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GABA protects pancreatic beta cells against apoptosis by increasing SIRT1 expression and activity

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ABSTRACT

We have previously shown that GABA protects pancreatic islet cells against apoptosis and exerts anti-inflammatory effects. Notably, GABA inhibited the activation of NF-κB in both islet cells and lymphocytes. NF-κB activation is detrimental to beta cells by promoting apoptosis. However, the mechanisms by which GABA mediates these effects are unknown. Because the above-mentioned effects mimic the activity of sirtuin 1 (SIRT1) in beta cells, we investigated whether it is involved. SIRT1 is an NAD^+-dependent deacetylase that enhances insulin secretion, and counteracts inflammatory signals in beta cells. We found that the incubation of a clonal beta-cell line (rat INS-1) with GABA increased the expression of SIRT1, as did GABA receptor agonists acting on either type A or B receptors. NAD^+ (an essential cofactor of SIRT1) was also increased. GABA augmented SIRT1 enzymatic activity, which resulted in deacetylation of the p65 component of NF-κB, and this is known to interfere with the activation this pathway. GABA increased insulin production and reduced drug-induced apoptosis, and these actions were reversed by SIRT1 inhibitors. We examined whether SIRT1 is similarly induced in newly isolated human islet cells. Indeed, GABA increased both NAD^+ and SIRT1 (but not sirtuins 2, 3 and 6). It protected human islet cells against spontaneous apoptosis in culture, and this was negated by a SIRT1 inhibitor. Thus, our findings suggest that major beneficial effects of GABA on beta cells are due to increased SIRT1 and NAD^+, and point to a new pathway for diabetes therapy.

1. Introduction

Gamma-aminobutyric acid (GABA) therapy markedly attenuates insulitis and systemic inflammation in mice [1–3]. Furthermore, we and others [1,4–6] have reported that GABA prevents the apoptosis of both rodent and human islet β cells. We investigated the mechanisms of action of GABA and established that it inhibits NF-κB activation [4]. This is highly important because NF-κB activation initiates both innate and adaptive immunity [7], and it promotes apoptosis of β cells [8–11]. Thus, recent findings point to GABA as a major drug to protect β cells against injury, and to inhibit inflammation and autoimmunity. However, it is unclear how it mediates these effects.

Interestingly, the effects of GABA treatment have many similarities with the actions of sirtuin 1 (SIRT1) in the endocrine pancreas. SIRT1 is an NAD^+-dependent deacetylase expressed in both the nucleus and the cytoplasm, which exerts positive effects in pancreatic β cells [12–15]. It protects these cells against oxidative stress and cytokine toxicity [16–19]. Notably, it inhibits NF-κB signaling by directly deacetylating the p65 subunit of NF-κB complex [14,20,21]. The importance of SIRT1 is highlighted by the finding

Abbreviations: Ac-p65, acetylated p65 (NF-kappaB component); AROS, active regulator of SIRT1; GABA, gamma-aminobutyric acid; $GABA_AR$, GABA type A receptor; $GABA_BR$, GABA type B receptor; SIRT1, sirtuin 1; STZ, streptozotocin.

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that patients with a SIRT1 mutation developed type 1 diabetes (T1D) [22]. Notably, conditional knockout of SIRT1 in mice results in β-cell dysfunction and reduces insulin secretion [23], whereas overexpression increases insulin secretion [24]. We hypothesized that GABA actions in β cells are mediated by this enzyme, and the goal of this study was to investigate this hypothesis.

The activity of SIRT1 is dependent on the availability of its cofactor, NAD^+ [12,17,18]. Thus, NAD^+ levels must be taken into consideration when assessing sirtuin activity. Pancreatic β cells are highly sensitive to metabolic stress, and sirtuins act as energy/nutrient stress sensors by responding to NAD^+ levels [17]. The NAD^+ level is subject to complex regulation, and is reduced in aged individuals [17].

Here, we found that GABA increases both the expression of SIRT1 and the levels of NAD^+ in a rat insulinoma cell line (INS-1) and in freshly isolated human islet cells. We document that SIRT1 plays a key role in mediating the beneficial effects of GABA on these cells, including increased insulin secretion and reduced susceptibility to apoptosis under various conditions. This points to GABA as a major inducer of SIRT1 in β cells, which protects against β-cell stress and potentially diabetes.

2. Materials and methods

2.1. Human islets isolation

Pancreata from deceased human donors were retrieved after consent was obtained by Transplant Quebec (Montreal, Canada). Human islets were isolated as described [4].

2.2. Cell culture

Islets were cultured in CMRL 1066 medium (5.56 mmol/l glucose) with 10% heat-inactivated FBS and glutamine, in ventilated Eppendorf tubes. Rat insulinoma cells INS-1 were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, HEPES and β-mercaptoethanol. Agonists and antagonists of GABA receptors type A ($GABA_AR$) and type B ($GABA_BR$) were from Sigma. Picrotoxin ($GABA_AR$ antagonist; 100 μM), saclofen ($GABA_BR$ antagonist; 100 μM), and muscimol ($GABA_AR$ agonist; 20 μM) were employed as described in our previous publications (1, 4, 5). Baclofen ($GABA_BR$ agonist) was applied to cultures at a concentration of 100 μM.

The following were added to cultures where indicated: A specific inhibitor of SIRT1, EX527 (200 nM); a selective inhibitor of SIRT1/SIRT2, sirtinol (4 μM); and an inhibitor of non-NAD^+-dependent histone deacetylases, panobinostat (20 nM).These compounds were all purchased from Selleckchem (Houston, TX), and were added at inhibitory concentrations as described by the manufacturer or in the literature.

2.3. Apoptosis

Apoptotic and live cells were identified by flow cytometric analysis by Annexin V and propidium iodide staining, as we have described [4]. Live cells are negative for both stains, whereas apoptotic cells are positive for annexin V.

2.4. qPCR for SIRT1

Human pancreatic islets were treated (or not) with 100 μM GABA in vitro for 2 h before RNA extraction using Qiagen's RNA-Easy extraction kit. After testing RNA quality in Agilent 2100 Bioanalyzer, the samples were converted to cDNA using SABiosciences (QIAGEN Inc., Toronto, Canada) First Strand kit and processed in qPCR. Human gene primers were designed using Primer 3 tool: SIRT1, sense GGACATGCCAGAGTCCAAGT, antisense GCTGGTGGAACAATTCCTGT; SIRT2, sense ATAACCCACACCCAGCG TAG, antisense AATGTCTTCTGCCCATCCAG; SIRT3, sense CATGA GCTGCAGTGACTGGT, antisense GAGCTTGCCGTTCAACTAGG; SIR T6, sense GCCTCTGACTTGCTGTGTTG, antisense AAGGCAGTGCAAG CCTCTAC; AROS, sense GAAGACGAAGGCAATTCAGG, antisense CTGCTGGCTCACAGACTCAG; HPRT1, sense ACGTCTTGCTCGAGAT GTGA, antisense, AATCCAGCAGGTCAGCAAAG; RPLP0, sense TCG ACAATGGCAGCATCTAC, antisense ATCCGTCTCCACAGACAAGG.

Expression of the house-keeping genes RPLP0 and HPR1 was used for normalizing the expression data of sirtuins and AROS. The data was calculated as ΔΔCt, with a twofold increase in expression considered positive.

2.5. Western blotting

The effect of GABA on the SIRT1 protein expression was evaluated by Western blotting using anti-SIRT1 and anti-β-actin mouse monoclonal antibodies from Abcam (Toronto, Canada) and anti-mouse IgG-HRP from Promega (Madison, WI). The cells were exposed to 100 μM GABA or specific agonists of $GABA_AR$ or $GABA_BR$ for 48 h with daily refreshment of the medium (including GABA or other agonists).

2.6. NAD^+ assay

NAD^+ levels were measured according to the protocol of the CycLex NAD^+/NADH Colorimetric Assay kit (Cedarlane, Toronto, Canada), with slight modifications. Briefly, cultured INS-1 cells or human islets were treated with or without GABA for 45 min and NAD^+ was extracted as recommended by the manufacturer. Briefly, cells ($1–5 \times 10^6$ cells) were extracted with 0.5 M $HClO_4$, incubated on ice for 30 min, and the precipitated protein was pelleted and used in protein assay. The supernatant was neutralized with 0.8 M potassium carbonate after removing the pelleted protein. The precipitated potassium perchlorate was removed by centrifugation and the clear extract was analyzed in enzyme assay within 2 h. The spectrophotometric assay was based on the enzymatic reduction of NAD+ into NADH by alcohol dehydrogenase. The enzyme assay data was normalized either by the corresponding protein concentration (in islets) or by the cell number (INS-1).

2.7. NF-κB deacetylation

SIRT1 removes an acetyl group from RelA p65. Enzyme activity was based on the estimation of the number of Acetyl-p65 (Ac-p65)-positive cells by flow cytometry. INS-1 cells were treated with 20 nM panobinostat to inhibit histone deacetylases overnight. The next day they were challenged with TNFα (3 ng/ml) for 30 min to activate NF-κB, then treated (or not) with 100 μM GABA in the presence or absence of SIRT1 inhibitor EX527 added at 200 nM. After 3 h the cells were harvested, fixed-permeabilized using a kit from BD Biosciences, and stained for SIRT1 and Ac-p65. Rabbit polyclonal anti-NF-κB p65 (acetyl K310) and mouse monoclonal anti-SIRT1 (both from Abcam, Toronto, Canada) react with mouse, rat, and human proteins. The numbers of Ac-$p65^+SIRT1^+$ and Ac-$p65^-SIRT1^+$ cells were compared by flow cytometry.

2.8. Insulin secretion

INS-1 cells were serum-starved overnight, rinsed, and treated or not with 100 μM GABA and 200 nM SIRT1 inhibitor EX527. The glucose concentrations were 1.4 and 11 mM. The conditioned medium was harvested after 1 h and insulin release was measured using an ELISA kit from Mercodia Inc. (Winston Salem, NC, USA).

2.9. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 program (GraphPad Software, Inc., California). The data are presented as the mean ± SEM, and the difference between groups was analyzed by ANOVA, and $p < 0.05$ was considered significant.

3. Results and discussion

3.1. GABA and other GABA receptor agonists increase the expression of SIRT1

Our previous findings revealed an anti-apoptotic and anti-inflammatory role of GABA on islet cells, in vitro and in vivo [1,4,5], but the molecular mechanisms behind these effects are unknown. Here, we examined the potential role of SIRT1. The activity of SIRT1 is regulated by several factors, including its level of expression and degradation, the availability of its essential substrate NAD^+, and in some cells the presence AROS [17,18,25]. Here, we studied the effect of GABA on these parameters.

GABA treatment increased the levels of SIRT1 protein in INS-1 cells, as detected by Western blotting (Fig. 1A and B). SIRT1 was induced by treatment with either GABA, muscimol that is a specific GABA type A receptor ($GABA_AR$) agonist, or baclofen that is a specific GABA type B receptor ($GABA_BR$) agonist. This suggests that both types of GABA receptor mediate this effect.

3.2. GABA treatment elevates the level of NAD^+ in INS-1 cells

The activity of SIRT1 depends on the availability of its rate-limiting substrate, NAD^+. We found that GABA-treatment of INS-1 cells significantly increased intracellular NAD+ levels (Fig. 2A and B). Interestingly, this effect was blocked by both a $GABA_AR$ antagonist (picrotoxin) and a $GABA_BR$ antagonist (saclofen) (Fig. 2B), suggesting that both receptors are involved.

Thus, two essential conditions for the increased activity of SIRT1 are met in the GABA-treated cells, i.e., increased SIRT1 protein and increased supply of its NAD^+ cofactor.

3.3. GABA treatment activates SIRT1

In these experiments, we demonstrate that SIRT1 deacetylase enzyme activity is increased by GABA. Activation of SIRT1 should result in deacetylation of the protein substrates of this enzyme, such as the p65 component of the transcription factor NF-κB. Deacetylation of p65 inhibits NF-κB pathway activation [21]. Here, we used a flow cytometric method to detect acetylated p65 (Ac-p65). We observed that GABA treatment of INS-1 cells promoted deacetylation of the p65 NF-κB component, such that a much larger number of cells exhibited negative staining for the acetylated form (Fig. 2C). This effect appears to depend on SIRT1 activity, because it is completely reversed by EX527, a specific inhibitor of SIRT1 (Fig. 2C). Note that in these experiments the cells were pre-treated with the inhibitor panobinostat to block histone deacetylases that are independent of NAD^+ (HDACs). Similar results were observed in cells treated with SRT1720, a low-molecular weight activator of SIRT1, in the absence of GABA (not shown). Previously we have reported that GABA exerts anti-inflammatory effect and inhibits the activity of NF-κB [4]. The current findings suggest that SIRT1 was responsible for the inactivation of this pathway.

Because activation of NF-κB has been linked to the apoptosis of β cells, SIRT1 activation may represent an important protective pathway against inflammatory mediators. To our knowledge this is the first report that GABA can inhibit NF-κB in a SIRT1-dependent fashion, and it may have important therapeutic implications. It is possible that GABA also protects β cells against apoptosis by other mechanisms (not NF-κB related), since we have demonstrated the activation of the PI3K-Akt and CREB-IRS-2 proliferation and survival signaling pathways [5]. Establishing the relative role these pathways in β-cell apoptosis requires further investigation.

3.4. The anti-apoptotic effects of GABA depend on the activity of SIRT1

As noted previously, the ability of GABA to protect β cells against apoptosis is well documented. Here, we aimed to demonstrate that this anti-apoptotic activity is dependent on SIRT1. We show that GABA prevents apoptosis of INS-1 cells in response to tacrolimus (FK506; a commonly used immunosuppressive drug) (Fig. 3A). We have previously demonstrated the toxic effects of tacrolimus on islet cells [4], and this is relevant to islet transplantation because this drug has been used to prevent rejection. GABA also reduced apoptosis induced by streptozotocin (STZ) (Fig. 3B). Notably, STZ causes severe oxidative stress in rodent β cells leading to apoptosis, and it is interesting that this was partially reversed. Two inhibitors of SIRT1, EX527 and sirtinol, negated the protective effect of GABA against apoptosis (Fig. 3A and B), supporting a role for SIRT1 in the anti-apoptotic effect.

3.5. GABA stimulates insulin release

Brief exposure of INS-1 cells to GABA induced release of insulin at a low glucose concentration (Fig. 3C). This effect was not related to a change in cell viability due to the short incubation time. Inhibition of SIRT1 with EX527 completely abolished the effect of GABA. We have previously shown that GABA increases insulin secretion [4,5]. Our current work suggests that increased

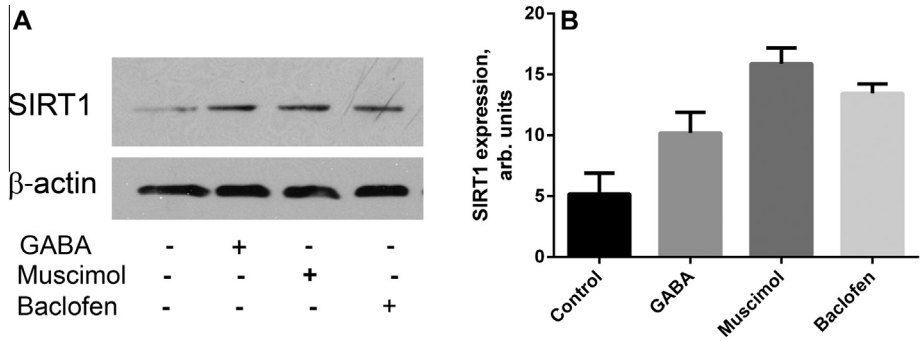

Fig. 1. GABA increases SIRT1 protein expression in INS-1 cells. (A) Western blot shows that GABA and agonists of GABA type A receptor (muscimol) and GABA type B receptor (baclofen) increase SIRT1 protein in INS-1 cells treated with 100 μM of GABA or the other agonists for 48 h. The image is representative of the two independent experiments. (B) Quantification of SIRT1 expression by INS-1 cells, expressed in arbitrary units as the mean ± SEM. All GABA receptor agonists increased SIRT1 expression ($p < 0.05$ versus control), indicating that both types of GABA receptors mediate this effect. Two experiments yielded similar results.

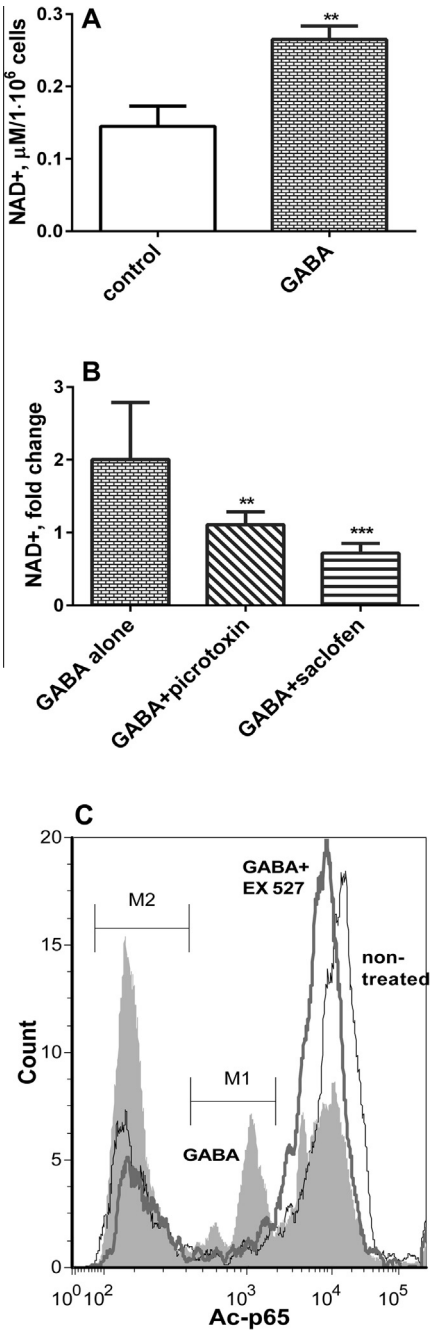

Fig. 2. GABA elevates the level NAD$^+$ in pancreatic cells and promotes activation of SIRT1. (A) INS-1 cells were treated or not with 100 μM GABA for 45 min and NAD$^+$ was extracted as described in Section 2. The data represent the mean ± SEM ($^{**}p < 0.01$for GABA versus control), and is representative of 5 independent experiments. (B) Performed as above, but the cells were pre-treated (or not) with antagonists of the GABA receptors, i.e., picrotoxin (type A receptor) or saclofen (type B receptor). The data are presented as a fold change over the level of NAD$^+$ in the corresponding control cells lacking GABA but having all other components, and represent the mean ± SEM of two independent experiments ($^{**}p < 0.01$ and $^{***}p < 0.001$ versus GABA alone). (C) Flow cytometric analysis of NF-κB p65 deacetylation. INS-1 cells were pretreated with TNF-α to activate NF-κB. The cells were then treated with 100 μM GABA alone (gray filled) or with 200 nM EX527 (gray line) for 16 h. The black line represents cells treated only with TNF-α. GABA increases the number of cells negative for acetylated p65 (Ac-p65), while decreasing the number of bright Ac-p65$^+$ cells. The SIRT1 inhibitor EX527 eliminates the effect of GABA. Thus, GABA increases deacetylation of NF-κB p65, which is known to inactivate NF-κB, and this is blocked by the SIRT1 inhibitor. The data are representative of two independent experiments.

GABA-induced insulin secretion is dependent on SIRT1 activity. The exact pathways leading from GABA receptor activation, increased SIRT1 activity and insulin production are likely complex and merit further investigation.

3.6. GABA induces SIRT1 and NAD$^+$ in human islets cells, and this protects against spontaneous apoptosis

The studies outlined above demonstrate a SIRT1 dependency in a clonal β-cell line. However, it was important to demonstrate that human islet cells are similarly responsive. SIRT1 is by far the sirtuin that has been most closely linked to metabolic disorders and diabetes. However, we also wished to examine the expression of other sirtuins, notably SIRT2, SIRT3 and SIRT6 that also appear to play a role in metabolism [13,14]. To examine multiple sirtuins, we incubated human islet cells with GABA and performed qPCR analysis for levels of mRNA (Fig. 4A). We found significant elevation of SIRT1, whereas SIRT2, 3, 6 were not elevated or even slightly depressed. Furthermore, we observed that the expression of AROS, an activator of SIRT1 in some cells, was also enhanced. This suggests that GABA stimulation has some selectivity for SIRT1.

We examined whether GABA could increase NAD+, and it did in a way similar to INS-1 cells (Fig. 4B). We also examined apoptosis, by an assay we have previously utilized [4]. Human islet cells undergo apoptosis spontaneously in culture, and this can be detected by staining with Annexin V and analyzed by flow cytometry. Here, as reported previously [4], the addition of GABA to cultures prevented apoptosis (Fig. 4C). Note that GABA is degraded in culture and was replaced daily. A SIRT1 inhibitor (EX527) almost completely negated the anti-apoptotic activity of GABA (Fig. 4C). Thus, our findings in human islet cells closely match those obtained with INS-1 cells, in terms of SIRT1 enhancement and activity.

In summary, this study demonstrates that GABA enhances SIRT1 and NAD$^+$ levels in islet β cells. This was apparent in both a clonal β-cell line (INS-1), and freshly isolated human islet cells. Importantly, our findings suggest that some previously reported effects of GABA on β cells are due to increased SIRT1 activity. This includes the ability of GABA to protect against apoptosis and to increase insulin secretion. The enhancement of SIRT1 expression appears to be dependent on both $GABA_AR$ and $GABA_BR$ activation, since specific agonists of these receptors have similar activity. Furthermore, specific antagonists of either $GABA_AR$ or $GABA_BR$ were able to reduce GABA-mediated responses. The fact that both SIRT1 and NAD$^+$ are elevated is important, since the activity of this enzyme is dependent on the availability of NAD$^+$. A key observation from this study relates to the prevention of NF-κB activation. We had previously reported this activity, but the mechanism was unknown. Our data suggests that GABA treatment inactivates NF-κB through the SIRT1-mediated deacetylation of the NF-κB p65 component.

A question for future consideration is how the effects of GABA compare to well-known SIRT1 activators such as resveratrol and synthetic compounds. In this respect, the fact that GABA acts through cell-surface receptors is of some interest. Outside the brain, GABA receptors are expressed by a restricted number of cell types, such as islet cells, lymphocytes and hepatocytes [26–28]. Thus, increased SIRT1 activity in response to GABA is likely to be more restricted and specific than resveratrol, or other SIRT1-activating small drugs, especially as orally administered GABA does not cross the blood–brain barrier. Furthermore, GABA has the additional effect of increasing NAD$^+$ levels, at least in islet cells. There is likely to be a complex interplay of pathways due to concurrent GABA receptor activation, and SIRT1-mediated deacetylation of

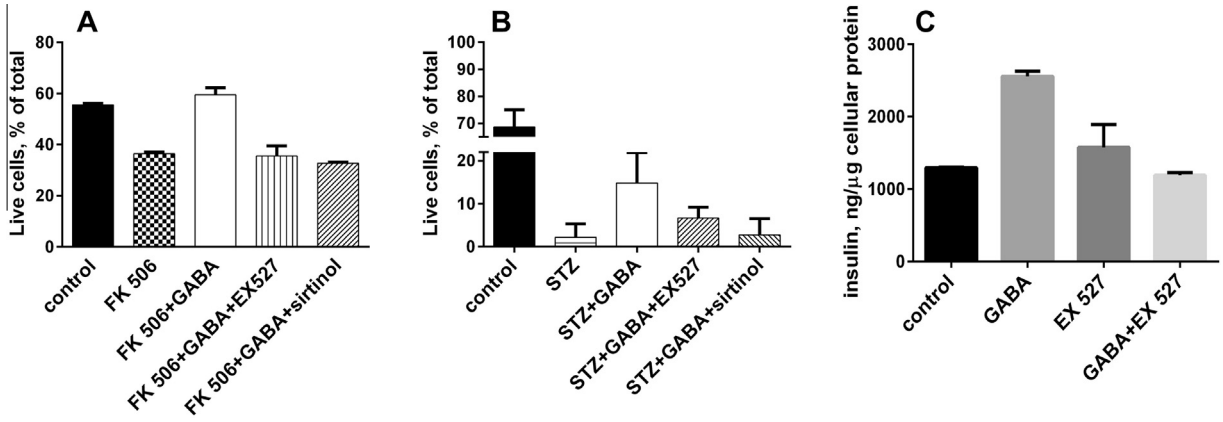

Fig. 3. SIRT1 is involved in GABA-mediated protection against apoptosis and induction of insulin secretion. INS-1 cells are protected from apoptosis induced by tacrolimus (FK506; panel A) or streptozotocin (STZ, panel B) by GABA, and the protective effect is reversed by EX527 (SIRT1 inhibitor) or sirtinol (SIRT1 and SIRT2 inhibitor). The cells were incubated with GABA (100 μM), EX527 (200 nM) or sirtinol (4 μM) as indicated, for 1 h before adding 1 μM FK506, and cell viability was tested after 48 h. Similarly, STZ (2 mM) was added after the cells were incubated with other reagents for 1 h, but was replaced with the original medium containing all additives and no STZ after 1 h incubation. Viable cells were counted after 24 h. The results are representative of two experiments. (C) GABA increased insulin secretion and this was blocked by EX527. INS-1 cells were serum-starved for 16 h, and incubated for 1 h in medium containing 1.4 mM glucose, 100 μM GABA, and 200 nM EX527, as indicated. Insulin levels were quantified by ELISA (mean ± SEM).

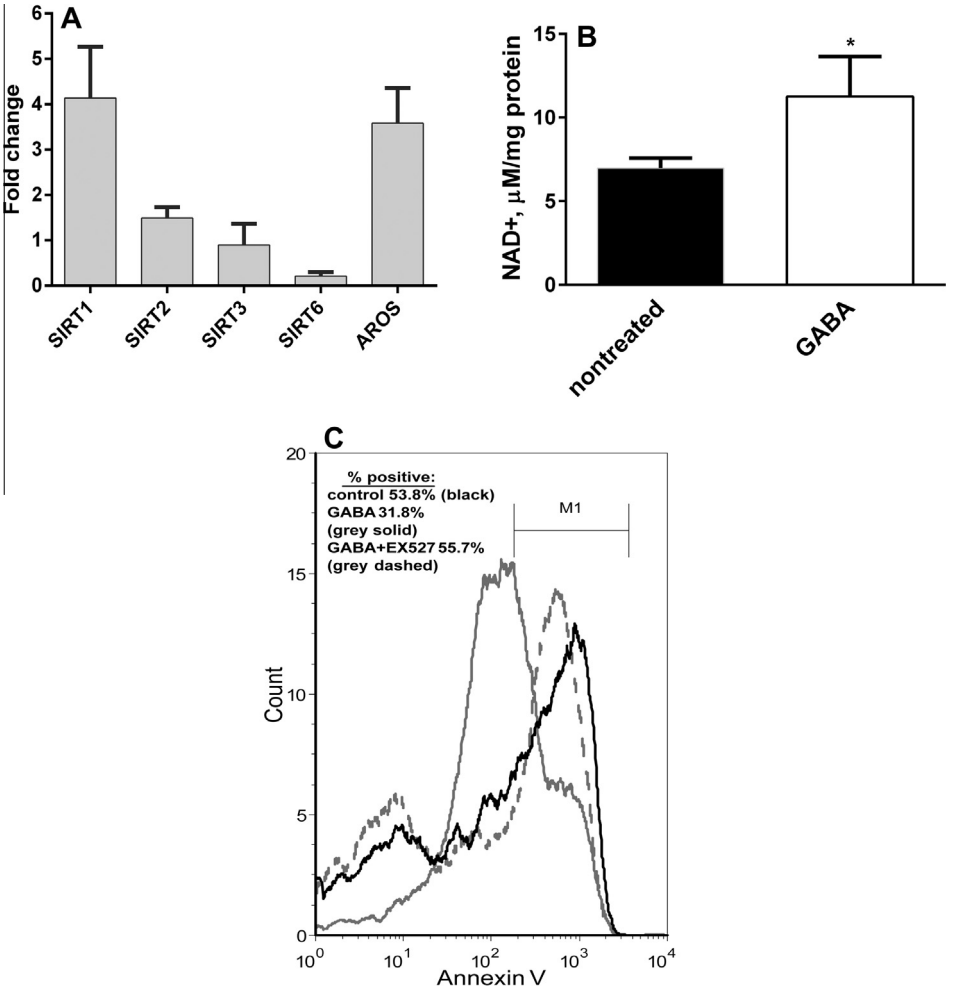

Fig. 4. In human islets, GABA increases SIRT1 and NAD^+, and reduces apoptosis. (A) Quantitative PCR (qPCR) analysis of sirtuin mRNA expression in human islet cells. GABA treatment in vitro induces expression of SIRT1 and AROS, but not of SIRT2, 3 and 6 (mean ± SEM of 3 human islet donors). Human islets were treated with 100 μm GABA for 2 h before the RNA extraction. The expression of SIRT1 and AROS was increased approximately fourfold in the GABA-treated islets compared to the non-treated ones ($p < 0.01$ for SIRT1 or AROS versus the other sirtuins). (B) NAD^+ is increased. 1 h treatment with 100 μM GABA increases the level of NAD^+ in human islets (d 3 in culture). The enzyme assay data is calculated per mg of cellular protein (*$p < 0.05$ for GABA versus nontreated). (C) GABA reduced the percentage of apoptotic islet cells and this was reversed by EX527. Cultured islets were treated or not with 100 μM GABA for 72 h. 200 nM SIRT1 inhibitor EX527 was added as indicated. The number of apoptotic cells was determined by flow cytometry analysis of annexin V staining (representative of the two experiments).

mediators involved in metabolic control and inflammation. Our study points to potential benefits of GABA administration in islet-cell physiology, inflammatory diseases, and the treatment of diabetes.

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