangles (\blacktriangle), control; open squares (\square), xanthine (50 μ M) plus xanthine oxidase (0.2 mU/3.5 ml); filled diamonds (\blacklozenge), xanthine/xanthine oxidase plus 50 μ M genipin. Data are the means \pm SEM of three independent experiments with three replicates per experiment. Right panels (**C** and **E**): filled triangles (\blacktriangle), control; filled diamonds (\blacklozenge), plus genipin. Data are the means \pm SEM of 8 replicates.

discover a UCP2 inhibitor capable of working in intact cells. Such an agent would be an extremely useful investigational tool for studying a number of aspects of UCP2 biology. In addition, a UCP2 inhibitor could theoretically represent a lead compound for agents aimed at improving β cell function in type 2 diabetes.

Results

Stimulation of insulin secretion by *Gardenia* extract and genipin

The extract of *Gardenia jasminoides* Ellis fruits has been used over the years in traditional Chinese medicine to treat symptoms of type 2 diabetes (Danbuo, 1984; Wang, 1991). We evaluated *Gardenia* extract and observed that it dose dependently stimulated insulin secretion by pancreatic islets derived from wild-

type (WT) but not from UCP2-deficient mice (data not shown). Because UCP2 was required for this effect, we assumed that *Gardenia* extract contained a UCP2 inhibitor. To identify the active molecule, *Gardenia* extract was fractionated using silica gel chromatography (1:1 hexane and ethyl acetate as eluant). Fractions were assessed for UCP2-dependent stimulation of insulin secretion. Using proton NMR and mass spectroscopic analysis, genipin was identified as a molecule within the active fraction. The chemical structure of genipin was confirmed by comparison with an authentic sample (Wako Chemicals USA, Inc.). Genipin, obtained from the commercial supplier noted above, was further tested for stimulation of insulin secretion (detailed studies for genipin presented later). Genipin stimulated insulin secretion, and this stimulation occurred with wild-type but not UCP2-deficient islets. Figure 1A shows the chemical structure of genipin (Djerassi et al., 1961).

Genipin inhibits superoxide-activated, UCP2-mediated mitochondrial proton leak

In order to assess the effect of genipin on superoxide-induced UCP2-mediated proton leak, mitochondria were isolated from kidneys of WT and UCP2-deficient mice, and proton leak was measured in the presence or absence of exogenous superoxide and genipin (Krauss et al., 2003). Exogenous superoxide was generated in situ by adding the enzyme xanthine oxidase and its substrate xanthine (+X/XO). In WT mitochondria, superoxide activated proton leak as indicated by the leftward shift of the proton leak titration curve (Figure 1B, +X/XO [□] versus control [▲]). Addition of genipin completely abolished superoxide-induced proton leak (Figure 1B, +genipin, +X/XO [◆] versus +X/XO [□]). In mitochondria lacking UCP2, addition of superoxide had no effect on proton leak (Figure 1D, +X/XO [□] versus control [▲]). Genipin was without effect on proton leak when either UCP2 (Figure 1D, +genipin, +X/XO [◆] versus +X/XO [□]), or superoxide (Figure 1C, +genipin [◆] versus control [▲]), or both were absent (Figure 1E, +genipin [◆] versus control [▲]). These results demonstrate that genipin inhibits superoxide-induced, UCP2-mediated proton leak. However, unknown from these studies is whether genipin inhibits UCP2 activity directly or whether genipin interferes in some way with the pathway by which superoxide activates UCP2.

Genipin does not scavenge superoxide

Genipin could inhibit UCP2-mediated proton leak by scavenging superoxide. To evaluate this possibility, we assessed the effects of genipin on an in vitro, cell-free superoxide generating system (xanthine + xanthine oxidase plus salmon sperm DNA plus hydroethidine) (Zhao et al., 2003). Genipin did not decrease levels of superoxide as observed by ethidium fluorescence, whereas addition of the enzyme superoxide dismutase (MnSOD) completely abolished this signal (data not shown). Therefore, genipin does not act as a superoxide scavenger.

Genipin inhibits HNE-activated, UCP2-mediated mitochondrial proton leak

Recently, it has been proposed that superoxide-dependent activation of UCP-mediated proton leak occurs via aldehydic lipid peroxidation intermediates such as 4-hydroxynonenal (HNE) (Murphy et al., 2003), which has been shown to directly stimulate UCP-dependent proton leak in isolated mitochondria (Echtay et al., 2003). According to this model, aldehydic peroxidation products such as HNE are generated as downstream products in an activation cascade, proximal to UCP2, and act as activators of UCP2-mediated proton leak (Echtay et al., 2003; Murphy et al., 2003). Thus, genipin could inhibit UCP2-mediated proton leak by interfering in a step in the pathway by which superoxide activates UCP2. In order to assess this possibility, we studied the effect of genipin on HNE-induced UCP2-mediated proton leak in mitochondria isolated from kidney of WT and UCP2-deficient mice (Echtay et al., 2003). In WT mitochondria, HNE activated proton leak (Figure 2A, +HNE [□] versus control [▲]). Preincubation of mitochondria with genipin completely abolished HNE-induced proton leak (Figure 2A, +genipin, +HNE [◆] versus +HNE [□]). In mitochondria lacking UCP2, addition of HNE had no effect on proton leak (Figure 2B, +HNE [□] versus control [▲]). Consistent with experiments shown in Figure 1, genipin had little or no effect on proton leak in mitochondria lacking UCP2 (Figure 2B, +genipin, +HNE [◆] versus +HNE [□]). Taken together, these

results demonstrate that genipin inhibits HNE-induced, UCP2-mediated proton leak. Thus, genipin does not interfere with the pathway of activation of UCPs by superoxide but most likely inhibits UCP2 activity directly.

Genipin does not affect other mitochondrial transport functions in liver mitochondria

In order to assess the potential effect of genipin on other mitochondrial carrier proteins, many of which are 20%–30% identical at the amino acid level to UCP2, and to assess effects on mitochondrial integrity in general, we measured respiration in isolated mouse liver mitochondria using various respiratory substrates. During state 3 respiration, fuel oxidation is coupled by ATP synthase to the availability of exogenously added ADP (ADP stimulates proton transport by ATP synthase, reducing backpressure on proton pumps in the electron transport chain, hence stimulating respiration). Therefore, rates of respiration in state 3 are dependent upon continued entry of fuels via various mitochondrial carriers as well as the exchange of extramitochondrial ADP for matrix ATP by the adenine nucleotide carriers. Note that UCP1 and its homologs, UCP2 and 3, are not expressed in liver under normal physiological conditions (Pecqueur et al., 2001; Vidal-Puig et al., 1997), therefore, any observed effects on liver mitochondrial activity would be UCP-independent. Using succinate (Figure 2C) or malate-glutamate (Figure 2D) as substrate, respiration in states 3 and 4 was assessed. In addition, total mitochondrial respiratory capacity was determined by measuring respiration following addition of 1 μ M FCCP (a chemical uncoupler that stimulates proton leak). Our results demonstrate that genipin does not acutely affect state 3, state 4, or total (FCCP-stimulated) respiration, indicating that genipin does not affect the activity of the adenine nucleotide carrier or other mitochondrial carriers involved in the metabolism of the substrates tested.

Genipin increases mitochondrial membrane potential, increases ATP levels, closes K_{ATP} channels, and stimulates insulin secretion in a UCP2-dependent manner

We previously demonstrated that UCP2 regulates mitochondrial membrane potential in intact cells. This was shown by demonstrating that lack of UCP2, or removal of endogenous superoxide (a UCP2 activator), increases mitochondrial membrane potential in dispersed islet cells (Krauss et al., 2003). In the present study, we investigated whether genipin induced changes in mitochondrial membrane potential in dispersed islet cells, as measured by the mitochondrial membrane potential-sensitive fluorescent dye, TMRM (Figure 3A). Addition of genipin to dispersed islet cells isolated from WT animals induced a rapid increase in mitochondrial TMRM fluorescence over time. In contrast, addition of genipin to dispersed islet cells lacking UCP2 had no effect on mitochondrial TMRM fluorescence. The detected TMRM signal was greatly reduced by addition of the chemical uncoupler, FCCP, indicating that it accurately reported mitochondrial membrane potential. Thus, addition of genipin induced a rapid increase in mitochondrial membrane potential in dispersed islet cells, and this effect required the presence of UCP2. These findings demonstrate that genipin inhibits UCP2-mediated proton leak, which then leads to an increase in mitochondrial membrane potential.

An increase in mitochondrial membrane potential is predicted to stimulate ATP production by ATP synthase and increase

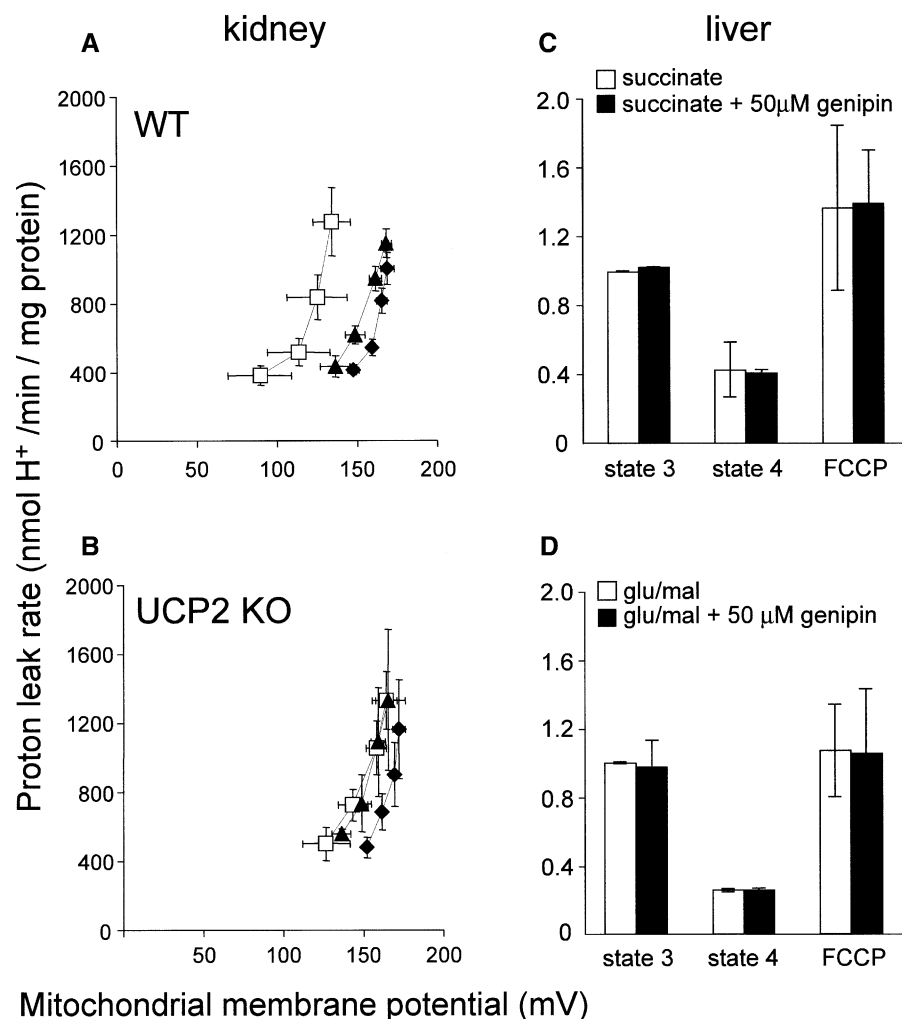


Figure 2. Effect of genipin on HNE-induced proton conductance of kidney mitochondria isolated from wild-type and UCP2-deficient mice and effect of genipin on state 3, state 4, and FCCP-uncoupled respiration in liver mitochondria isolated from wild-type mice

Effect of genipin on HNE-induced proton conductance of kidney mitochondria isolated from WT (A) and UCP2-deficient mice (B) and effect of genipin on state 3, state 4 and FCCP-uncoupled respiration in liver mitochondria isolated from WT mice (C and D). A and B) Proton leak titration was performed with addition of 35 μ M HNE essentially as described previously (Echtay et al., 2003) and in Experimental Procedures, and with or without 50 μ M genipin. Graphs show the rate of proton leak as a function of its driving force, mitochondrial membrane potential. Filled triangles (\blacktriangle), control; open squares (\square), HNE (35 μ M); filled diamonds (\blacklozenge), HNE plus 50 μ M genipin. Proton leak data are the means \pm SEM of three independent experiments with three replicates per experiment.

C and D) Potential effects of genipin on state 3 and state 4 respiration in isolated liver mitochondria was assessed in the presence of 4 mM succinate (C) or 8 mM glutamate/malate (D), with or without 50 μ M genipin. State 3 and 4 respiration was measured within 20 min of genipin addition, and data were normalized to the state 3 condition for each substrate without genipin present. Data are the means \pm SEM of three independent experiments with three replicates per experiment.

levels of ATP. This was tested by assessing effects of genipin on ATP levels in incubated islets. As is shown in Figure 3B, genipin increased ATP levels in WT islets but not in islets isolated from UCP2-deficient mice. In contrast, 20 mM glucose increased ATP levels in both WT and UCP2-deficient islets. Thus, acute inhibition of UCP2 increases ATP levels in islet cells.

In pancreatic β cells, ATP closes plasma membrane K_{ATP} channels which depolarizes the cell, opening voltage gated calcium channels, ultimately stimulating insulin secretion. In the present study, we assessed whether acute inhibition of UCP2 by genipin would, as predicted, close K_{ATP} channels. To address this issue, currents (Figure 3C(ii), bottom tracing) were recorded in response to alternate ± 20 mV voltage steps (Figure 3C(ii), top tracing) from a holding potential of -70 mV, using the “perforated-patch” configuration (Sakura et al., 1998). To evaluate the density of K_{ATP} channels, currents (pA) were recorded in the presence of zero glucose and were normalized to cell capacitance (pF) to control for possible differences in cell size. These studies indicated that the density of K_{ATP} channels is similar in wild-type versus UCP2-deficient cells (pA/pF, mean \pm SE, WT = 12.8 ± 1.8 [n = 5], UCP2 $^{-/-}$ = 13.2 ± 2.2 [n = 5]). Representative current traces showing the effects of glucose, genipin, and tolbutamide (specific K_{ATP} channel blocker) are shown in

Figure 3C(i) and the summary of these recordings is shown in Figure 3C(iii). As can be seen, genipin closed K_{ATP} channels in WT but not in UCP2-deficient cells. The absence of an effect of genipin in cells lacking UCP2 argues against a direct effect of genipin on K_{ATP} channels. In contrast, both 10 mM glucose and tolbutamide closed K_{ATP} channels in both WT and UCP2 $^{-/-}$ cells, indicating that K_{ATP} channels function normally in UCP2-deficient cells. Thus, inhibition of UCP2 by genipin increases mitochondrial membrane potential, increasing the level of ATP, which then closes K_{ATP} channels.

UCP2 is a negative regulator of insulin secretion (Zhang et al., 2001). Thus, deficiency of UCP2 increases insulin secretion in vitro and in vivo (Krauss et al., 2003; Zhang et al., 2001). In the studies above, we show that genipin inhibits UCP2 activity in isolated mitochondria and intact cells. Thus, it is predicted that genipin would stimulate insulin secretion. We therefore assessed whether genipin affects insulin secretion in islets (Figure 4). Genipin dose dependently increased insulin secretion in WT islets (Figure 4, left panel). In contrast, genipin had no effect in islets deficient in UCP2 (Figure 4, right panel). Taken together, these studies show that genipin acutely inhibits UCP2 activity, which then increases mitochondrial membrane potential, increases ATP levels, closes K_{ATP} channels, and consequently, stimulates insulin secretion.

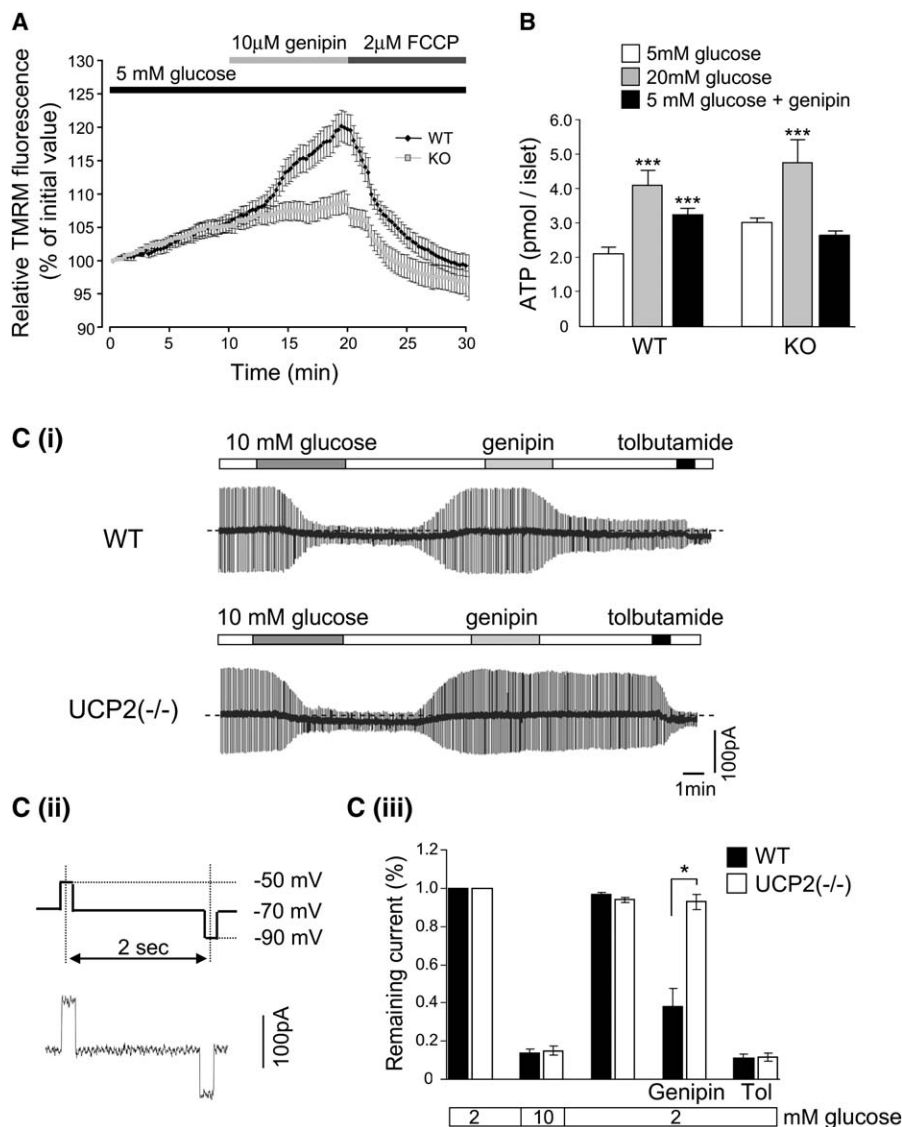


Figure 3. Effects of genipin on islet cell mitochondrial membrane potential and K_{ATP} channel activity

A) Effect of genipin on mitochondrial membrane potential in islet cells. Relative mitochondrial membrane potential was calculated based on TMRM fluorescence in WT and UCP2-deficient dispersed islet cells as described in [Supplemental Data](#). TMRM-loaded cells were imaged for 10 min prior to addition of 10 μM Genipin and then for a further 10 min before and then 10 min after addition of 2 μM FCCCP as indicated. Results are expressed as the means \pm SEM of three individual experiments (≥ 12 cells per experiment).

B) Glucose- and genipin-induced changes in ATP levels in wild-type and UCP2-deficient islets. Following overnight culture, islets were preincubated for 30 min in 5 mM glucose and then incubated again in KRHB buffer containing 5 mM, 20 mM glucose, or 5 mM glucose plus 10 μM genipin. Islet ATP content was determined by luciferin-luciferase bioluminescent assay as described in [Experimental Procedures](#). Results are expressed as means \pm SEM of three individual experiments with three replicates per experiment. $***p < 0.001$, treated versus untreated control (i.e., 5 mM glucose group) (unpaired, two-tailed t test).

C) Effect of glucose (10 mM) and genipin (10 μM) on K_{ATP} channel currents in islet cells. Electrophysiological recordings were performed in the “perforated patch” mode as described in [Experimental Procedures](#). Currents were recorded in response to alternate voltage steps (± 20 mV, 200 ms) from a holding potential of -70 mV. **[C(i)]** Representative current traces recorded in response to alternate voltage steps (± 20 mV, 200 ms) from a holding potential of -70 mV. **[C(ii)]** Two alternate voltage steps (above) and the current responses (below) in a wild-type cell exposed to 2 mM glucose. **[C(iii)]** Summary of K_{ATP} channel currents in response to glucose, genipin, and tolbutamide. Current amplitudes for each condition were averaged over 1–3 min intervals during which current amplitudes were stable. The current amplitude was normalized to its own basal value (2 mM glucose) and was then plotted as remaining current (%). Data represent mean \pm SEM (WT cells, $n = 6$; UCP2 $^{-/-}$ cells, $n = 5$). $*p < 0.05$, wild-type versus UCP2 $^{-/-}$ cells (unpaired, two-tailed t test).

Genipin reverses high glucose- and obesity-induced β cell dysfunction

Impaired secretion of insulin by pancreatic β cells, together with resistance to insulin action, causes type 2 diabetes. A large body of work has established that chronic hyperglycemia and obesity cause loss of glucose-stimulated insulin secretion (GSIS) ([Matthews and Clark, 1997](#); [Robertson, 2000](#)). We have shown that deficiency of UCP2, or removal of endogenously produced superoxide (a UCP2 activator), prevents high glucose- and obesity-induced loss of GSIS ([Krauss et al., 2003](#)). Here, we assessed the effect of genipin on high glucose- and obesity-induced β cell dysfunction. Pancreatic islets were isolated from WT ([Figure 5A](#)) or UCP2 KO mice ([Figure 5B](#)) and subjected to chronic incubations (48 hr) in low (5.5 mM) or high (25 mM) glucose. Following chronic incubation, islets were washed and insulin-secretion studies were performed using three different concentrations of glucose (5.5, 12.5, and 25 mM), with or without genipin present (5 μM).

WT islets incubated chronically at low glucose released increasing amounts of insulin in response to increasing concentrations of glucose ([Figure 5A](#), left panel). Following chronic incubation in high glucose, islets increased basal insulin release but were completely unresponsive to glucose stimulation ([Figure 5A](#), middle panel). These data are consistent with other reports and our previous results ([Krauss et al., 2003](#)) showing that chronic high glucose increases basal insulin secretion and, at the same time, causes complete loss of glucose responsiveness. To assess whether genipin could restore high glucose-induced loss of glucose responsiveness, we acutely added 5 μM genipin to islets during the insulin-release portion of the study. Acute addition of genipin reversed the high glucose-induced block in GSIS ([Figure 5A](#), right panel). To see whether this effect of genipin was mediated by UCP2, islets were isolated from UCP2 KO mice and then incubated in chronic high glucose. Following chronic incubation in low glucose (5.5 mM), UCP2 KO islets, like WT islets, released increasing amounts of insulin in response

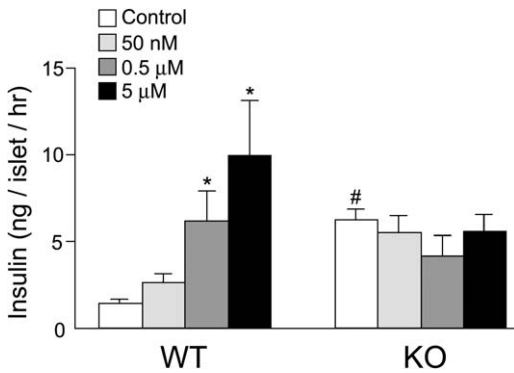


Figure 4. Effect of genipin on insulin secretion from isolated islets

Pancreatic islets were isolated from WT and UCP2 KO mice. Islets were treated with different doses of genipin, which was present during the one hour insulin release study. Concentration of glucose was 5.5 mM. Results are the means \pm SEM of four independent experiments with eight replicates per experiment. * $p < 0.05$, treated versus untreated control; # $p < 0.05$, UCP2-deficient control versus WT control (unpaired, two-tailed t test).

to increasing concentrations of glucose (Figure 5B, left panel). Unlike WT islets, however, islets from UCP2 KO mice, when incubated chronically at 25 mM glucose, retained glucose responsiveness (Figure 5B, middle panel). In addition, genipin reduced the elevation in basal insulin secretion. Their pattern of response was similar to that observed in WT islets incubated with genipin (Figure 5A, right panel). Importantly, in UCP2 KO islets, genipin had no additional beneficial effect on GSIS (Figure 5B, right panel), indicating that improved glucose responsiveness is caused by genipin-dependent inhibition of UCP2.

Because absence of UCP2 in *ob/ob* islets restores glucose sensing, and because removing the activator of UCP2 (superoxide) in dysfunctional islets similarly restores islet function (Krauss et al., 2003; Zhang et al., 2001), we reasoned that genipin (as an inhibitor of UCP2) would reverse obesity-induced β cell dysfunction. Thus, we studied insulin secretion in islets from *ob/ob* mice and from *ob/ob* mice lacking UCP2 in the absence or presence of genipin. Islets from *ob/ob* mice showed elevated basal insulin secretion and loss of glucose responsiveness compared with WT islets (Figure 5C, first set of bars, versus Figure 5A, left panel), similar to what was observed in WT islets following chronic high-glucose treatment (Figure 5A, middle panel). Genipin acutely restored glucose sensing in *ob/ob* islets (Figure 5C, second set of bars). In addition, genipin reduced the elevation in basal insulin secretion. Islets isolated from *ob/ob* mice lacking UCP2 were responsive to glucose (Figure 5C, third set of bars), similar to *ob/ob* islets treated with genipin, and also similar to WT islets treated with genipin (Figure 5A, right panel) or UCP2-deficient islets during exposure to high glucose (Figure 5B, middle panel). Of note, islets from *ob/ob* mice lacking UCP2 treated with genipin did not improve glucose sensing above the level seen in islets lacking UCP2 alone (Figure 5C, last set of bars). Therefore, genipin acutely improves the significant obesity-induced impairment of GSIS.

A genipin derivative, 1, 10-anhydrogenipin (AG), inhibits proton leak, inhibits K_{ATP} channels, and stimulates insulin secretion in a UCP2-dependent manner

Genipin is a naturally occurring protein cross-linking agent (Fujikawa et al., 1988; Huang et al., 1998). To investigate if this action

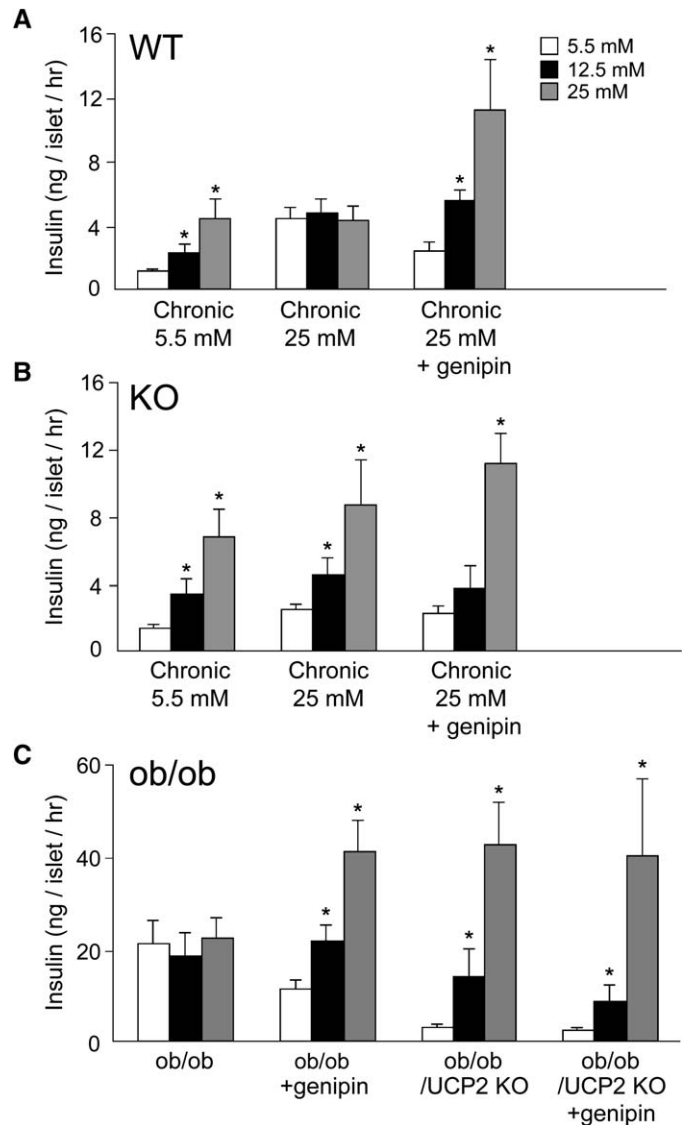


Figure 5. Effect of genipin on chronic high glucose- and obesity-induced β cell dysfunction

Pancreatic islets were isolated from WT (A) or UCP2 KO mice (B) and subjected to chronic incubations at low (5.5 mM) and high (25 mM) glucose. Islets were incubated for a total of 48 hr and washed, and then insulin-secretion studies were performed using three different concentrations of glucose (5.5, 12.5, and 25 mM) with or without 5 μ M genipin. In a separate experiment, islets were isolated from *ob/ob* mice (C). *ob/ob* islets were incubated in RPMI (11 mM glucose) overnight, then washed, and insulin-secretion studies were performed using three different concentrations of glucose (5.5, 12.5, and 25 mM) with or without 5 μ M genipin. In (A) and (B), results are the means \pm SEM of four independent experiments with eight replicates per experiment. In (C), results are the means \pm SEM of 10–20 replicates. * $p < 0.05$, 12.5 mM or 25 mM glucose versus 5.5 mM glucose (unpaired, two-tailed t test).

of genipin is required for inhibition of UCP2, we set out to synthesize a derivative of genipin that lacks cross-linking activity. It has been reported that genipin can be dimerized by equimolar amounts of glycine to form the blue pigment, genipocyanin G1, a highly conjugated dimeric adduct (Figure 6A, structure 1) (Fujikawa et al., 1987). It has also been reported that cytochrome c can be cross-linked by genipin to form oligomers and that the cross-linking process likely involves formation of complexes in which two primary amine groups from separate proteins react

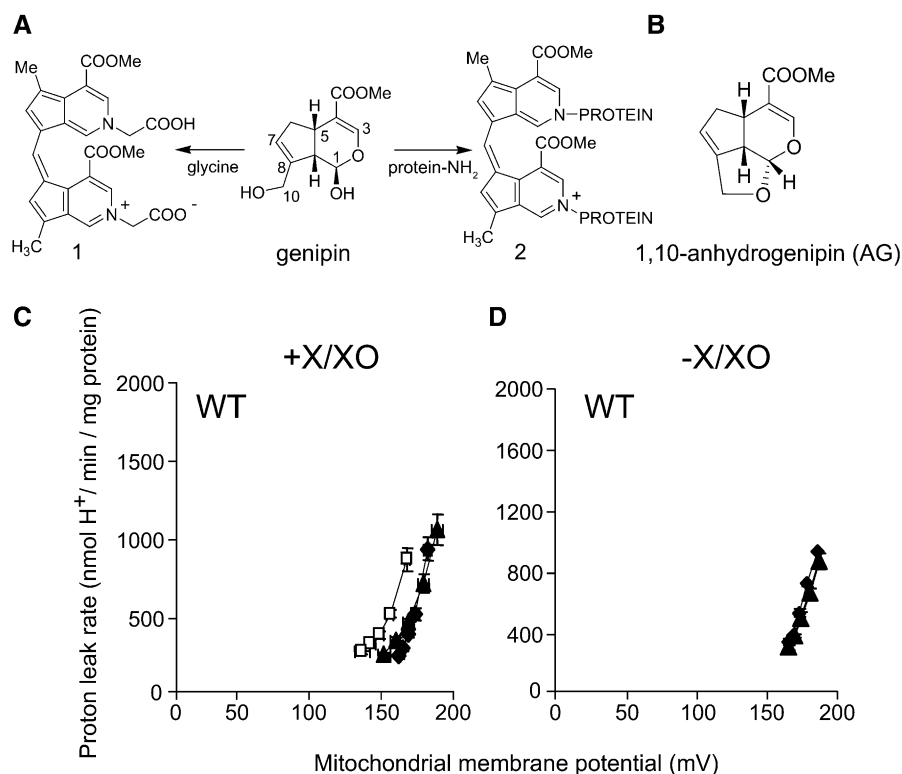


Figure 6. Effects of the genipin derivative, 1, 10-anhydrogenipin on superoxide-dependent proton leak of kidney mitochondria

A) Proposed mechanism by which genipin cross-links proteins, **(B)** chemical structure of the genipin derivative, 1, 10-anhydrogenipin (AG), and **(C and D)** effect of AG on superoxide-dependent proton leak of kidney mitochondria isolated from WT mice. Proton leak titrations were performed in the presence **(C)** and absence **(D)** of a superoxide-generating system (xanthine plus xanthine oxidase) with or without 50 μ M AG. Left panel **(C)**: filled triangles (\blacktriangle), control; open squares (\square), xanthine plus xanthine oxidase (X/XO); filled diamonds (\blacklozenge), AG plus X/XO. Right panel **(D)**: filled triangles (\blacktriangle), control; filled diamonds (\blacklozenge), plus AG. Proton leak data are the means \pm SEM of six to eight replicates.

with genipin (Figure 6A, structure 2) (Fujikawa et al., 1988). As the C10 primary alcohol and the C1 hemiacetal of genipin are likely required for the formation of the oligomeric products (Touyama et al., 1994), it is conceivable that genipin derivatives lacking these two active sites may lose cross-linking activity. To confirm this prediction, a dehydrated derivative, 1,10-anhydrogenipin (AG, Figure 6B) was prepared from genipin. We performed a simple color assay by treating the synthetic derivative, AG, or genipin, with an equimolar amount of glycine in pH 7 buffer at 80°C. After 4 hr, the AG solution, but not the genipin solution, was still clear, which indicates that dimerization to form conjugated genipocyanin G1-type blue pigments did not occur with AG. To confirm that AG lacks protein cross-linking activity, the previously described cytochrome c cross-linking assay was performed (Fujikawa et al., 1988) with genipin and AG. This study, shown in the Supplemental Data available with this article online, revealed that genipin but not AG oligomerizes cytochrome c. Thus, as predicted, AG lacks protein cross-linking activity.

We then assessed the effects of AG on (1) UCP2-mediated proton leak in isolated mitochondria, on (2) K_{ATP} channel activity in dispersed islet cells, and on (3) insulin secretion from isolated islets. As previously observed, superoxide stimulates proton leak (Figure 6C, +X/XO [\square] versus control [\blacktriangle]). As with genipin, AG inhibited superoxide-stimulated proton leak (Figure 6C, +AG, +X/XO [\blacklozenge] versus +X/XO [\square]). Also, like genipin, AG had no effect on proton leak when superoxide was absent (Figure 6D, +AG [\blacklozenge] versus control [\blacktriangle]). As shown in Figure 7A, AG inhibited K_{ATP} channel activity in wild-type cells but not in UCP2-deficient cells. Finally, as shown in Figure 7B, AG stimulated insulin secretion in wild-type islets but not in UCP2-deficient islets. Comparison between the effects of genipin and AG, at the same doses, on K_{ATP} channel activity (10 μ M) and on insulin secretion (5 μ M, genipin data from Figure 4 plotted again in Figure 7B to facilitate

comparison) raises the possibility that AG, while having the same activities as genipin, may be less potent. This apparent decrease in potency could be due to loss of cross-linking activity but more likely is related to other properties of AG that may affect its solubility, its ability to cross membranes, or possibly its interaction with UCP2. Additional studies are necessary to differentiate these possibilities. Despite these issues, these findings suggest that protein cross-linking activity is not required for inhibition of UCP2 by genipin-related compounds.

Discussion

In the present study, we identified a molecule, genipin, which inhibits UCP2-mediated proton leak. In isolated kidney mitochondria, genipin specifically inhibits UCP2-mediated proton leak. In pancreatic islets, genipin increases mitochondrial membrane potential, increases ATP levels, closes plasma membrane K_{ATP} channels, and stimulates insulin secretion in a UCP2-dependent manner. Importantly, acute inhibition of UCP2 by genipin reverses high glucose- and obesity-induced β cell dysfunction.

Prior to this discovery, purine nucleotides (ATP, ADP, GTP, and GDP) were the only known inhibitors of UCPs (Echtay et al., 2001; Jaburek et al., 1999; Zackova et al., 2003). However, purine nucleotides are impermeable to cell membranes and therefore are not useful for studies in whole cells or animals. Superoxide is an important, physiologically relevant regulator of UCPs (Echtay et al., 2003). Recently, it has been proposed that superoxide-dependent activation of UCP-mediated proton leak occurs via aldehydic lipid peroxidation intermediates such as 4-hydroxynonenal (HNE) (Murphy et al., 2003), which have been directly shown to stimulate UCP-dependent proton leak in isolated mitochondria (Echtay et al., 2003). Our results show that genipin inhibits HNE-induced, UCP2-mediated proton

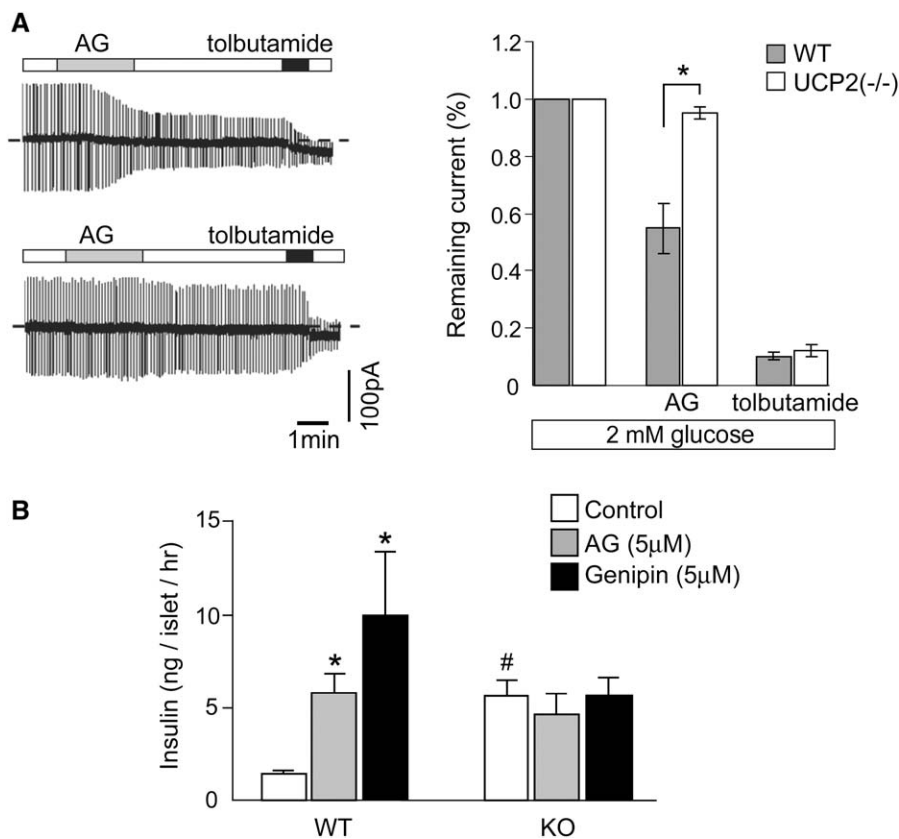


Figure 7. Effects of 1, 10-anhydrogenipin on K_{ATP} channel currents and insulin secretion

A) Effects of AG on K_{ATP} channel currents. Electrophysiological recordings were performed in the “perforated patch” modes as was done in Figure 3C. Left panel: representative tracings from wild-type (WT) and UCP2^{-/-} cells. In sequential order (as indicated), cells were exposed to 2 mM glucose → AG (10 μM) plus 2 mM glucose → 2 mM glucose → tolbutamide (0.2 mM) plus 2 mM glucose → 2 mM glucose. Right panel: summary of K_{ATP} channel currents in response to AG and tolbutamide. Current amplitudes for each condition were averaged over 1–3 min intervals during which current amplitudes were stable. The current amplitude was normalized to its own basal value (2 mM glucose) and was then plotted as remaining current (%). Data represent mean ± SEM (WT cells, n = 5; UCP2^{-/-} cells, n = 4). *p < 0.05, wild-type versus UCP2^{-/-} cells (unpaired, two-tailed t test).

B) Effects of AG on Insulin secretion. Pancreatic islets were isolated from WT and UCP2 KO mice. Islets were treated with vehicle (control), genipin (5 μM) or AG (5 μM) during the 1 hr insulin release study. Concentration of glucose was 5.5 mM. Note, genipin data shown above is from Figure 4 and is plotted again here to facilitate comparison between AG and genipin. Results are the means ± SEM of four independent experiments with eight replicates per experiment. *p < 0.05, treated versus untreated control; #p < 0.05, UCP2-deficient control versus WT control (unpaired, two-tailed t test).

leak, suggesting that genipin does not inhibit UCP2 by interfering with upstream components of the suggested superoxide activation pathway. Thus, we speculate that genipin inhibits UCP2-mediated proton leak by directly interacting with UCP2. Given the high degree of homology between UCP2 and UCP3, genipin may also inhibit UCP3-mediated proton leak. Our preliminary studies indicate that this could be the case. However, additional work is required to confirm this possibility.

Genipin is a naturally occurring cross-linking agent. It is theoretically possible that the cross-linking activity of genipin could be required for inhibition of UCP2. In addition, the cross-linking activity could produce adverse, nonspecific effects due to interaction with other proteins. For these reasons, we synthesized a genipin derivative, AG (Figure 6B), that lacks protein cross-linking activity. Of interest, AG inhibits UCP2-mediated proton leak, closes K_{ATP} channels, and stimulates insulin secretion in a UCP2-dependent fashion. These results suggest that the cross-linking activity of genipin is not required for its biological activity as a UCP inhibitor.

A PubMed (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>) search for papers on genipin suggests a variety of activities for this compound. It is possible that these other activities are secondary to inhibition of UCP2. This possibility could be critically tested by using UCP2-deficient animals and cells to determine if the presence of UCP2 was required for these effects. Alternatively, and more likely given what is known about the biology of UCP2, these other activities are probably unrelated to inhibition of UCP2. This contention is supported by the fact that most of these other activities are observed at concentrations of genipin significantly higher than those employed in the present study. This raises the possibility that these other actions may be medi-

ated by the cross-linking activity of genipin. If this is the case, then derivatives of genipin, like AG, that lack cross-linking activity but retain UCP2-inhibiting activity, could be useful UCP2 inhibitors.

β cells sense glucose through its metabolism and the resulting increase in ATP, which closes K_{ATP} channels depolarizing the cell, increasing Ca^{2+} influx, ultimately stimulating insulin secretion. Uncoupling protein 2 (UCP2) mediates mitochondrial proton leak, decreasing ATP production. It has previously been established that UCP2 negatively regulates glucose-stimulated insulin secretion (Chan et al., 2001; Zhang et al., 2001). Of pathophysiologic significance, UCP2 was markedly upregulated in islets of *ob/ob* mice (Zhang et al., 2001), a model of obesity-induced diabetes. Importantly, *ob/ob* mice lacking UCP2 had restored first-phase insulin secretion, increased serum insulin levels, and greatly decreased levels of glycemia (Zhang et al., 2001). These results establish UCP2 as a critical link between obesity, β cell dysfunction, and type 2 diabetes. Recently, we have demonstrated that endogenously produced superoxide activates UCP2 (Krauss et al., 2003). Interruption of the superoxide-UCP2 pathway improves high glucose- and obesity-induced impairment of glucose-stimulated insulin secretion (GSIS) (Krauss et al., 2003). In the present study, we demonstrate that acute exposure to genipin reverses high glucose- and obesity-induced loss of GSIS. A novel feature of the present study is the rapidity of this effect. Importantly, genipin is without effect in islets lacking UCP2, indicating that genipin’s ability to reverse high glucose- and obesity-induced β cell dysfunction is explained by its ability to inhibit UCP2.

Exposure to either chronic high glucose or the obese state produces an elevation in basal insulin secretion, in addition to

causing loss of glucose sensing. This has previously been observed by us (Krauss et al., 2003) and others (Bjorklund et al., 2000; Patane et al., 2002). Chronic exposure to free fatty acids similarly elevates basal insulin secretion while causing loss of glucose sensing (Joseph et al., 2004). The reason why basal secretion is elevated in these states of β cell dysfunction is not known. It is interesting to note that inhibition of UCP2 by means of gene knockout (Joseph et al., 2004; Krauss et al., 2003), removal of superoxide (Krauss et al., 2003), or genipin (present study) improves both abnormalities (i.e., the increased basal insulin secretion and the loss of glucose-stimulated insulin secretion). Just as it is unknown why these states cause an increase in basal insulin secretion, it is similarly unknown why inhibition of UCP2 corrects this abnormality. One possible explanation is that increased UCP2 activity, associated with states of β cell dysfunction, impairs the ability of the cell to keep cytosolic Ca^{2+} concentrations low. This could happen because increased UCP2 activity reduces ATP levels, which powers Ca^{2+} -ATPases to pump Ca^{2+} out of the β cell and also into the endoplasmic reticulum (Rizzuto and Pozzan, 2006). In addition, increased UCP2 activity reduces the mitochondrial membrane potential, the driving force for Ca^{2+} entry into mitochondria. Consistent with this possibility, it has previously been shown that chronic hyperglycemia markedly elevates basal cytosolic Ca^{2+} concentrations (Bjorklund et al., 2000; Taylor et al., 2005). If this hypothesis is correct, then inhibition of UCP2 in states of β cell dysfunction would be expected to reduce basal cytosolic Ca^{2+} concentrations and hence basal insulin secretion while, at the same time, restoring glucose-stimulated insulin secretion. This hypothesis, regarding the role of increased UCP2 activity in augmenting basal insulin secretion in states of β cell dysfunction, will need to be evaluated in future studies.

Impaired glucose-stimulated insulin secretion by pancreatic β cells, together with resistance to insulin action, causes type 2 diabetes. Current treatment for diabetes mellitus begins with diet and exercise. However, compliance can be poor and, as the disease progresses, treatment with sulfonylureas, thiazolidinediones, or metformin, is often necessary. Of interest, none of these pharmacologic treatments are specifically directed at a pathogenic cause of pancreatic β cell dysfunction. Also, in many patients, these pharmaceutical agents prove inadequate for maintaining blood glucose at an acceptable level and injection with insulin is necessary. Therefore, a need exists for new therapies, and in particular, agents that specifically target pancreatic β cell dysfunction. Given that increased UCP2 activity in pancreatic β cells causes high glucose- and obesity-induced loss of glucose sensing, and that genipin inhibits UCP2 activity and acutely improves high glucose- and obesity-induced β cell dysfunction, genipin or analogs of genipin represent lead compounds that comprise a novel starting point for the development of therapies aimed at a pathogenic cause of pancreatic β cell dysfunction.

Experimental procedures

Isolation of mitochondria, proton leak titrations, and mitochondrial respiration studies

See [Supplemental Data](#) for detailed methodology.

Preparation of dispersed islet cells and imaging of mitochondrial membrane potential

See [Supplemental Data](#) for detailed methodology.

Patch-clamp electrophysiology

Experiments were carried out on dispersed islet cells, using the perforated-patch configuration, which retains cell metabolism intact (Sakura et al., 1998). Currents flowing through K_{ATP} channels were monitored using alternate 20 mV pulses of 200 ms duration from a holding potential of -70 mV. Pulses were applied every 2 s. Whole-cell currents were recorded using a MultiClamp 700B Amplifier and pClamp 9.2 software (Axon Instrument). Recording electrodes had resistances of 2.5–4 M Ω when filled with the following solution: containing 70 mM K_2SO_4 , 10 mM NaCl, 10 mM KCl, 21 mM MgCl_2 , 10 mM HEPES, and 40 mM sucrose (pH 7.2 with KOH). For perforated-patch recordings, 0.15–0.2 mg/ml amphotericin B was added to the pipette solution. The standard external solution consisted of: 140 mM NaCl, 5.6 mM KCl, 1.2 mM MgCl_2 , 2.6 mM CaCl_2 , 10 mM HEPES, and 2 mM glucose (pH 7.4 with NaOH). Bath solutions containing either 2 mM glucose, 10 mM glucose, or 2 mM glucose plus 10 μM genipin, 10 μM AG (1,10-anhydrogenipin), or 0.2 mM tolbutamide were applied to the recording chamber through perfusion. All experiments were carried out at room temperature (22°C–24°C).

ATP measurements

Following overnight culture in RPMI 1640 (11 mM glucose, 7.5% FCS, and 1% penicillin-streptomycin), batches of five size-matched islets were preincubated for 30 min in 1 ml KRHB containing 5 mM glucose and then incubated for 1 hr in 1 ml KRHB containing either 5 or 20 mM glucose or 5 mM glucose plus 10 μM genipin. At the end of this incubation, islets were disrupted with trichloroacetic acid (5% vol/vol final) and vortexed for 10 s. Following a 5 min incubation on ice, extracts were centrifuged (10,000 \times g, 5 min) at 4°C. The supernatant (400 μl) was mixed with 1.5 ml of diethyl ether and the ether phase containing the trichloroacetic acid was discarded. This step was repeated a further three times. Extracts were diluted with 400 μl of buffer containing 20 mM HEPES, 3 mM MgCl_2 (pH 7.75), and stored at -80°C . ATP was measured in duplicate using a luciferin-luciferase bioluminescent assay (Sigma). Light emission was recorded for 30 s using a photon counting luminometer (Victor²; PerkinElmer, Wellesley, Massachusetts). ATP concentration was obtained by comparison to known standards (0–100 pmol) prepared in parallel with the cell extracts.

Islet insulin secretion

Islets were isolated as previously described (Krauss et al., 2003) and incubated overnight in RPMI 1640 (11 mM glucose, supplemented with 7.5% FCS and 1% penicillin-streptomycin). On the next day, islets were transferred into DMEM (5.5 mM glucose plus 0.1% gelatin) with or without genipin (0 nM, 50 nM, 0.5 μM , or 5 μM) or the derivative AG (5 μM). After 1 hr of incubation, medium was removed for measurement of insulin concentrations. Insulin levels in the medium were determined as described previously (Zhang et al., 2001) except that a rat-insulin ELISA kit (Crystal Chem Inc., Chicago, Illinois) was used.

Islet incubation in chronic high glucose

Isolation of islets and incubation conditions

Islets were isolated and incubated overnight as described above. On the next day, all islets were transferred to RPMI 1640 (5.5 or 25 mM glucose, 7.5% FCS) and incubated for 48 hr at 37°C. After chronic incubation, all islets were washed three times with DMEM. Medium-sized islets (of the same size) were used for determination of insulin release. For each of the different conditions (chronic incubation at 5.5 and 25 mM glucose), three islets were transferred to an Eppendorf tube containing 1 ml of fresh DMEM (5.5, 12.5, and 25 mM glucose plus 0.1% gelatin) with or without 5 μM genipin and incubated at 37°C for 1 hr. For each condition in every experiment, 5–8 replicates were studied (i.e., 5–8 Eppendorf tubes, each containing 3 islets). After 1 hr of incubation, an aliquot of medium was removed for insulin assay.

Insulin-secretion studies in *ob/ob* islets

ob/ob mice lacking UCP2 were created as described previously (Zhang et al., 2001; Krauss et al., 2003). Male mice aged 16–20 weeks were used for islet studies. For each genotype, islets from three mice were pooled for insulin-release studies. After being isolated and incubated overnight in RPMI, 11 mM glucose, the islets were washed three times with DMEM and then placed into Eppendorf tubes (1 islet per tube), with each tube containing 1 ml of fresh DMEM (5.5, 12.5, and 25 mM glucose plus 0.1% gelatin) with or without 5 μM genipin, and incubated at 37°C for 1 hr. Islets of similar size were

used for determination of insulin release with 5–10 replicates analyzed per condition. Multiple experiments were performed. The results of a representative experiment are shown in Figure 5C.

Genipin and 1,10-anhydrogenipin

Genipin was obtained from Wako Chemicals USA (<http://www.wakouusa.com>, catalog # 078-0302). Preparation of 1,10-anhydrogenipin (AG): Genipin (5 g, 22.1 mmol) and triphenylphosphine (6085 mg, 23.2 mmol) were dissolved in 110 ml of distilled methylene chloride. To the solution at 0°C was added diisopropyl azodicarboxylate (4.60 ml, 23.2 mmol) dropwise. The resulting mixture was stirred at the same temperature for 30 min. Concentration in vacuo and purification on silica gel using hexane/EtOAc (10:1–4:1) as eluant afforded 1.9 g of AG as a white solid (41% yield, >99% purity). IR (neat): 2950, 1700, 1634, 1437, 1287, 1189, 1075, 1038, 891 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3): δ 7.48 (1 H, s), 5.54 (1H, br s), 5.30 (1H, d, $J = 4.4$ Hz), 4.45 (1H, br d, $J = 12.8$ Hz), 4.36 (1H, br d, $J = 12.8$ Hz), 3.71 (3H, s), 3.62 (1H, br s), 3.27 (1H, dd, $J = 14.4, 6.8$ Hz), 3.22 (1H, br d, $J = 16.0$ Hz), 2.69 (1H, br d, $J = 16.0$ Hz) ppm; ^{13}C NMR (100 MHz, CDCl_3): δ 167.7, 153.0, 143.3, 121.6, 107.7, 96.0, 64.7, 54.5, 51.0, 46.2, 32.1 ppm; HRMS (CI): calculated. for $\text{C}_{11}\text{H}_{12}\text{O}_4$ 208.0736, found 208.0696.

Supplemental data

Supplemental data include Supplemental Experimental Procedures, one figure, and Supplemental References and can be found with this article online at <http://www.cellmetabolism.org/cgi/content/full/3/6/417/DC1/>.

Acknowledgments

This work was supported by grants from the National Institutes of Health (to B.B.L.) and the American Diabetes Association (Junior Faculty Award), National Natural Science Foundation of China (Outstanding Young Scientist Award 30225037), IRP0430, and National Basic Research Foundation of China (973 Program, 2004CB518603) (to C.-Y.Z.). J.A.P., Jr. thanks the National Institutes of Health (GM-62842) and Bristol-Myers Squibb (Unrestricted Grant in Synthetic Organic Chemistry) for research support.

Received: September 1, 2005

Revised: December 3, 2005

Accepted: April 10, 2006

Published: June 6, 2006

References

- Bjorklund, A., Lansner, A., and Grill, V.E. (2000). Glucose-induced $[\text{Ca}^{2+}]_i$ abnormalities in human pancreatic islets: important role of overstimulation. *Diabetes* 49, 1840–1848.
- Chan, C.B., De Leo, D., Joseph, J.W., McQuaid, T.S., Ha, X.F., Xu, F., Tsushima, R.G., Pennefather, P.S., Salapatek, A.M., and Wheeler, M.B. (2001). Increased uncoupling protein-2 levels in beta-cells are associated with impaired glucose-stimulated insulin secretion: mechanism of action. *Diabetes* 50, 1302–1310.
- Danbuo, Y. (1984). *Zabing Guagayao*, Second edition. Y. Wang, ed. (P. R. China: People's Health Publishers), p. 414.
- Djerassi, C., Nakano, T., James, A.N., Zalkow, L.H., Eisenbraun, E.J., and Shooley, J.N. (1961). Terpenoid. XLVII. The structure of genipin. *J. Org. Chem.* 26, 1192–1206.
- Echtay, K.S., Esteves, T.C., Pakay, J.L., Jekabsons, M.B., Lambert, A.J., Portero-Otin, M., Pamplona, R., Vidal-Puig, A.J., Wang, S., Roebuck, S.J., and Brand, M.D. (2003). A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. *EMBO J.* 22, 4103–4110.
- Echtay, K.S., Roussel, D., St-Pierre, J., Jekabsons, M.B., Cadenas, S., Stuart, J.A., Harper, J.A., Roebuck, S.J., Morrison, A., Pickering, S., et al. (2002). Superoxide activates mitochondrial uncoupling proteins. *Nature* 415, 96–99.
- Echtay, K.S., Winkler, E., Frischmuth, K., and Klingenberg, M. (2001). Uncoupling proteins 2 and 3 are highly active H(+) transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone). *Proc. Natl. Acad. Sci. USA* 98, 1416–1421.
- Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyrueis, C., Bouillaud, F., Seldin, M.F., Surwit, R.S., Ricquier, D., and Warden, C.H. (1997). Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat. Genet.* 15, 269–272.
- Fujikawa, S., Fukui, Y., and Koga, K. (1987). Structure of genipocyanin G1, a spontaneous reaction product between genipin and glycine. *Tetrahedron Lett.* 28, 4699–4700.
- Fujikawa, S., Nakamura, S., and Koga, K. (1988). Genipin, a new type of protein crosslinking reagent from gardenia fruits. *Agric. Biol. Chem.* 52, 869–870.
- Huang, L.L., Sung, H.W., Tsai, C.C., and Huang, D.M. (1998). Biocompatibility study of a biological tissue fixed with a naturally occurring crosslinking reagent. *J. Biomed. Mater. Res.* 42, 568–576.
- Jaburek, M., Varecha, M., Gimeno, R.E., Dembski, M., Jezek, P., Zhang, M., Burn, P., Tartaglia, L.A., and Garlid, K.D. (1999). Transport function and regulation of mitochondrial uncoupling proteins 2 and 3. *J. Biol. Chem.* 274, 26003–26007.
- Joseph, J.W., Koshkin, V., Saleh, M.C., Sivitz, W.I., Zhang, C.Y., Lowell, B.B., Chan, C.B., and Wheeler, M.B. (2004). Free fatty acid-induced beta-cell defects are dependent on uncoupling protein 2 expression. *J. Biol. Chem.* 279, 51049–51056.
- Joseph, J.W., Koshkin, V., Zhang, C.Y., Wang, J., Lowell, B.B., Chan, C.B., and Wheeler, M.B. (2002). Uncoupling protein 2 knockout mice have enhanced insulin secretory capacity after a high-fat diet. *Diabetes* 51, 3211–3219.
- Kassis, N., Bernard, C., Pusterla, A., Casteilla, L., Penicaud, L., Richard, D., Ricquier, D., and Ktorza, A. (2000). Correlation between pancreatic islet uncoupling protein-2 (UCP2) mRNA concentration and insulin status in rats. *Int. J. Exp. Diabetes Res.* 1, 185–193.
- Krauss, S., Zhang, C.Y., and Lowell, B.B. (2002). A significant portion of mitochondrial proton leak in intact thymocytes depends on expression of UCP2. *Proc. Natl. Acad. Sci. USA* 99, 118–122.
- Krauss, S., Zhang, C.Y., Scorrano, L., Dalgaard, L.T., St-Pierre, J., Grey, S.T., and Lowell, B.B. (2003). Superoxide-mediated activation of uncoupling protein 2 causes pancreatic beta cell dysfunction. *J. Clin. Invest.* 112, 1831–1842.
- Krempler, F., Esterbauer, H., Weitgasser, R., Ebenbichler, C., Patsch, J.R., Miller, K., Xie, M., Linnemayr, V., Oberkofler, H., and Patsch, W. (2002). A functional polymorphism in the promoter of UCP2 enhances obesity risk but reduces type 2 diabetes risk in obese middle-aged humans. *Diabetes* 51, 3331–3335.
- Laybutt, D.R., Sharma, A., Sgroi, D.C., Gaudet, J., Bonner-Weir, S., and Weir, G.C. (2002). Genetic regulation of metabolic pathways in beta-cells disrupted by hyperglycemia. *J. Biol. Chem.* 277, 10912–10921.
- Matthews, D.R., and Clark, A. (1997). *Beta-cell defects and pancreatic abnormalities in non-insulin-dependent diabetes mellitus* (Oxford, United Kingdom: Blackwell Science).
- Murphy, M.P., Echtay, K.S., Blaikie, F.H., Asin-Cayuela, J., Cocheme, H.M., Green, K., Buckingham, J.A., Taylor, E.R., Hurrell, F., Hughes, G., et al. (2003). Superoxide activates uncoupling proteins by generating carbon-centered radicals and initiating lipid peroxidation: studies using a mitochondria-targeted spin trap derived from alpha-phenyl-N-tert-butyl nitron. *J. Biol. Chem.* 278, 48534–48545.
- Patane, G., Anello, M., Piro, S., Vigneri, R., Purrello, F., and Rabuazzo, A.M. (2002). Role of ATP production and uncoupling protein-2 in the insulin secretory defect induced by chronic exposure to high glucose or free fatty acids and effects of peroxisome proliferator-activated receptor-gamma inhibition. *Diabetes* 51, 2749–2756.
- Pecqueur, C., Alves-Guerra, M.C., Gelly, C., Levi-Meyrueis, C., Couplan, E., Collins, S., Ricquier, D., Bouillaud, F., and Miroux, B. (2001). Uncoupling protein 2, in vivo distribution, induction upon oxidative stress, and evidence for translational regulation. *J. Biol. Chem.* 276, 8705–8712.

- Rizzuto, R., and Pozzan, T. (2006). Microdomains of intracellular Ca²⁺: molecular determinants and functional consequences. *Physiol. Rev.* 86, 369–408.
- Robertson, R.P. (2000). Glucose toxicity of the beta cell: cellular and molecular mechanisms. In *Diabetes mellitus. A fundamental and clinical text*, S.T.D. Le Roith and J.M. Olefsky, eds. (Philadelphia, Pennsylvania: Lippincott Williams & Wilkins), pp. 125–132.
- Sakura, H., Ashcroft, S.J., Terauchi, Y., Kadowaki, T., and Ashcroft, F.M. (1998). Glucose modulation of ATP-sensitive K-currents in wild-type, homozygous and heterozygous glucokinase knock-out mice. *Diabetologia* 41, 654–659.
- Sasahara, M., Nishi, M., Kawashima, H., Ueda, K., Sakagashira, S., Furuta, H., Matsumoto, E., Hanabusa, T., Sasaki, H., and Nanjo, K. (2004). Uncoupling protein 2 promoter polymorphism -866G/A affects its expression in beta-cells and modulates clinical profiles of Japanese type 2 diabetic patients. *Diabetes* 53, 482–485.
- Sesti, G., Cardellini, M., Marini, M.A., Frontoni, S., D'Adamo, M., Del Guerra, S., Lauro, D., De Nicolais, P., Sbraccia, P., Del Prato, S., et al. (2003). A common polymorphism in the promoter of UCP2 contributes to the variation in insulin secretion in glucose-tolerant subjects. *Diabetes* 52, 1280–1283.
- Taylor, J.T., Huang, L., Keyser, B.M., Zhuang, H., Clarkson, C.W., and Li, M. (2005). Role of high-voltage-activated calcium channels in glucose-regulated beta-cell calcium homeostasis and insulin release. *Am. J. Physiol. Endocrinol. Metab.* 289, E900–E908.
- Touyama, R., Inoue, K., Takeda, Y., Yatsuzuka, M., Ikumoto, T., Moritome, N., Shingu, T., Yoloi, T., and Inouye, H. (1994). *Chem. Pharm. Bull. (Tokyo)* 42, 1571–1578.
- Vidal-Puig, A., Solanes, G., Grujic, D., Flier, J.S., and Lowell, B.B. (1997). UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. *Biochem. Biophys. Res. Commun.* 235, 79–82.
- Wang, X. (1991). *Zhongyi Neike Bianzheng Xue*, First edition. C. Li, ed. (P. R. China: People's Health Publishers), pp. 238–241.
- Winzell, M.S., Svensson, H., Enerback, S., Ravnskjaer, K., Mandrup, S., Esser, V., Arner, P., Alves-Guerra, M.C., Miroux, B., Sandler, F., et al. (2003). Pancreatic beta-cell lipotoxicity induced by overexpression of hormone-sensitive lipase. *Diabetes* 52, 2057–2065.
- Zackova, M., Skobisova, E., Urbankova, E., and Jezek, P. (2003). Activating omega-6 polyunsaturated fatty acids and inhibitory purine nucleotides are high affinity ligands for novel mitochondrial uncoupling proteins UCP2 and UCP3. *J. Biol. Chem.* 278, 20761–20769.
- Zhang, C.Y., Baffy, G., Perret, P., Krauss, S., Peroni, O., Grujic, D., Hagen, T., Vidal-Puig, A.J., Boss, O., Kim, Y.B., et al. (2001). Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell* 105, 745–755.
- Zhao, H., Kalivendi, S., Zhang, H., Joseph, J., Nithipatikom, K., Vasquez-Vivar, J., and Kalyanaraman, B. (2003). Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic. Biol. Med.* 34, 1359–1368.