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Stachydrine Ameliorates High-Glucose Induced Endothelial Cell Senescence and SIRT1 Downregulation

Luigi Servillo,[1] Nunzia D'Onofrio,[1] Lara Longobardi,[2] Ivana Sirangelo,[1] Alfonso Giovane,[1] Domenico Cautela,[3] Domenico Castaldo,[3] Antonio Giordano,[4,5] and Maria Luisa Balestrieri[1]*

[1]*Department of Biochemistry, Biophysics and General Pathology, Second University of Naples, Naples, Italy*
[2]*Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, 27599*
[3]*Stazione Sperimentale per le Industrie delle Essenze e dei derivati dagli Agrumi (SSEA), Azienda Speciale della Camera di Commercio di Reggio Calabria, RC, Italy*
[4]*Sbarro Research Institute, College of Science and Technology, Temple University, Philadelphia, Pennsylvania, 19122*
[5]*Department of Medicine, Surgery & Neuroscience, University of Siena, Italy*

ABSTRACT

Hyperglycaemia, a characteristic feature of diabetes mellitus, induces endothelial dysfunction and vascular complications by accelerating endothelial cell (EC) senescence and limiting the proliferative potential of these cells. Here we aimed to investigate the effect of stachydrine, a proline betaine present in considerable quantities in juices from fruits of the *Citrus* genus, on EC under high-glucose stimulation, and its underlying mechanism. The senescence model of EC was set up by treating cells with high-glucose (30 mM) for different times. Dose-dependent (0.001–1 mM) evaluation of cell viability revealed that stachydrine does not affect cell proliferation with a similar trend up to 72 h. Noticeable, stachydrine (0.1 mM) significantly attenuated the high-glucose induced EC growth arrest and senescence. Indeed, co-treatment with high-glucose and stachydrine for 48 h kept the percentage of EC in the G_0/G_1 cell cycle phase near to control values and significantly reduced cell senescence. Western blot analysis and confocal-laser scanning microscopy revealed that stachydrine also blocked the high-glucose induced upregulation of $p16^{INK4A}$ and downregulation of SIRT1 expression and enzyme activity. Taken together, results here presented are the first evidence that stachydrine, a naturally occurring compound abundant in citrus fruit juices, inhibits the deleterious effect of high-glucose on EC and acts through the modulation of SIRT1 pathway. These results may open new prospective in the identification of stachydrine as an important component of healthier eating patterns in prevention of cardiovascular diseases. J. Cell. Biochem. 114: 2522–2530, 2013.

KEY WORDS: STACHYDRINE; HYPERGLYCAEMIA; ENDOTHELIAL CELLS; SENESCENCE

Stachydrine (*N*,*N*-dimethyl-L-proline or proline betaine), a well-known constituent of Leonurus heterophyllus sweet, has been found to be present at not negligible concentrations in juices from fruits of the *Citrus* genus along with L-proline, *N*-methyl-L-proline (hygric acid), 4-hydroxy-L-prolinebetaine (betonicine), 4-hydroxy-L-proline, *N*-methyl nicotinic acid (trigonelline), and choline [Servillo et al., 2011a,b]. In particular, highest stachydrine contents were found in the juices of the red and yellow oranges (548 and 486 mg/L, respectively) followed by chinotto (*Citrus myrtifolia*) juice (455 mg/L), bitter orange juice (418 mg/L), mandarin juice (342 mg/L), lemon juice (332 mg/L), and grapefruit juice (246 mg/L) [Servillo et al., 2011a]. Other studies also reported the presence of stachydrine in orange juices, making this compound one of the major compounds in orange juice after sugars and organic acids [Zeisel et al., 2003]. Stachydrine, as all betaines, is a quaternary ammonium compound. Betaines are

Abbreviations: ADMA, asymmetric dimethylarginine; CVD, cardiovascular diseases; DDAH, dimethylarginine dimethylaminohydrolase; EC, endothelial cells; EPC, endothelial progenitor cells; ET-1, endothelin-1; FOXO, forkhead box class O; GabaBet, gaba-betaine; IL-1α, interleukin-1α; LPS, lipopolysaccharide; SIRT1, silent information regulator 1; Stachy, stachydrine; TXB(2), thromboxane B(2).

The authors declare that they have no conflict of interests.

*Correspondence to: Maria Luisa Balestrieri, Department of Biochemistry, Biophysics and General Pathology, Second University of Naples; Via L. de Crecchio 7; Naples 80138, Italy. E-mail: marialuisa.balestrieri@unina2.it

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ubiquitous in the vegetal world and tend to accumulate in the cytoplasm and intercellular fluids where they play an important role in protecting proteins, nucleic acids, and cell membranes against abiotic stress [Hasegawa et al., 2000; Kavi et al., 2005; Stret et al., 2006]. Being compatible with cellular metabolism even at molar concentrations, betaines are generally referred to as compatible osmolytes [Hasegawa et al., 2000]. The role of stachydrine, as well as other betaines, in the vegetal world has been fully established. However, despite its abundance not only in Chinese herb Leonurus heterophyllus sweet but also in the juices from fruits of the *Citrus* genus, little is known about its potential role as nutritional factor. It is recognized that a healthy diet rich in fruit and vegetables is associated with lower rates of cancers, diabetes, and cardiovascular diseases (CVD) [Riboli and Norat, 2003; Mente et al., 2009]. In particular, several in vitro and clinical studies demonstrate that vegetable and fruit consumption or Mediterranean diet can be determinant in the prevention of CVD and diabetes [Ignarro et al., 2007; Berg et al., 2008; Fiorito et al., 2008; Thomazella et al., 2011].

To date, only few in vitro studies provide evidence that stachydrine protects endothelial against the injury induced by anoxia-reoxygenation [Yin et al., 2010] and inhibits secretion of IL-1α, TXB(2), ET-1, and E-selectin in LPS-induced endothelial cells [Hu et al., 2012]. In humans, proline betaine concentrations were reported to be increased in plasma and urine after orange juice consumption [Atkinson et al., 2007]. Interestingly, metabolomic profiling studies report increased levels of stachydrine in patients with primary dilated cardiomyopathy [Alexander et al., 2011] and indentified this compound as a putative biomarker of citrus consumption [Heinzmann et al., 2010].

The functional alterations that occur at endothelial level during hyperglycaemia, a characteristic feature of diabetes mellitus and vascular complications in diabetes, comprise changes in vaso-regulation, enhanced generation of reactive oxygen intermediates, inflammatory activation, and altered barrier function [Balestrieri et al., 2010; Eringa et al., 2013]. In particular, hyperglycaemia accelerates EC senescence which limits the proliferative potential [Chen et al., 2002; Yokoi et al., 2006; Balestrieri et al., 2013; Mortuza et al., 2013]. In EC and endothelial progenitor cells (EPC), the hyperglycemia accelerated aging-like process is mediated by the downregulation of the silent information regulator (Sirt) 1 (SIRT1), causing reduction of mitochondrial antioxidant enzyme in a p300 and FOXO1 mediated pathway [Balestrieri et al., 2008a,2013; Mortuza et al., 2013]. Although it is important to ascertain the effect of dietary patterns with respect to the prevention of diabetes and its vascular complication, a comprehensive understanding of the mechanism of action of a single nutrient is a critical step to unveil novel beneficial nutrients. In this context, the findings that stachydrine can be taken daily through the diet in conspicuous amounts [Zeisel et al., 2003; Servillo et al., 2011a,b], led us to investigate whether this compound is able to interfere with the mechanisms responsible for the hyperglycaemia-induced endothelial dysfunction. To this end, here, using a model with high-glucose induced EC damage, we examined the effect of stachydrine on the EC proliferative capacity and senescence and uncovered the possible relevant mechanism.

MATERIALS AND METHODS

CHEMICALS

Stachydrine (*N*,*N*-dimethyl-L-proline) was purchased from Extra-synthese (Genay, France). XTT assay kit, D-glucose, senescence cell staining kit, propidium iodide, antibody against γ-tubulin, and antibody against vimentin were from Sigma–Aldrich (Milan, Italy). Phosphate-buffer saline (PBS) was from Lonza (Verviers, Belgium). Antibody against $p16^{INK4A}$ and the secondary antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, Texas). Antibody against SIRT1 was from Abcam (Cambridge, UK). Alexafluor 633 and Alexafluor 488 were from Alexa, Minimum Essential Medium, L-glutamine, penicillin, streptomycin, amphotericin B, fetal bovine serum (FBS), and trypsin–EDTA were from Invitrogen (Carlsbad, CA). Fluor de Lys kit (AK-555) was from Biomol, Enzo Life Sciences (Lausen, Switzerland).

ENDOTHELIAL CELL CULTURE AND TREATMENT

EC (CPAE, CCL-209) were obtained from the American Type Culture Collection. EC, used between passage 18 and 26 population doublings, were grown in Minimum Essential Medium containing 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin at 37°C in a fully humidified atmosphere of 5% CO_2. High-glucose treatment was performed treating EC with 30 mM D-glucose (H-Gluc). EC were also treated with high-glucose in the presence of 0.1 mM stachydrine (H-Gluc + Stachy). Controls were performed in the presence of media with normal glucose alone (5.5 mM) (CTR), or with 0.1 mM stachydrine (Stachy). Stachydrine was added 6 h before culturing in high-glucose. Osmotic control was performed by treatment with 30 mM L-glucose.

CELL PROLIFERATION ASSAY

The cell proliferation was determined by XTT after treatment with high-glucose (30 mM) in the presence or absence of stachydrine (0.1 mM) up to 72 h. Briefly, EC were seeded in 96-well at a density of 4,000 cells/well and incubated for 12, 24, 48, and 72 h at 37°C with high-glucose alone or with high-glucose in the presence of stachydrine (0.1 mM). Control cells received normal glucose concentration or stachydrine (0.1 mM) alone. At the end of incubations, XTT assay was performed following manufacturer's procedure. The absorbance was measured at 492 nm in a spectrophotometer (TECAN Infinite 2000). The negative control wells contained medium only.

β-GALACTOSIDASE ACTIVITY ASSAY

Senescence-associated β-galactosidase (SA-β-Gal) activity was determined by senescence cells histochemical staining kit following manufacturer's procedure. Briefly, EC, e grown in 6-well plates (5×10^4 cells/well), were incubated for 48 h as described above. Stained cells were viewed under a Nikon Eclipse TE300 Inverted microscope (Nikon Instruments, Inc., Melville, NY). Results were expressed as the percentage of SA-β-galactosidase positive cells/field.

CELL CYCLE ANALYSIS

Fluorescence-activated cell sorting (FACS) analysis was used to monitor the effect of stachydrine on the cell cycle in high-glucose treated EC. 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 cold phosphate-buffered saline (PBS). EC were then incubated with RNAse (2.5 μg/ml) and propidium iodide (50 μg/ml) for 45 min at 4°C. DNA content was measured by FACSCalibur instrument (BD Biosciences) by acquiring at least 10,000 events. The analysis of the cell cycle was performed by using Cell-Quested software (Becton Dickinson). Based on the cell cycles analysis, the proliferation index is expressed as the result of the following equation [Wang and Ren, 2006]:

$$\text{Proliferation Index} = \frac{[S + G2/M]}{[G0/G1 + S + G2/M]} \times 100$$

WESTERN BLOT ANALYSIS

Control and treated cells were lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM sodium chloride, 1% Nonidet P-40, 1 mM EDTA, 1 mM sodium orthovanate, 1 mM sodium fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM PMSF) for 30 min at 4°C. Cell lysates were centrifuged at 10,000 × g for 10 min and supernatants were collected. Protein content was determined by the Lowry method [Lowry et al., 1951]. Approximately, 70 μg of protein extracts were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto membranes by Trans-Blot Turbo Transfer System (BioRad). Membranes were incubated overnight at 4°C with antibodies against $p16^{INK4A}$ (mouse, 1:250), against γ-tubulin (mouse, 1:1,000). After incubation with secondary 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 Pharmacia).

CONFOCAL LASER-SCANNING MICROSCOPY

Confocal microscope analysis of was performed as described [Balestrieri et al., 2013]. Briefly, fixed and permeabilized EC were incubated with specific antibodies against Vimentin (1:1,000) and against SIRT1 (1:500). Secondary antibodies were Alexafluor 633 (1:1,000) or Alexafluor 488 (1:1,000). Microscopy analyses were performed using Zeiss LSM 510 confocal microscope equipped with a plan-apochromat X 63 (NA 1.4) oil immersion objective. The fluorescence of the Alexa 488 and Alexa 633 was collected in multi-track mode using a BP505-530 and a LP650 as emission filters, respectively. The fluorescence intensity was evaluated with ImageJ software and expressed as arbitrary fluorescence units (AFU).

SIRT1 ASSAY

Deacetylation activity of SIRT1 was measured using the Fluor de Lys kit (AK-555) following the manufacturer's protocol. Fluorescence was measured by excitation at 360 nm and emission at 460 nm and enzymatic activity was expressed as relative fluorescence units (RFU)/mg protein.

STATISTICAL ANALYSIS

The data shown were mean values of at least four independent experiments and expressed as mean ± SD. Statistical analysis was performed by the Student's *t*-test. Statistical significance was set at a level of $P < 0.05$.

RESULTS

STACHYDRINE ATTENUATED THE CYTOTOXIC EFFECT OF HIGH-GLUCOSE ON EC

In order to evaluate the effect of stachydrine (Fig. 1) on EC proliferative capacity, cells were exposed to increasing concentrations of stachydrine (final concentrations of 0.001, 0.01, 0.1, and 1 mM) for 12, 24, 48, and 72 h. Dose–response results showed that stachydrine does not affect cell viability with a similar trend among 12, 24, 48, and 72 h (Fig. 2, panel A). Therefore, based on these and previous results [Yin et al., 2010], we chose 0.1 mM as the concentration of stachydrine. In line with the previous studies [Zanetti et al., 2001; Chen et al., 2007], high-glucose (30 mM) inhibits EC proliferation in a time-dependent manner with a significant effect starting at 48 h ($P < 0.05$) (Fig. 2, panel B). Indeed, after 48 and 72 h of treatment with high-glucose, EC viability was inhibited by 35% and 48%, respectively, compared to control cells ($P < 0.05$) (Fig. 2, panel B). The effect of high-glucose was significantly reversed by administration of stachydrine (0.1 mM). Indeed, interestingly, cell viability increased by twofold in EC treaded with high-glucose in the presence of stachydrine (H-Gluc + Stachy) ($P < 0.01$ vs. high-glucose treated cells). Cell cycle distribution, measured by flow cytometry indicated that high-glucose treatment arrested cells in the G_0/G_1 phase at 48 h of treatment ($P < 0.05$ vs. control cells) (Fig. 3, panel A and B). This effect was blocked by co-treatment with stachydrine which kept the percentage of cells in the G_0/G_1 phase near to control values. More in detail, the percentage of cells in G_0/G_1 phase was 71.50% in high-glucose treated cells ($P < 0.05$ vs. control cells) and 61.44% in cells co-treated with high-glucose and stachydrine ($P < 0.05$ vs. high-glucose treated cells). Similarly, the cell proliferation index of the EC treated with high-glucose was significantly lower than that of the control cells at 48 h (28.5 ± 1.1% vs. 36 ± 2.1% in control cells, $P < 0.05$) and returned to near control value in the presence of stachydrine (34.1 ± 0.9% vs. 28.5 ± 1.1% in high-glucose, $P < 0.05$) (Fig. 3, panel C). Based on these results, the time of 48 h was chosen to elucidate the mechanism by which stachydrine reduces the high-glucose induced toxicity.

INHIBITION OF HIGH-GLUCOSE INDUCED EC SENESCENCE

Accordingly to previous studies [Yuan et al., 2010] the percentage of senescent EC reached a significant level at 48 h after the treatment with high-glucose (Fig. 4, panel A and B). Indeed, high-glucose significantly enhanced SA β-gal activity compared to the control

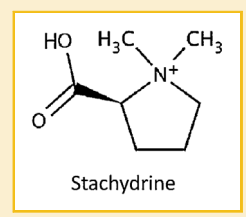

Fig. 1. Chemical structure of stachydrine.

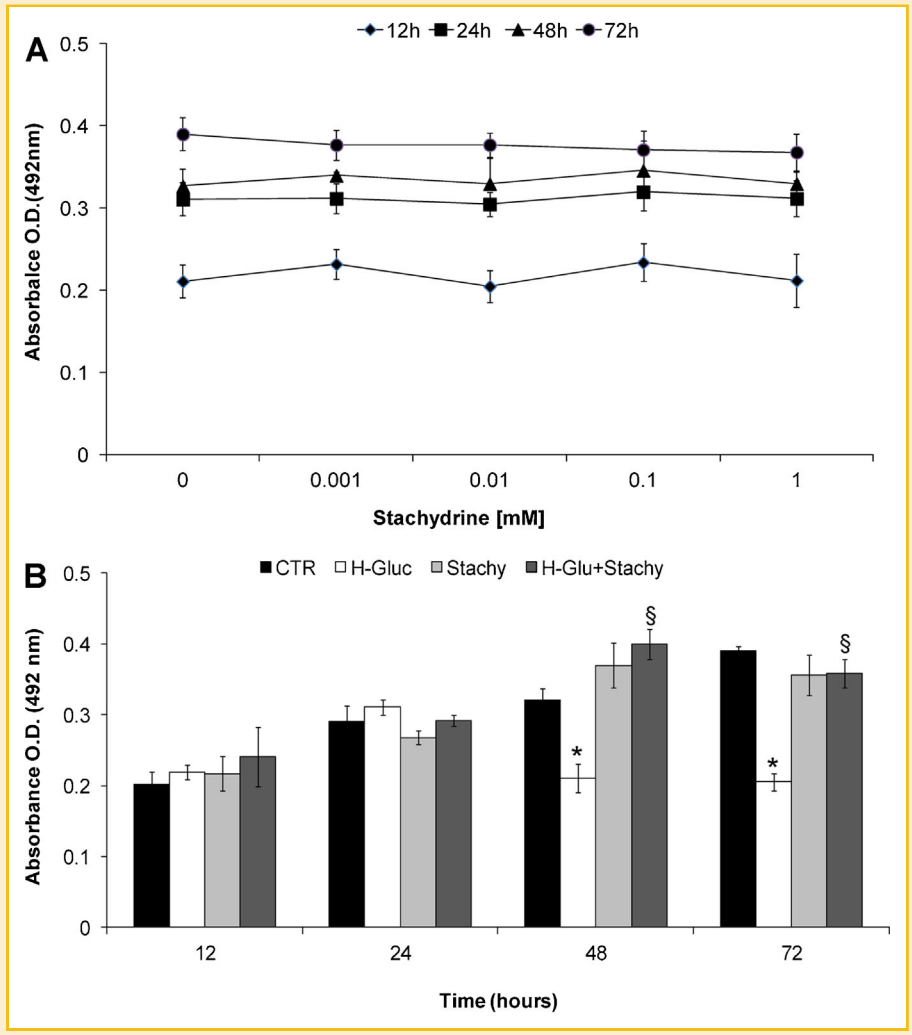

Fig. 2. Time-dependent and dose–response effects of stachydrine on EC cell proliferation. A: Dose–response of stachydrine on EC viability. B: Time course: cells were treated for different times (12, 24, 48, and 72 h) with high-glucose (H-Gluc) (30 mM), high-glucose in the presence of stachydrine (H-Gluc + Stachy), media alone (CTR), or stachydrine alone (Stachy). Results are expressed as mean ± SD of six independent experiments. $^{*}P < 0.05$ versus CTR, $^{\S}P < 0.01$ versus H-Gluc.

cells (32.42 ± 2.78% vs. 11.25 ± 1.48% in control cells, $P < 0.01$) (Fig. 4, panel A and B). On the contrary, the presence of stachydrine during the treatment with high-glucose prevented cell senescence (10.95 ± 2.77% vs. 32.42 ± 2.78% in high-glucose) (Fig. 4, panel A and B).

MODULATION OF P16^{INK4A} PROTEIN EXPRESSION

The effect of stachydrine on the high-glucose induced EC senescence was supported by the parallel change in the expression of p16^{INK4A}, a protein implicated in the senescence program (Fig. 4, panel C and D). Indeed, the expression of p16^{INK4A}, which was up-regulated by high-glucose (1.16 ± 0.04 arbitrary units vs. 0.77 ± 0.05 arbitrary units in control cells $P < 0.05$), was comparable to that observed in control cells when the culture with high-glucose for 48 h was performed in the presence of stachydrine ($P < 0.05$ vs. high-glucose) (Fig. 4, panel C and D).

REGULATORY EFFECT ON SIRT1 EXPRESSION AND ACTIVITY

Downregulation of SIRT1 expression is associated with glucose toxicity either in EC [Yuan et al., 2010] and in EPC [Balestrieri et al., 2008a,2013; Mortuza et al., 2013]. Accordingly, SIRT1 expression was significantly downregulated in a time-dependent manner by high-glucose (Fig. 5). Indeed, confocal laser-scanning microscopy analysis of SIRT1 expression revealed a significant decrease of the fluorescence intensity starting from 24 h after high-glucose culture (24.4 ± 1.5 vs. 35.1 ± 1.9 AFU in control cells, $P < 0.05$). After 48 and 72 h, SIRT1 fluorescence intensity was even lower than that observed after 24 h of treatment with high-glucose (21.6 ± 1.3 and 18.1 ± 1.1 vs. 35.1 ± 1.9 AFU in control cells, respectively) with concomitant changes in cell shape and morphology. When EC were cultured for 48 h with high-glucose plus stachydrine, results indicated that stachydrine modulates either SIRT1 protein expression and activity (Fig. 6). Specifically, as

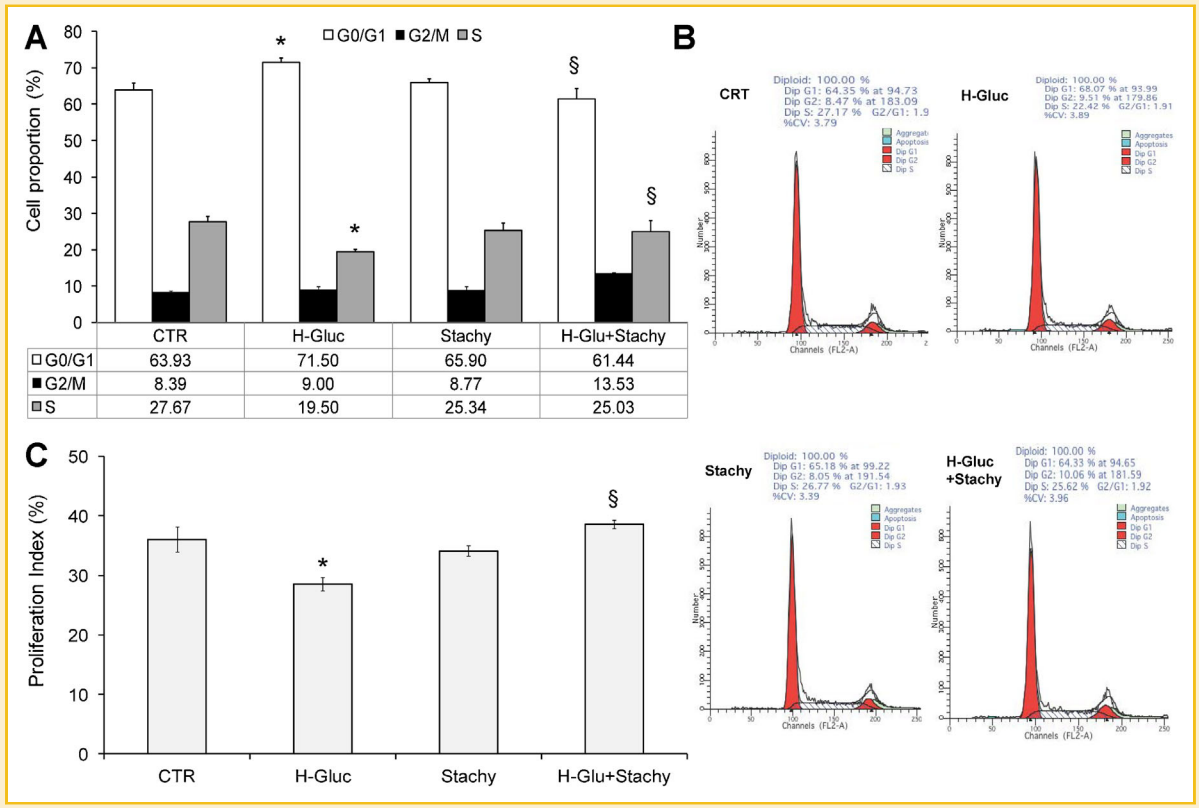

Fig. 3. Effects of stachydrine on cell cycle distribution. A: Percentage of EC proportion after culture for 48 h with high-glucose (H-Gluc), high-glucose in the presence of stachydrine (H-Gluc + Stachy), media alone (CTR), or stachydrine (Stachy). Results are the mean ± SD of five independent experiments. B: Representative FACS cell cycle plots. C: Percentage of EC proliferation index. Results are the mean ± SD of five independent experiments. $^{*}P < 0.05$ versus CTR, $^{\S}P < 0.01$ versus H-Gluc.

demonstrated by confocal laser-scanning microscopy analysis, SIRT1 fluorescence intensity in EC co-treated with high-glucose and stachydrine was comparable to that of control cells (Fig. 6, panel A and B). Similarly to SIRT1 protein expression levels, the analysis of the enzyme activity showed the same results (Fig. 6, panel C). The SIRT1 activity was inhibited by high-glucose (410 ± 33 vs. 950 ± 57 RFU in control cells, $P < 0.05$) and the high-glucose inhibitory effect was prevented by stachydrine co-treatment (690 ± 65 vs. 410 ± 33 RFU in high-glucose cells) ($P < 0.05$).

DISCUSSION

The major findings of the present study are that stachydrine ameliorates the detrimental effect of high-glucose on EC and that its beneficial effect occurs through the modulation of cell senescence signaling and SIRT1 expression. Notably, stachydrine, which showed no cytotoxicity at the concentration used of 0.1 mM, can be assumed through the consumption of a little amount of citrus juices [Servillo et al., 2011a,b]. Indeed, the concentration of 0.1 mM (about 15 mg/L) is the plasma physiological concentration that could be reached after the consumption of just one glass (200 ml) of citrus juice. More in detail, based on the content of stachydrine in orange juice (about 500 mg/L) [Servillo et al., 2011a], one glass of orange juice contains about 100 mg of stachydrine which, if completely absorbed in the intestinal tract, roughly corresponds to a plasma concentration of about 20 mg/L.

Metabolomic studies revealed that this proline betaine, a free radical scavenger that exerts protective effects in the ischaemic myocardium [Liu et al., 2009], was increased significantly in patients with primary dilated cardiomiopathy [Alexander et al., 2011]. To date, although stachydrine can be daily introduced through *Citrus* juice consumption [Servillo et al., 2011a,b], only few in vitro studies describe its effect on the pathophysiologic mechanisms of major human diseases, such as cancer and CVD. In particular, it is known that stachydrine exerts beneficial effect during the injury induced by anoxia-reoxygenation [Yin et al., 2010] and the LPS-induced release of proinflammatory mediators in endothelial cells [Hu et al., 2012]. Endothelial dysfunction and reduced new blood vessel growth represent the most dangerous factors contributing to vascular complications in diabetes [Sheetz and King, 2002; Fadini et al., 2005; Balestrieri et al., 2013]. To date, hyperglycaemia-induced senescence is well recognized as one of the major causal factors in the development of endothelial dysfunction. In the present study, as previously described [Kuki et al., 2006; Yuan et al., 2010], a high-glucose (30 mM) cell culture model was used to simulate clinical hyperglycemia for the in vitro evaluation of the effect of stachydrine on high-glucose induced cytotoxicity. In line with previous studies [Kuki et al., 2006; Yuan et al., 2010], the results revealed that

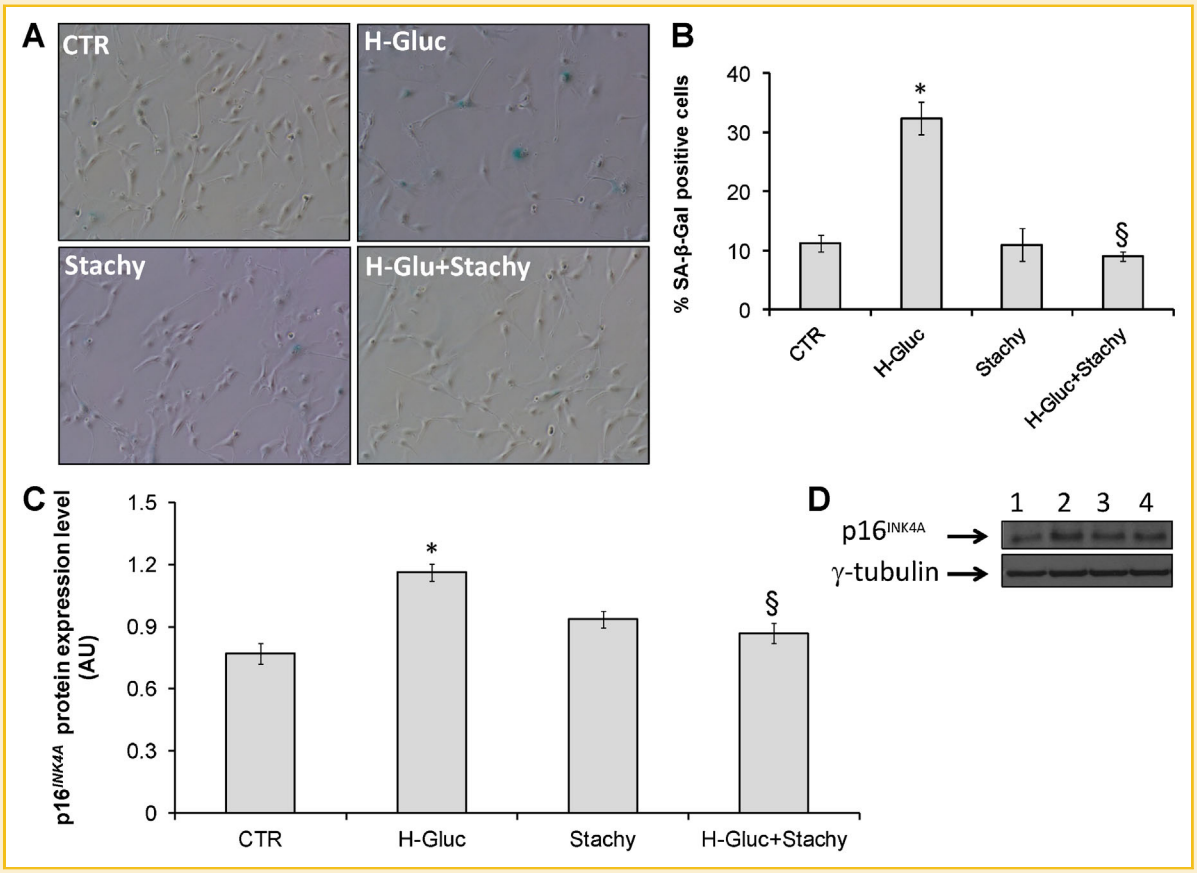

Fig. 4. Effect of stachydrine on high-glucose induced EC senescence. A: Representative images of SA-β-galactosidase staining (blue) of EC grown for 48 h with high-glucose (H-Gluc), high-glucose plus stachydrine (H-Gluc + Stachy), media alone (CTR), or stachydrine (Stachy). B: Percentage of cells positive to SA-β-galactosidase staining. Values are mean ± SD of five independent experiments. $^{*}P < 0.01$ versus CTR, $^{\S}P < 0.01$ versus H-Gluc. C: Western blot analysis of $p16^{INK4A}$ protein expression. Expression of $p16^{INK4A}$ (normalized to γ-tubulin) was measured by immunoblotting after EC treatment for 48 h with media alone (CTR), high-glucose (H-Gluc), stachydrine (Stachy), high-glucose plus stachydrine (H-Gluc + Stachy). Protein level values were expressed as arbitrary units (AU). D: Representative Western blot gel imaging: lane 1, CTR; lane 2, H-Gluc; lane 3, Stachy; lane 4, H-Gluc + Stachy. Data are the mean ± SD of four independent experiments. $^{*}P < 0.05$ versus CTR, $^{\S}P < 0.05$ versus H-Gluc.

incubation of the EC with high-glucose (30 mM) for 48 h determined a consistent reduction of cell viability by arresting cells in the G_0/G_1 cell cycle phase and increasing the percentage of senescent cells. These results confirmed the detrimental role of hyperglycaemia in the EC functionality. However, at the same time, the co-incubation with stachydrine significantly improved EC viability and inhibited cell senescence by modulating $p16^{INK4A}$, tumor suppressor protein known to transduce senescence-signals and mediate entrance of cell into senescence [Lundberg et al., 2000; Balestrieri et al., 2008b]. These results suggest that stachydrine exerts a beneficial effect on hyperglycaemia-induced senescence through regulating $p16^{INK4A}$ pathway. Another key regulator of this pathway is SIRT1, an important player in calorie restricted mediated life span extension [Cohen et al., 2004], which exerts protective effects against endothelial dysfunction by preventing stress-induced premature senescence [Ota et al., 2007]. Results of this study showed that stachydrine counteracts the detrimental effects of high-glucose by both downregulating $p16^{INK4A}$ protein levels and by preventing the inhibition of SIRT1 activity and expression. It is not clear, at the moment, the mechanism by which stachydrine modulates $p16^{INK4A}$ and SIRT1 expressions. Multiple regulatory mechanisms, not approached in this study, act in concert in the signal transduction pathway responsible for SIRT1 regulation in the endothelial dysfunction during altered glucose homeostasis [Revollo and Li, 2013]. First, as an enzyme, the activity of SIRT1 can be directly regulated by substrate availability, post-translational modifications, interacting protein partners, or activators or repressors small molecules [Revollo and Li, 2013]. Second, the alteration of SIRT1 expression levels can be achieved through transcription factors, RNA binding proteins, miRNAs, or the ubiquitin–proteasome system [Revollo and Li, 2013]. Moreover, SIRT1 may function as positive modulator of the endothelial function by stimulating nitric oxide secretion [Yuan et al., 2010]. Many compounds are known to be modulators of SIRT1 [Dai et al., 2010; Sanchez-Fidalgo et al., 2012]. These include the stilbenes resveratrol and piceatannol, the chalcones butein and isoliquiritigenin, and the lavones fisetin and quercetin [Dai et al., 2010; Sanchez-Fidalgo et al., 2012]. In addition, the modulation of SIRT1 expression in high-glucose cultured EC has also been achieved by BTM-0512, a novel

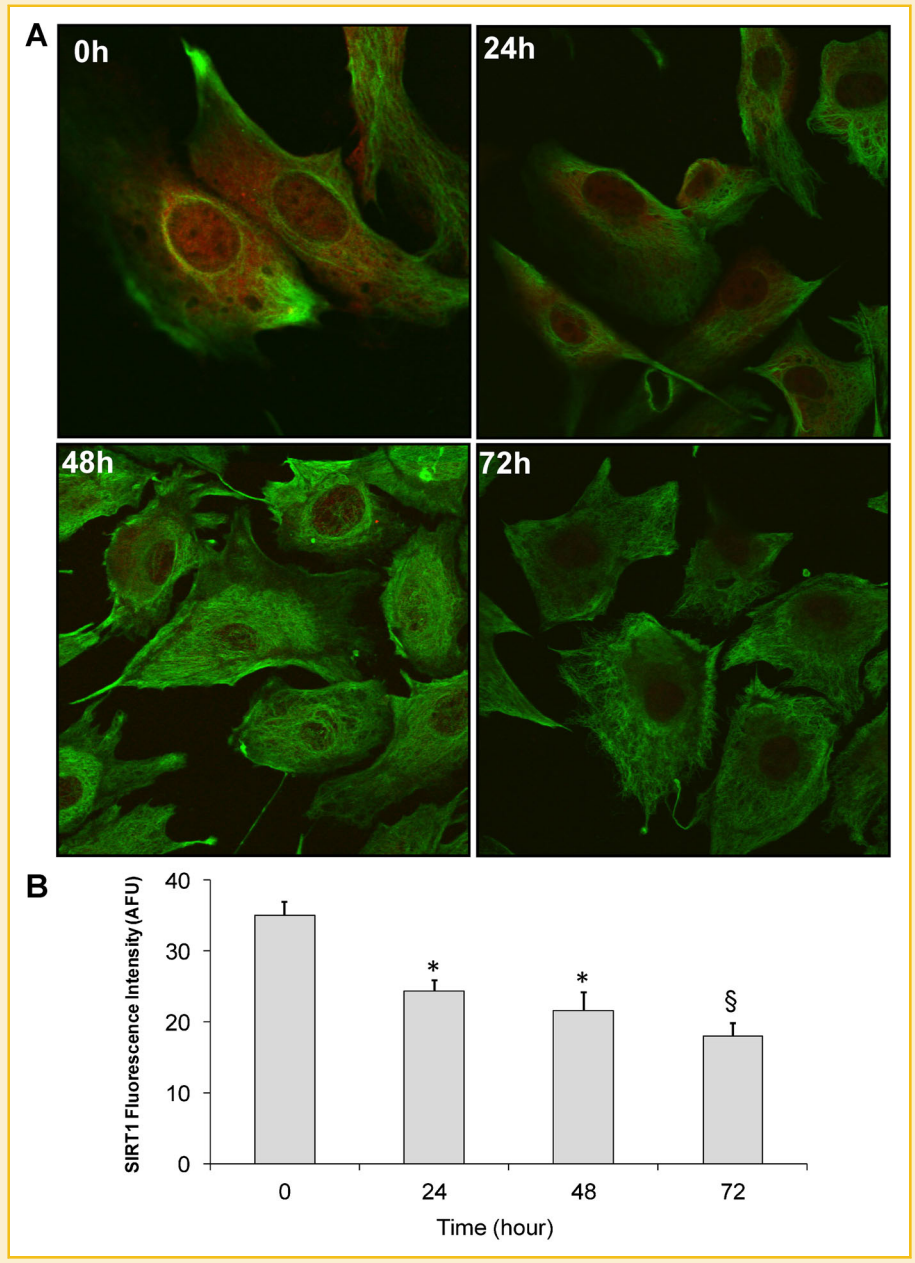

Fig. 5. Evaluation of SIRT1 expression in EC. A: Representative confocal images of EC during a time-dependent culture in the presence of high-glucose. Secondary antibodies were Alexa Fluor 488 and Alexa Fluor 633. B: Immunofluorescence analysis of SIRT1 (red) and vimentin (green) protein levels. The fluorescence intensity was calculated with Image J software analysis and expressed as AFU. Alexa 488 and Alexa 633 fluorescence emissions were acquired in multi-track mode using BP 505–530 and LP650 filters, respectively. The mean ($n = 4$) of the unspecific fluorescence of the secondary antibodies was calculated with ImageJ software and subtracted. Data are mean ± SD of six independent experiments with $^{*}P < 0.05$ versus CTR, $^{\S}P < 0.01$ versus CTR.

derivative of resveratrol, which has been shown to be able to exert a beneficial effect on high glucose-induced cellular senescence through to the activation of SIRT1 and dimethylarginine dimethylaminohydrolase (DDAH)/asymmetric dimethylarginine (ADMA) pathway [Yuan et al., 2010]. It is clear that dysregulation SIRT1 can lead to a multiple pathological conditions and molecules able to modulate it and mimic calorie restriction metabolically are potentially new targets for the prevention and treatment of age-related diseases. Our previous results [Servillo et al., 2013] together with present results indicate that fruits and vegetables, two diet components determinant in the prevention of CVD, are a source of AMDA and stachydrine. It is tempting to speculate that these two compounds, as well as other betaines identified in citus juice [Napoli et al., 2008; Servillo et al., 2013], can contribute to the fine direct/indirect regulation the endothelial function via the modulation of nitric oxide synthase and cellular senescence pathway. However, before drawing a firm

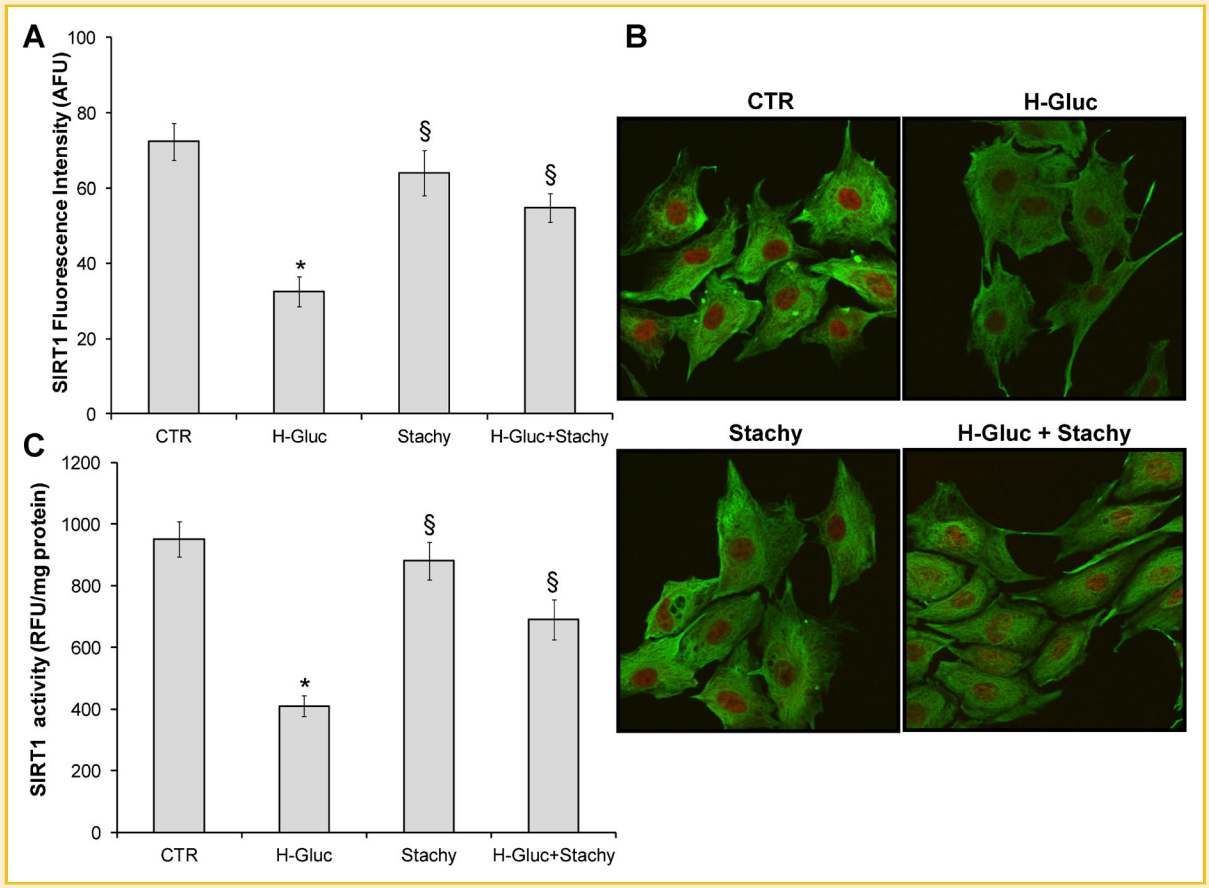

Fig. 6. Effect of stachydrine on high-glucose induced SIRT1 downregulation. A: Immunofluorescence analysis of SIRT1 (Alexa Fluor 633, red) and vimentin (Alexa Fluor 488, green) protein levels was performed with Image J software analysis and expressed as arbitrary fluorescence units (AFU). B: Representative confocal images of EC during a time-dependent culture in the presence of high-glucose showing changes in cell morphology and SIRT1 expression. C: SIRT1 activity was determined by Fluor de Lys kit and expressed as relative fluorescence units (RFU)/mg protein. Data are mean ± SD of five independent experiments with $^{*}P < 0.05$ versus CTR, $^{\S}P < 0.05$ versus H-Gluc. Control, CTR; high-glucose, H-Gluc; stachydrine, Stachy; high-glucose with stachydrine, H-Gluc + Stachy.

conclusion, further long-term in vivo studies or animal studies, a more suitable method for investigation of vascular pathophysiology, are needed to have an accurate estimation of the extent of intestinal adsorption and to confirm the beneficial effect of on the hyperglycaemia-induced endothelial dysfunction. In the present study, although the in vitro 48 h high-glucose treatment of EC reflects only the temporary effect of high-glucose on EC, we clearly established that stachydrine, an abundant component of citrus juices, is able to prevent the high-glucose cytotoxicity in EC by acting on the senescence and SIRT1 pathways.

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