Gaba-betaine modulates SIRT1 and p16^{INK4A} expression during high-glucose induced endothelial cell senescence

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Abstract

Gaba-betaine (γ-aminobutyric acid betaine, GabaBet), a betaine formed by trimethyllysine (TML), is a precursor of the L-carnitine biosynthesis. Betaine, quaternary ammonium compounds, are naturally occurring osmoprotectants or compatible solutes with a widespread distribution in plant and animal kingdoms. Among betaines, stachydrine (N,N-dimethyl-L-proline) (ProBet), abundant in citrus fruit juices, inhibits the deleterious effect of high-glucose (hGluc) on endothelial cells (EC). Hyperglycaemia induces endothelial dysfunction and vascular complications by promoting EC senescence, altering antioxidant enzyme involved in the system defence against reactive oxygen species (ROS), and limiting the cell proliferative potential. This study was designed to determine the possible effect of GabaBet against the hGluc-induced oxidative injury. Evaluation of cell viability revealed that GabaBet does not affect cell proliferation from 0.001 to 1mM up to 72 h. Of interest, co-treatment for 48 h with GabaBet (0.1 mM) and hGluc (30 mM) (GabaBet+hGluc) attenuated the EC growth arrest in the G0/G1 cell cycle phase. GabaBet counteracted the hGluc induced upregulation of p16^{INK4A} and the increased superoxide dismutase activity. Moreover, the downregulation of sirtuin 1 (SIRT1) expression, occurring under hGluc conditions, is significantly blocked by GabaBet. On the whole, results show that beneficial effects of GabaBet in the prevention of hGluc-induced endothelial senescence is paralleled by the modulation of SIRT1 and p16^{INK4A} expression levels.

Introduction

Gaba-betaine (GabaBet) (Figure 1), or γ-aminobutyric acid betaine, originates from the trimethyllysine (TML) released upon protein degradation. In mammals, GabaBet is the precursor of the L-carnitine biosynthesis, as the hydroxylation of the GabaBet produces 3-hydroxy-4-*N,N,N*-trimethylaminobutyrate, better known as carnitine.[1,2] GabaBet has been shown to be involved both in L-carnitine synthesis and in an alternative pathway of gut microbiota-dependent conversion of carnitine into the pro-atherogenic metabolite trimethylamine-*N*-oxide (TMAO).[3] At the same time, experimental data also indicate that GabaBet itself possesses cardioprotective activity.[4] Indeed, previous studies indicated that mildronate, an inhibitor of carnitine biosynthesis and uptake, induces an increase in GabaBet contents and reduces atherosclerosis in apoE/LDLR$^{-/-}$ mice.[5] Moreover, marked elevations of GabaBet plasma levels following the administration of mildronate are associate with the improved endothelial function during hyperglycaemia and hypertension.[6-8] In particular, increased GabaBet levels with preserved L-carnitine content in vascular tissues attenuated the development of endothelial dysfunction induced by high-glucose and had no impact on endothelial dysfunction induced by triglycerides or lysophosphatidylcholine.[7] The enhanced reactive oxygen species (ROS) generation and depletion of nitric oxide (NO) bioavailability alter the endothelium functionality during hyperglycaemia, a key feature of diabetes and its vascular complications.[9,10] The hyperglycaemia-induced endothelial dysfunction is also mediated by downregulation of sirtuins, proteins that take part to the mechanisms regulating vascular dysfunction related to aging, inflammation, and diabetes.[11,12] At vascular level, the effects of sirtuin 1 (SIRT1) are due to the deacetylation of multiple targets, including endothelial nitric oxide synthase, peroxisome proliferator-activated receptor-γ coactivator 1-α, p53, forkhead Box O family, and nuclear factor-kappa B (NF-κB).[11,12] Sirtuin 6 (SIRT6) plays a key role in cardiac hypertrophy, heart failure, myocardial hypoxic damage, metabolism and endothelial inflammation.[13,14] Indeed, SIRT6 deficiency in EC resulted in an increased expression of pro-inflammatory cytokines, such as interleukin 1β, and increased transcriptional activity of NF-κB.[14] Evidence about the role of SIRT6 in the prevention of the deleterious effect of high-glucose (hGluc) on EC and endothelial progenitor cell (EPC) indicate that the downregulation of SIRT6 is paralleled by the increased expression of NF-κB.[15]

Plasma betaine levels are reported to be reduced in insulin-resistant subjects and to closely correlate with insulin sensitivity.[16] Moreover, stachydrine, the betaine of proline very abundant in citrus juice, which differs from GabaBet for its cyclic structure,[17] has been demonstrated to counteract the hGluc induced endothelial cell senescence via SIRT1 downregulation.[18] Another betaine, the 2-mercapto histidine betaine (ESH), namely ergothioneine, also protects EC against the hGluc induced endothelial senescence *via* the regulation of SIRT1/SIRT6 expression and their cellular targets, NF-κB and p66^{Shc}.[10,19] In the light of these observations, we sought to investigate the possible effect of GabaBet on the biochemical mechanism(s) responsible for the hyperclycaemia-induced endothelial senescence.

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Materials and Methods

Endothelial cell culture

EC were obtained from the American Type Culture Collection (CPAE, CCL-209). EC, used between passage 18 and 26 population doublings, were grown in Minimum Essential Medium (MEM, with Earle's Salts; Gibco, Life Technologies) containing 20% FBS (Performance Plus, United States, Gibco), 1% NEAA (Non-Essential Amino Acid solution 100X, Lonza), and 1% Pen-Strep (10.000 units/mL penicillin and 10.000 mg/mL streptomycin, Gibco) at 37°C in a fully humidified atmosphere of 5% CO_2.

Endothelial cell treatments

Sub-confluent EC were seeded 12h before each experiment. GabaBet (Sigma-Aldrich, Milan, Italy) (up to 1 mM) was added before starting the short-term treatment with hGluc (30 mM) in complete culture medium (GabaBet+hGluc), according to previous studies on stachydrine.[18] The effect of GabaBet itself was evaluated by treating EC with GabaBet alone (GabaBet) in medium containing normal glucose (5.5 mM). Control cells (Ctr) were incubated only with normal glucose. Osmotic control was performed by treatment with 25 mM L-glucose.

Cell viability assays

The cell viability was determined by MTT assay after treatment with hGluc (30 mM) in the presence or absence of GabaBet (0.1 mM) up to 72 h. Briefly, EC were seeded in 96 well-plate at a density of 1×10^5 cells /well and incubated up to 72 h at 37°C with hGluc or with hGluc in the presence of GabaBet (0.1 mM) (GabaBet+hGluc). Control cells (Ctr) received normal glucose concentration (5.5 mM) or GabaBet (0.1 mM) alone (GabaBet). At the end of incubations, MTT assay was performed following manufacturer's procedure. Absorbance rate was measured at 570 nm using a microplate reader (BIO-RAD). Cell growth data were expressed as percentage of the control. The negative control wells contained only medium. Cell viability was also determined by Trypan Blue assay (Sigma-Aldrich) and expressed as percentage (%) of cell viability following manufacturer's instructions.

Cell cycle analysis

The effect of GabaBet on the cell cycle in hGluc treated EC was monitored by fluorescence-activated cell sorting (FACS) analysis. To this end, EC were grown in 60 mm plates (1×10^5 cells/well) and incubated for 48 h as described above. After 48 h, EC were collected and then centrifuged for 5 min at 300 × g. Cells were fixed with 3% formaldehyde, permeabilized for 5 min with 0.1% Triton X-100, and washed twice with PBS. EC were then incubated with RNAse (2.5 mg/mL) and propidium iodide (50 mg/mL) for 45 min at 4°C. DNA content was measured by FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) by acquiring at least 10,000 events. The cell cycle analysis was performed by using Cell-Quested software (Becton Dickinson, Milan, Italy). The proliferation index was calculated as previously described.[18]

Superoxide dismutase activity

Quantification of superoxide dismutase (SOD) activity in EC under hGluc conditions, with or without GabaBet was performed by Cayman Chemical SOD Assay kit. This assay kit utilizes a tetrazolium salt for detection of radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine. SOD activity is standardized using the cytochrome c and xanthine oxidase coupled assay. Briefly, EC were grown in 60 mm plates (1×10^5 cells/well) and incubated for 48 h as described above. At the end of the treatment, EC were collected and cytosolic superoxide dismutase was assayed spectrophotometrically at 460 nm in the cell lysates. Triplicate aliquots of SOD standards (10 µL) or EC lysates (10 µL) were placed in 96-well microplate. SOD activity (U/µg of protein) was calculated according to the formula: % inhibition=(A control-A sample)/A control×100.

Western blot analysis

Cells were washed twice with 1X cold phosphate-buffered saline (PBS) and scraped in lysis buffer (50 mM Tris, pH 7.4, 150 mM sodium chloride, 1% Nonidet P-40, 1 mM EDTA, 1mM sodium orthovanadate, 1 mM sodium fluoride, 1 mg/mL leupeptin, 1 mg/mL aprotinin, 1 mg/mL pepstatin A, 1 mM phenylmethylsulfonyl fluoride). Scraped cells were sonicated for 10-15 s to complete cell lysis. After 30 min of gently shaking at 4°C, cell lysates were centrifuged at 10.000 × g for 10 min and supernatants were collected. Aliquots of lysate, approximately 50 µg, were fractionated on 15% ($p16^{INK4A}$) or 7% (SIRT1) SDS-polyacrylamide gel electrophoresis and transferred on nitrocellulose membranes. Membranes were blocked in 5% w/v milk, 1X TBS, 0.1% Tween-20 at 25°C for 1 h with gentle shaking, and then incubated overnight at 4°C with antibodies against SIRT1 (1:2000) (rabbit monoclonal, ab32441, Abcam Cambridge, UK), $p16^{INK4A}$ (mouse, 1:250, Santa Cruz Biotechnology, Inc. Dallas, TX, USA), or against ɣ-tubulin (mouse monoclonal, 1:1000, GTU-88, Sigma, St. Louis, MO, USA). After incubation with secondary

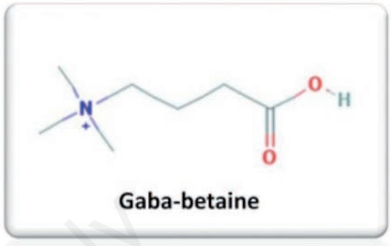

Figure 1. Chemical structure of Gaba-betaine.

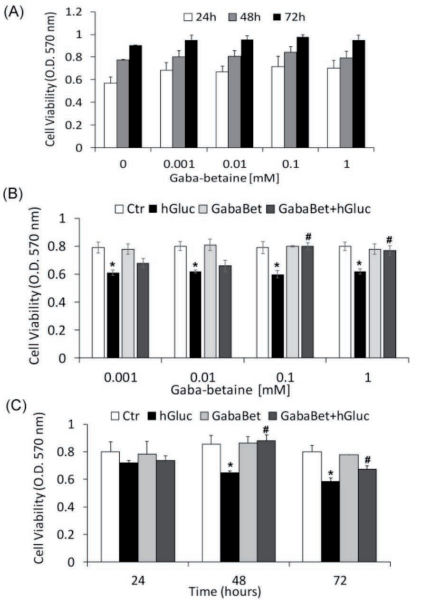

Figure 2. Time-dependent and dose-response effects of Gaba-betaine on EC viability. A) Dose-response effects of Gaba-betaine on endothelial cells (EC) viability. B) Dose-dependent effects of Gaba-betaine on high-glucose (hGluc)-induced cytotoxicity at 48 h. C) Effect of Gaba-betaine (0.1 mM) on EC treated for different times (24, 48 and 72 h) with high-glucose (hGluc) (30 mM). Control cells (Ctr) were incubated in media containing normal glucose (5.5 mM). Results are expressed as mean±standard deviation of five independent experiments. *P<0.05 vs Ctr; #P<0.05 *vs* hGluc.

antibodies (1:10:000), membranes were washed three times and bands were detected by the enhanced chemoluminescence kit (ECL, Amersham, Aylesbury, UK). Semiquantitative densitometry of Western blots was performed using a Scan LKB (Amersham).

Confocal laser scanning microscopy

EC cultured in 24-well plates containing microscope glass (12 mm) (Thermo Scientific) were fixed with 3% (v/v) paraformaldehyde for 20 min and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min at room temperature. Fixed and permeabilized EC were incubated with specific antibodies against vimentin (1:1000) and against SIRT1 (1:500). Secondary antibodies were Alexa fluor 633 (1:1000) or Alexa fluor 488 (1:1000). Microscopy analyses were performed using Zeiss LSM 700 confocal microscope equipped with a plan-apochromat X 63 (NA 1.4) oil immersion objective. The fluorescence intensity was evaluated with ImageJ software and results expressed as arbitrary fluorescence units (AFU).

Statistical analysis

Data are expressed as mean±SD. Statistical differences were assessed by Student's t-test and P<0.05 value was considered significant.

Results

Gaba-betaine blocks high-glucose cytotoxicity

MTT assay was used to evaluate the effect of increasing concentrations of GabaBet (0.001, 0.01, 0.1, 1 mM) on the EC proliferative capacity. Dose-dependent response showed that cell viability was not affected by GabaBet up to 72 h (Figure 2A). The highest efficiency in reducing the hGluc effects occurred at 0.1 and 1mM concentration (P<0.05 *vs* hGluc) (Figure 2B). Based on these results and for a better comparison with stachydrine[18], the GabaBet concentration of 0.1 mM was chosen for this study and, therefore, tested at different times of hGluc exposure. Results indicated that administration of GabaBet (0.1 mM) 12 h before hGluc treatment (GabaBet+hGluc) reversed the effect of hGluc on EC viability with a highest effect occurring at 48 h (P<0.05 *vs* hGluc treated cells) (Figure 2C). These results were confirmed by Trypan Blue dye exclusion assay indicating a percentage of cell viability of 86±8.3% in GabaBet+hGluc *vs* 63±5.2% in hGluc cells.

Effect of Gaba-betaine on cell cycle phases

Flow cytometry analysis showed that the hGluc- induced arrest in the G0/G1 phase at 48 h (P<0.05 *vs* control cells) (Figure 3A and B) was ameliorated by GabaBet co-treatment. More in details, in hGluc treated cells, the percentage of EC in G0/G1 phase was 69.25% ±2.9 (P<0.05 *vs* control cells) and 63.14%±2.3 in GabaBet+hGluc treated cells (P<0.05 *vs* hGluc). Moreover, the cell proliferation index in EC treated with hGluc, which was lower than that observed in the control cells (29.63%±0.9 *vs* 36.06%±0.7 in control cells, P<0.05), significantly increased in the presence of GabaBet (35.27%±0.8 *vs* 29.63%±0.9 in hGluc, P<0.05) (Figure 3C). Based on these results, the time of 48h was chosen to investigate the molecular mechanism(s) during the protective effect elicited by GabaBet.

Gaba-betaine effect on superoxide dismutase activity p16^{INK4A} expression

The antioxidant properties of GabaBet were tested by quantification of the superoxide dismutase (SOD) activity. Results showed that hGluc treatment induced an increased SOD activity compared to control cells (0.045 U/µg *vs* 0.033 U/µg in control cells, P<0.05) (Figure 4A). This effect was counteracted by GabaBet co- treatment (GabaBet+hGluc) (0.030±0.04 U/µg *vs* 0.045±0.03 in hGluc, P<0.05) (Figure 4A). Results also showed an increased expression level of p16^{INK4A}, a senescence related protein, in EC under hGluc conditions (0.9±0.05 arbitrary units vs. 0.75±0.05 arbitrary units in control cells, P<0.05). Interestingly, GabaBet treatment (GabaBet+hGluc) determined a significant reduction of the p16^{INK4A} expression levels compared to hGluc (0.69±0.05 arbitrary units vs. 0.9±0.05, P<0.05) (Figure 4B and C).

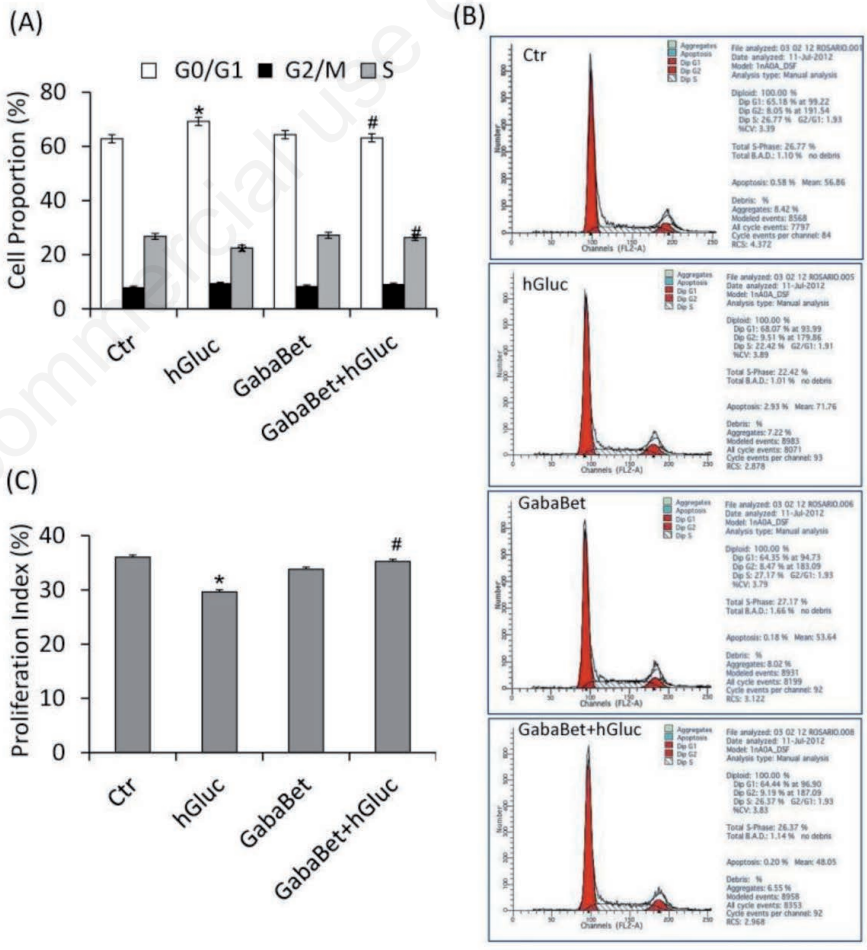

Figure 3. Effects of Gaba-betaine on cell cycle. A) Percentage of endothelial cells (EC) proportion after treatment for 48 h with high-glucose (hGluc), high-glucose in the presence of Gaba-betaine (GabaBet+hGluc), normal glucose (Ctr), or Gaba-betaine alone (GabaBet). Results are the mean±standard deviation of n=5 independent experiments. B) Representative fluorescence-activated cell sorting cycle plots. C) Percentage of EC proliferation index. Results are the mean±standard deviation of five independent experiments. *P<0.05 *vs* Ctr, #P<0.05 *vs* hGluc.

Sirtuin 1 modulation by Gaba-betaine

When we evaluated the possible effect of GabaBet on the downregulation of SIRT1 expression levels associated with hGluc toxicity,[20] both confocal laser-scanning microscopy and Western blot analyses revealed that GabaBet positively modulates SIRT1 expression levels in GabaBet+hGluc samples. Specifically, SIRT1 arbitrary fluorescence units (AFU) were significantly decreased by 48 h treatment with hGluc (34.96 ±4.63 *vs* 95.32±5.89 AFU in control cells, P<0.01) whereas GabaBet+hGluc cells showed a SIRT1 fluorescence intensity significantly higher than that observed in hGluc cells (83.54±2.93 *vs* 34.96±4.63 AFU in hGluc, P<0.01) (Figure 5A and B). The modulation of SIRT1 by GabaBet during hGluc treatment was confirmed by Western blot analysis showing that GabaBet treatment (GabaBet+hGluc) determined an upregulation of SIRT1 expression levels (0.97±0.05 arbitrary units vs. 0.83±0.05 arbitrary units hGluc, P<0.05) (Figure 5C and D).

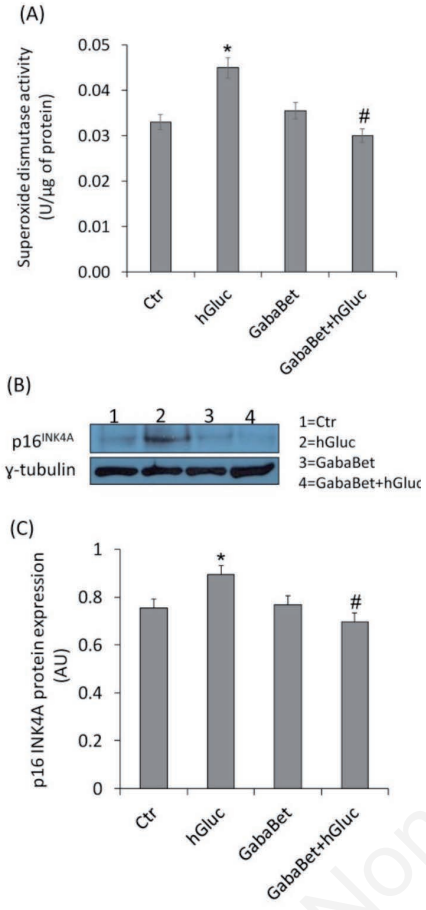

Figure 4. Gaba-betaine modulates superoxide dismutase (SOD) activity and p16INK4A protein expression. A) SOD activity was assessed in endothelial cells (EC) treated for 48 h with normal glucose (Ctr), high-glucose (hGluc), Gaba- betaine (GabaBet), high-glucose plus Gaba-betaine (GabaBet+hGluc). Results were expressed as units (U) of SOD/µg of proteins. Data are the mean±SD of five independent experiments. *P<0.05 *vs* Ctr, #P<0.05 vs hGluc. B) Representative Western blot gel imaging. C) Expression of p16INK4A measured by immunoblotting after treatment for 48 h with normal glucose (Ctr), high-glucose (hGluc), Gaba-betaine (GabaBet), high-glucose plus Gaba-betaine (GabaBet+hGluc). Protein level values were expressed as arbitrary units (AU). Data are the mean±standard deviation of four independent experiments. *P<0.05 vs Ctr, #P<0.05 *vs* hGluc.

Discussion

This study reports the evidence about the *in vitro* protective role GabaBet against the hGluc-induced oxidative stress and premature senescence in EC. The protective effects exerted by GabaBet are concomitant with the modulation of SIRT1 and p16INK4A expression levels. In particular, administration of GabaBet 12 h before hGluc treatment reversed the effect of hGluc on EC viability. More in details, cotreatment with GabaBet and hGluc affected the cell cycle distribution, as this treatment kept the percentage of cells in the G0/G1phase near to control values. In EC, GabaBet administration counteracted the oxidative stress induced by hGluc, by restoring the SOD activity and by negatively regulating p16INK4A expression, a known marker of cellular senescence.[21] At the same time, GabaBet prevented the hGluc-induced downregulation of SIRT1, a member. of the sirtuin family with multiple roles in the modulation of the vascular function.[11] Indeed, it is well known that SIRT1 prevents hydrogen peroxide-induced premature senescence of EC and protects blood vessels from hyperglycemia-induced endothelial dysfunction through a mechanism involving the downregulation of p66Shc expression.[22]

To date, findings about the protective cardiovascular effects of GabaBet are controversial. Intestinal microbiota metabolism of L-carnitine has been shown to promote atherosclerosis by producing the proatherogenic TMAO and GabaBet has been found to be a proatherogenic intermediate in gut microbial metabolism of L-carnitine to

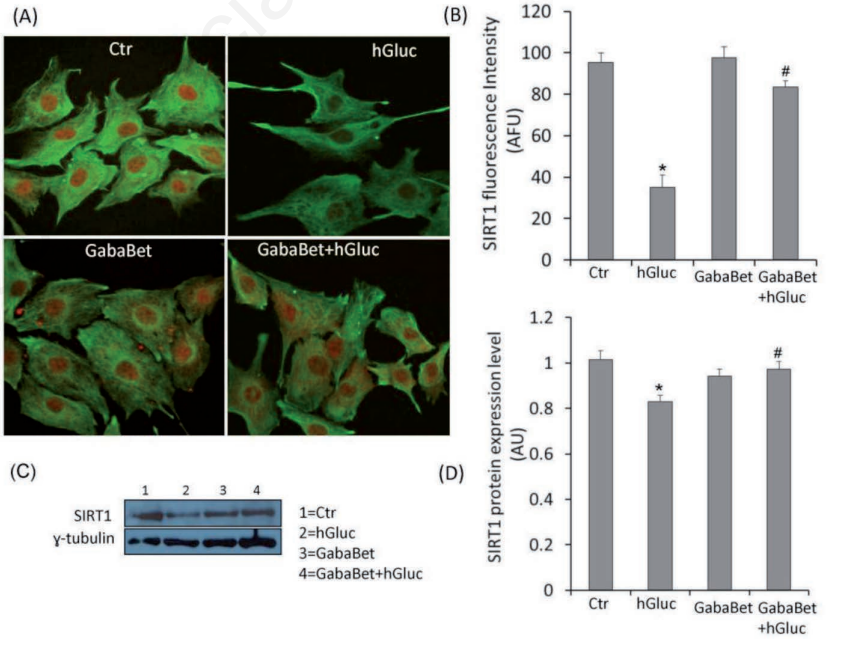

Figure 5. Effect of Gaba-betaine on high-glucose induced SIRT1 downregulation. A) Representative confocal images of sirtuin 1 (SIRT1) expression in EC treated for 48 h normal-glucose (Ctr), high-glucose (hGluc), Gaba-betaine (GabaBet), high-glucose plus Gaba-betaine (GabaBet+ hGluc). B) Immunofluorescence analyses of SIRT1 (Alexa Fluor 633, red) and vimentin (Alexa Fluor 488, green) were performed with ImageJ software analysis and expressed as arbitrary fluorescence units (AFU). *P<0.01 *vs* Ctr, #P<0.01 *vs* hGluc. C) Representative Western blot gel imaging of SIRT1 protein expression. D) Expression of SIRT1 (normalized to γ-tubulin) was measured by immunoblotting in Ctr, hGluc, GabaBet, and GabaBet+ hGluc samples. Protein level values were expressed as arbitrary units (AU). Data are the mean±standard deviation of four independent experiments. *P<0.05 *vs* Ctr, #P<0.05 vs hGluc.

TMAO.[3] Based on the relationship between GabaBet, carnitine, and TMAO, it has been hypothesized that intestinal microbiota may contribute to the well-established link between high levels of L-carnitine consumption and CVD risk and that GabaBet could be related to the presence of carotid atherosclerosis and its complications linked to high levels of TMAO.[3] A study conducted on a cohort of 264 patients with carotid atherosclerosis reported increased serum levels of GabaBet and TML, but not TMAO.[23] However, a direct role of GabaBet in atherogenesis has been questioned.[24] Indeed, results in in apoE/LDLR$^{-/-}$ mice model showed that decreased L-carnitine levels and increased GabaBet levels in vascular tissues are associated with anti-atherosclerotic effects.[5] Similarly, in animal model of hypertension, marked elevations of plasma GabaBet attenuate the development of endothelial dysfunction, suggesting that changes in vascular tissue levels of L-carnitine and GabaBet might have vasoprotective effects.[6] The unchanged L-carnitine content and the increased vascular levels of GabaBet counteracted the development of hGluc-induced endothelial dysfunction.[7] The cardioprotective effect of long-term administration of mildronate, an inhibitor of carnitine biosynthesis, is associated with a decreased free carnitine concentration and increased GabaBet concentration, which correlates with the cardioprotection of mildronate.[25,26]

Administration of betaine has an impact on carnitine metabolism, and this could further explain the link between betaine and lipid metabolism,[27] as evidenced by its ability to attenuate hepatic triglyceride accumulation.[28] Betaine may be particularly important for hepatic lipid metabolism, as betaine supplementation of mice with diet-induced obesity reduces hepatic lipids and improves whole body metabolism. Among other effects, plasma betaine levels in humans are inversely linked to triglycerides and non-high density lipoprotein (non-HDL) cholesterol, and positively to HDL cholesterol.[16]

Conclusions

In line with the antioxidant properties of stachydrine and other betaines, this study sheds light on the *in vitro* cytoprotective effect of GabaBet against hGluc-induced endothelial senescence. Evidence, here provided, that the *in vitro* endothelial beneficial effect of GabaBet against the hGluc detrimental effects occurs through the positive regulation of SIRT1 and negative modulation of p16^{INK4A} indicate that this betaine display effects similar to those elicited by stachydrine,[18,29] suggesting its potential role as natural bioactive compound in the prevention of hyperglycaemia-induced endothelial dysfunction. A deeper characterization of the metabolism and the molecular mechanism(s) of action of this betaine is critical to understand the differences with GabaBet intermediate in gut microbial metabolism of L-carnitine to TMAO.[3] Furthermore, future investigations might be helpful in the elucidation of the possible effect of GabaBet on SIRT6 modulation.[10] Indeed, both SIRT1 and SIRT6, showing an established role in the protection against CVD and hGluc-induced endothelial senescence,[30] could be crucial targets in the setting of natural bioactive compounds able to limit the cellular oxidative processes responsible for the onset of diabetes and its vascular complications.

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