

RESEARCH PAPER

Metformin modulates hyperglycaemia-induced endothelial senescence and apoptosis through SIRT1

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BACKGROUND AND PURPOSE

Endothelial dysfunction can be detected at an early stage in the development of diabetes-related microvascular disease and is associated with accelerated endothelial senescence and ageing. Hyperglycaemia-induced oxidative stress is a major contributing factor to the development of endothelial dysfunction. Clinical data indicate that the hypoglycaemic agent, metformin, has an endothelial protective action; however, its molecular and cellular mechanisms remain elusive. In the present study, we have investigated the protective effect of metformin during hyperglycaemia-induced senescence in mouse microvascular endothelial cells (MMECs).

EXPERIMENTAL APPROACH

MMECs were cultured in normal glucose (11 mM) and high glucose (HG; 40 mM) in the presence and absence of metformin (50 µM) for 72 h. The expression of sirtuin-1 (SIRT1) and senescence/apoptosis-associated markers was determined by immunoblotting and immunocyto techniques. SIRT1 expression was inhibited with appropriate siRNA.

KEY RESULTS

Exposure of MMECs to HG significantly reduced SIRT1 protein expression, increased forkhead box O1 (FoxO-1) and p53 acetylation, increased p21 and decreased Bcl2 expression. In addition, senescence-associated β-galactosidase activity in MMECs was increased in HG. Treatment with metformin attenuated the HG-induced reduction of SIRT1 expression, modulated the SIRT1 downstream targets FoxO-1 and p53/p21, and protected endothelial cells from HG-induced premature senescence. However, following gene knockdown of SIRT1 the effects of metformin were lost.

CONCLUSIONS AND IMPLICATIONS

HG-induced down-regulation of SIRT1 played a crucial role in diabetes-induced endothelial senescence. Furthermore, the protective effect of metformin against HG-induced endothelial dysfunction was partly due to its effects on SIRT1 expression and/or activity.

Abbreviations

AMPK, AMP-activated protein kinase; CVD, cardiovascular disease; eNOS, endothelial NOS; FoxO-1, forkhead box O1; LKB-1, liver kinase B-1; MMECs, mouse microvascular endothelial cells; ROS, reactive oxygen species; SIRT1, sirtuin 1

Introduction

Diabetes is a major global burden that currently affects more than 400 million people worldwide with a predicted >50% increase in prevalence by 2030 (Whiting *et al.*, 2011; Chen *et al.*, 2012). Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in patients with diabetes, and diabetes-associated vascular complications are the primary contribution to the higher mortality rate that is seen in diabetic subjects (Grundy *et al.*, 2002). Microvascular disease in particular has been linked to hyperglycaemia (DCCT, 1993). Furthermore, endothelial dysfunction is associated with the development of premature ageing-related changes in the vasculature that include arterial stiffening, impaired angiogenesis and defective vascular repair, and is regarded as an important causative factor in the onset of diabetes-associated CVD (Erusalimsky, 2009).

Endothelial senescence is a major contributor to vascular ageing (Erusalimsky, 2009; Wang and Bennett, 2012) and is accelerated by hyperglycaemia-induced generation of reactive oxygen species (ROS) (Triggle and Ding, 2010). The superoxide radical generated by NADPH oxidase system is both a major source of ROS and a major contributor to the development of diabetes-associated endothelial dysfunction (Ding *et al.*, 2007; Giacco and Brownlee, 2010; Triggle and Ding, 2010). Substantial evidence indicates that hyperglycaemia-induced ROS formation and decreased bioavailability of NO help promote the shortening of telomere length, increase DNA damage, increase genomic instability and growth arrest, thus resulting in premature senescence in endothelial cells (Hoffmann *et al.*, 2001; Hayashi *et al.*, 2006; Yokoi *et al.*, 2006; Ota *et al.*, 2008; Zhong *et al.*, 2010; Matsui-Hirai *et al.*, 2011). These changes result in an endothelial cell phenotype that is pro-inflammatory, pro-thrombotic and pro-atherosclerotic (Erusalimsky, 2009), and also negatively affects the vasodilatory, angiogenic and regenerative properties of endothelial cells, thus accelerating the development of diabetes-associated CVD (Testa and Ceriello, 2007).

Sirtuin 1 (SIRT1), the closest mammalian homologue of Sir2 (Frye, 1999), plays a crucial role in cell proliferation, angiogenesis and endothelium-dependent vasodilatation via deacetylation of endothelial NOS (eNOS), thus increasing the bioavailability of NO (Nisoli *et al.*, 2005; Mattagajasingh *et al.*, 2007; Potente *et al.*, 2007; Arunachalam *et al.*, 2010). Conversely, inhibition of SIRT1 increases NADPH oxidase activation and subunit expression, and reduces ACh-mediated endothelium-dependent vasodilatation in rat thoracic aorta (Zarzuelo *et al.*, 2013). Results from *in vitro* and *in vivo* studies indicate that SIRT1 plays a central role in the regulation of endothelial cell growth, senescence and apoptosis (Menghini *et al.*, 2009; Zhao *et al.*, 2010; Bai *et al.*, 2012). Furthermore, SIRT1 activation or overexpression confers protection against diabetes-induced hyperglycaemic memory and vascular dysfunction (Ota *et al.*, 2008; Orimo *et al.*, 2009; Zhou *et al.*, 2011; Paneni *et al.*, 2013). Polymorphisms in SIRT1 may also contribute to variations in glucose tolerance and increased risk of mortality in humans (Figarska *et al.*, 2013).

Metformin, a biguanide, is the most widely prescribed oral hypoglycaemic drug for the treatment of type 2 diabetes. Metformin lowers blood glucose and improves insulin sensi-

tivity by reducing hepatic gluconeogenesis via the activation of AMP-activated protein kinase (AMPK) as well as promoting AMPK-mediated translocation of glucose transporter type 4 (Yang and Holman, 2006; Viollet *et al.*, 2012). In addition to its hypoglycaemic actions in human subjects, metformin improves endothelial function (Mather *et al.*, 2001). Furthermore, in animal models of diabetes and in cell culture studies, metformin improves eNOS activity and endothelial function (Davis *et al.*, 2006; Matsumoto *et al.*, 2008; Sena *et al.*, 2011). SIRT1 activation by metformin significantly attenuates ROS-mediated activation of the transcription factor and regulator of inflammatory responses, NF- κ B, in bovine retinal capillary endothelial cells and retinal endothelial cells from diabetic rats (Zheng *et al.*, 2012). However, whether SIRT1 expression is critical for mediating the beneficial effects of metformin on microvascular endothelial function has not been clearly demonstrated. For instance, metformin has also been shown to activate an atypical protein kinase C-regulated cAMP response element-binding protein pathway, PKC-CBP, which results in the recruitment of neural stem cells and enhanced neural function (Wang *et al.*, 2012). Hence, metformin may act via multiple cellular targets to protect cell function. In the current study, we have tested, using a gene knockdown approach, the hypothesis that metformin modulated hyperglycaemia-induced premature senescence and apoptosis in mouse microvascular endothelial cells (MMECs) via a SIRT1-dependent signalling pathway(s).

Methods

Endothelial cell culture

MMECs were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM consisting of either normal glucose (NG; 11 mM) or high glucose (HG; 40 mM) for 72 h (media were changed every 24 h) as previously described (Ding *et al.*, 2007). Glucose concentrations were chosen on the basis of random glucose measurements taken from normoglycaemic mice, which approximate 11.0 mM, and type 2 diabetic *db/db* mice, which range from approximately 25 to 55 mM (Pannirselvam *et al.*, 2002; Semeniuk *et al.*, 2002), and as also detailed in the Jackson Laboratory Physiological Data Summary for C57BL/6J mice. For the studies comparing the effects of mannitol and 3-O-methyl-D-glucopyranoside (3-OMG), MMECs were incubated with different media that were defined as NG (glucose concentration of 11 mM), high glucose (HG; 40 mM), osmotic control (40 mM: 11 mM of glucose + 29 mM of D-mannitol) and non-metabolizable glucose (3-O-methyl glucose, 3-OMG; 40 mM: 11 mM of glucose + 29 mM of 3-OMG) for 72 h. Media were changed every 24 h.

SIRT1 gene silencing

MMECs grown in antibiotic-free DMEM on culture dishes and/or coverslips for 24 h were transfected with SIRT1 small-interfering (siRNA; 10 nM; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and non-targeted control (scrambled) siRNA using Opti-MEM® I reduced serum media and Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). After 72 h of transfection,

cells were washed and processed for immunoblotting and fluorescence studies as described next.

Cell culture treatment protocols

MMECs placed in culture media (DMEM) on culture dishes and/or coverslips were exposed to either NG or HG alone or with metformin (50 μ M) for 72 h. The concentration of metformin used in this study was estimated as that approximating peak plasma concentration in clinical use (Tucker *et al.*, 1981; Sambol *et al.*, 1996) and equivalent to the lowest concentration reported for a cell culture studies with bovine aortic endothelial cells (Davis *et al.*, 2006). For the SIRT1 knockdown studies, media were changed (either NG or HG) every 24 h, and treatment with or without metformin was initiated 12 h after transfection.

Immunoblotting

Immunoblotting was used to detect the expression of SIRT1, forkhead box O1 (FoxO-1), Ac-p53, p53, B-cell lymphoma 2 (Bcl-2; Cell Signaling Technology, Inc., Beverly, MA, USA), Ac-FoxO-1, p-FoxO-1, p21 and senescence marker protein-30 (SMP-30) (Santa Cruz Biotechnology, Inc.) as previously described (Arunachalam *et al.*, 2010). Briefly, cellular protein (50 μ g) was analysed by electrophoresis on SDS-PAGE gels and transblotted on nitrocellulose membrane. Membranes were blocked with 5% (w/v) non-fat milk or BSA in PBS containing 0.1% (v/v) Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) and incubated with relevant primary antibody (1:1000 dilution). After washing, bound antibody was detected using anti-rabbit/mouse antibody (1:5000 dilution) linked to HRP, and bound complexes were detected and documented using enhanced chemiluminescence method (Bio-Rad, Hercules, CA, USA) and Geliance imaging system (PerkinElmer, Waltham, MA, USA).

Measurement of ROS dihydroethidium (DHE) staining

DHE was used to monitor superoxide production. DHE (excitation/emission wavelengths of 535/610 nm for oxidized product) reacts with superoxide to yield ethidium, which binds to nuclear DNA and generates nuclear fluorescence. MMECs grown on coverslips were washed with PBS and incubated with DHE (5 mM, in ice-cold methanol) in a light-protected humidified chamber for 30 min at room temperature. Ethidium fluorescence was examined and imaged by fluorescence confocal microscopy (Carl Zeiss, Oberkochen, Germany), and comparative levels of fluorescence were analysed using ImageJ software [National Institutes of Health (NIH), Bethesda, MD, USA].

Immunofluorescence staining

Immunofluorescence staining was used to detect Ac-FoxO-1 and p21 levels in MMECs. Briefly, cells were grown on coverslips washed with 1 $\times$ PBS, fixed in 4% paraformaldehyde, permeabilized using 100% chilled methanol and incubated with blocking buffer for 1 h (5% goat serum in 1 $\times$ PBS). Cells were then incubated with primary antibodies (Ac-FoxO-1 and p21), followed by staining with appropriate secondary antibodies (Alexa Fluor 488/555; Cell Signaling Technology, Inc.) and DAPI (Invitrogen) to reveal the nuclei. Coverslips were

mounted in fluorescence mounting medium (Vector Lab, Burlingame, CA, USA), and the cells were viewed and photographed by confocal microscopy (Carl Zeiss).

Senescence-associated β -galactosidase staining

The senescence-associated β -galactosidase activity was detected in formaldehyde-fixed cells according to the manufacturer's instructions (Sigma-Aldrich). The assay is based on a histochemical stain for β -galactosidase activity at pH 6 and found to be specific for senescent endothelial cells, but not for quiescent or terminally differentiated cells. In brief, the culture media were removed and the cells were then washed once with PBS and fixed with the fixation buffer for 6–7 min at room temperature. After two additional washes with PBS, the staining solution [400 mM citric acid/sodium phosphate (pH 6.0), 1.5 M NaCl, 20 mM MgCl₂, 5-bromo-4-chloro-3-indolyl- β -D-galactoside, 500 mM potassium ferrocyanide] was added to each well. Cells were incubated at 37°C overnight and then observed and photographed under a microscope (Colibri; Carl Zeiss) for development of blue colour. The percentage of blue cells versus total cells was counted using ImageJ software (NIH).

Data analysis

Results are presented as mean $\pm$ SEM. All data were analysed by statistical software 'GraphPad Prism 5.0' (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis was performed using one-way ANOVA. *Post hoc* comparisons between the groups were performed by Bonferroni multiple comparisons test, with $P < 0.05$ used to indicate statistical significance.

Materials

Unless otherwise stated all chemicals used were of analytical grade and purchased from Sigma-Aldrich.

Results

Hyperglycaemia accelerates endothelial senescence via changes in expression of SIRT1 and downstream signalling targets

The effect of HG on SIRT1 expression and downstream signalling targets in MMECs was determined by immunoblotting (Figure 1A–F). Exposure of MMECs to HG for 72 h resulted in a significant decrease in SIRT1 protein expression, compared with MMECs that had been cultured for the same period of time in NG ($P < 0.05$; Figure 1A and C). The expression levels of phosphorylated and acetylated FoxO-1 and p21 proteins, the downstream targets of SIRT1 and important mediators of vascular senescence and apoptosis, were also determined. The decrease in expression of SIRT1 was accompanied by a decrease in p-FoxO-1 ($P < 0.05$; Figure 1B and E) and significant increases in p21 protein expression ($P < 0.05$; Figure 1A and D) and Ac-FoxO-1 ($P < 0.05$; Figure 1B and F) when compared with MMECs in NG. These results reveal that down-regulation of SIRT1 protein expression is associated with increased FoxO-1 acetylation and p21 expression in microvascular endothelial cells maintained in HG for 72 h.

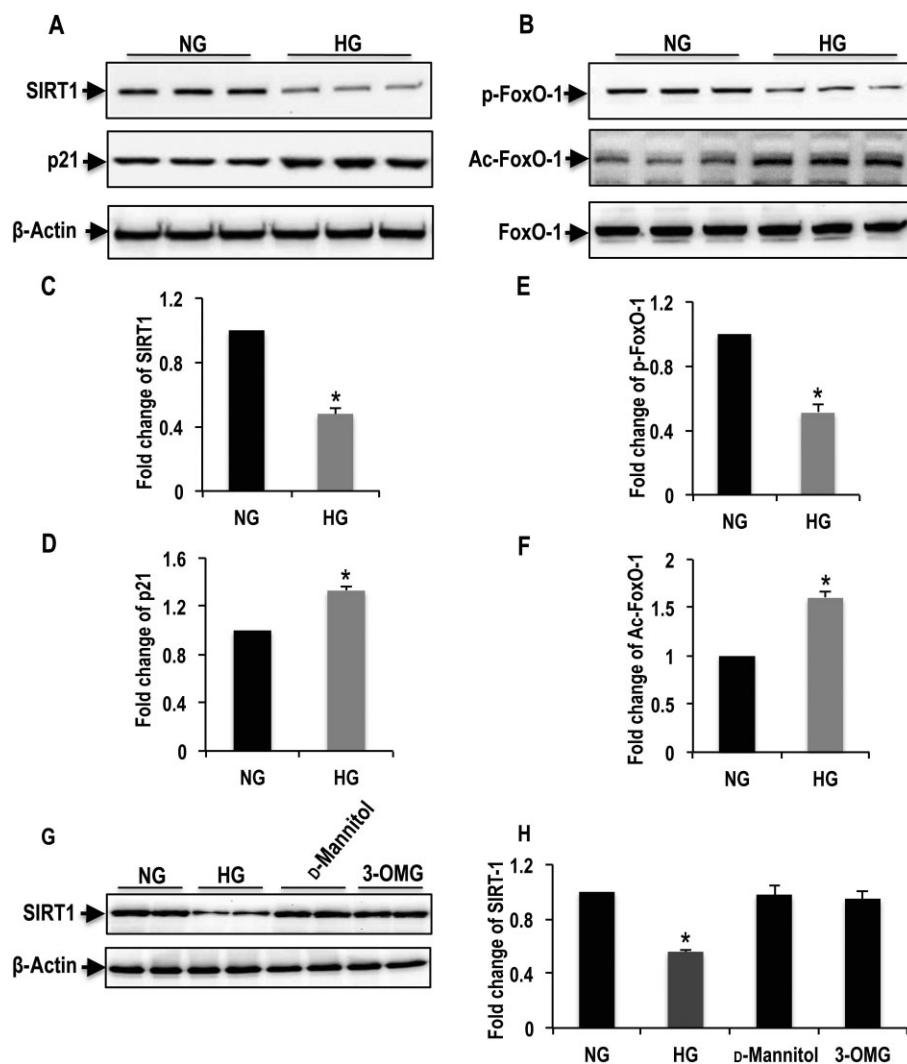


Figure 1

Effect of HG exposure on SIRT1, FoxO-1 and p21 expression in MMECs. MMECs were cultured either in NG (11 mM) or HG (40 mM) media for 72 h, and SIRT1, p21, p-FoxO-1, Ac-FoxO-1 and FoxO-1 protein levels were determined by immunoblotting (A, B). Results were normalized to controls, and histograms represent the relative intensity of SIRT1, p21, p-FoxO-1 and Ac-FoxO-1 (C–F). Values represent mean $\pm$ SEM ($n = 3$ –4 per group). * $P < 0.05$, significantly different from NG. For the studies comparing the effects of mannitol and 3-OMG, MMECs were also incubated with media consisting of NG (11 mM) or HG (40 mM), D-mannitol and 3-OMG for 72 h, and SIRT1 protein levels were determined by immunoblotting (G). Histogram represents the relative intensity of SIRT1 (H). Values represent mean $\pm$ SEM ($n = 3$ –4 per group). * $P < 0.05$, significantly different from NG.

Exposure of MMECs to D-mannitol (osmotic control) and the non-metabolizable glucose analogue, 3-OMG for 72 h, did not alter SIRT1 expression ($P < 0.05$; Figure 1G and H).

Metformin reduces HG-induced oxidative stress, senescence and apoptosis in microvascular endothelial cells

Experiments were designed to determine whether the protective effect of metformin in MMECs exposed to HG for 72 h was associated with SIRT1 activation. MMECs were maintained in media containing either NG or HG alone or with metformin and stained with DHE for the measurement of ROS (Figure 2A and B). The intracellular production of ROS

was increased (as shown by increased nuclear fluorescence) in MMECs in HG, compared with ROS-induced nuclear fluorescence in MMECs in NG. However, treatment of MMECs maintained in HG with metformin resulted in lower levels of ROS, compared with MMECs in HG that were not treated with metformin (Figure 2A). Senescence-associated β -galactosidase staining was increased in MMECs in HG, compared with MMECs maintained in NG (Figure 2C and D). Nonetheless, evidence of senescence was also present in the MMECs that were maintained in NG, suggesting that the effects of glucose were concentration-dependent. However, MMECs in HG that were treated with metformin showed reduced endothelial senescence, compared with untreated MMECs in HG.

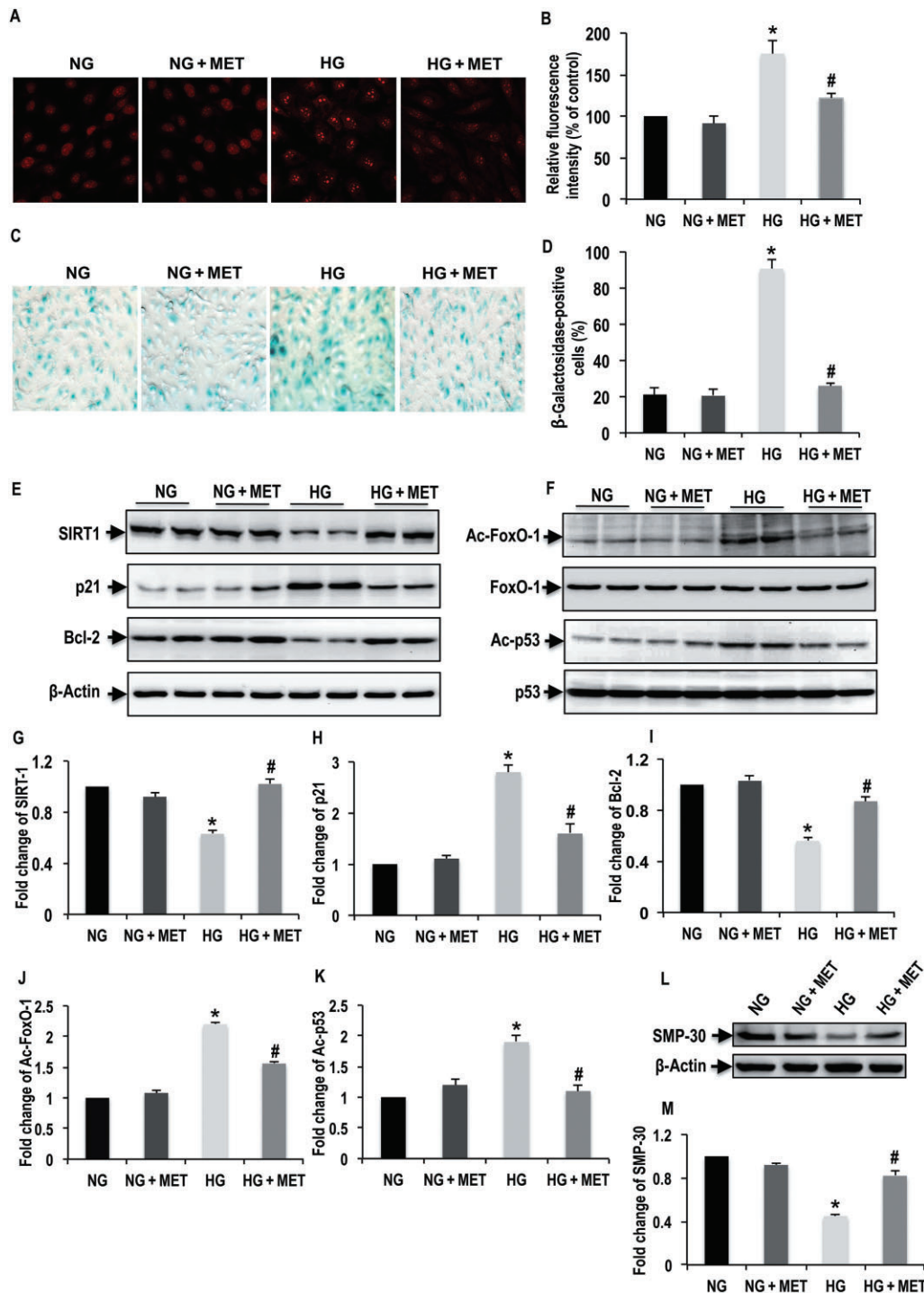


Figure 2

Effect of treatment with metformin on HG-induced oxidative stress, senescence, SIRT1 protein expression and its downstream signalling in MMECs. MMECs were cultured either in NG (11 mM) or HG (40 mM) media for 72 h in the presence or absence of metformin (MET; 50 μ M). Cells were fixed and stained with DHE to detect intracellular ROS levels (A; 63 $\times$ magnification). Panel B shows the relative intensity of ethidium fluorescence signal. Values represent mean $\pm$ SEM. * P < 0.05, significantly different from NG; # P < 0.05, significantly different from HG. Cells were fixed and stained for senescence-associated β -galactosidase activity (C; 20 $\times$ magnification). Histogram represents the percentage of senescence-associated β -galactosidase-positive cells (D). Values represent mean $\pm$ SEM. * P < 0.05, significantly different from NG; # P < 0.05, significantly different from HG. A representative image from three separate experiments is illustrated. Cell lysates were used to detect the SIRT1, p21, Bcl-2, Ac-FoxO-1, FoxO-1, Ac-p53, p53 and SMP-30 protein levels by immunoblotting (E–F and L). Results were normalized to controls, and histograms represent the relative intensity of SIRT1, p21, Bcl-2, Ac-FoxO-1, Ac-p53 and SMP-30 levels (G–K and M). Values represent mean $\pm$ SEM (n = 3–4 per group). * P < 0.05, significantly different from NG; # P < 0.05, significantly different from HG.

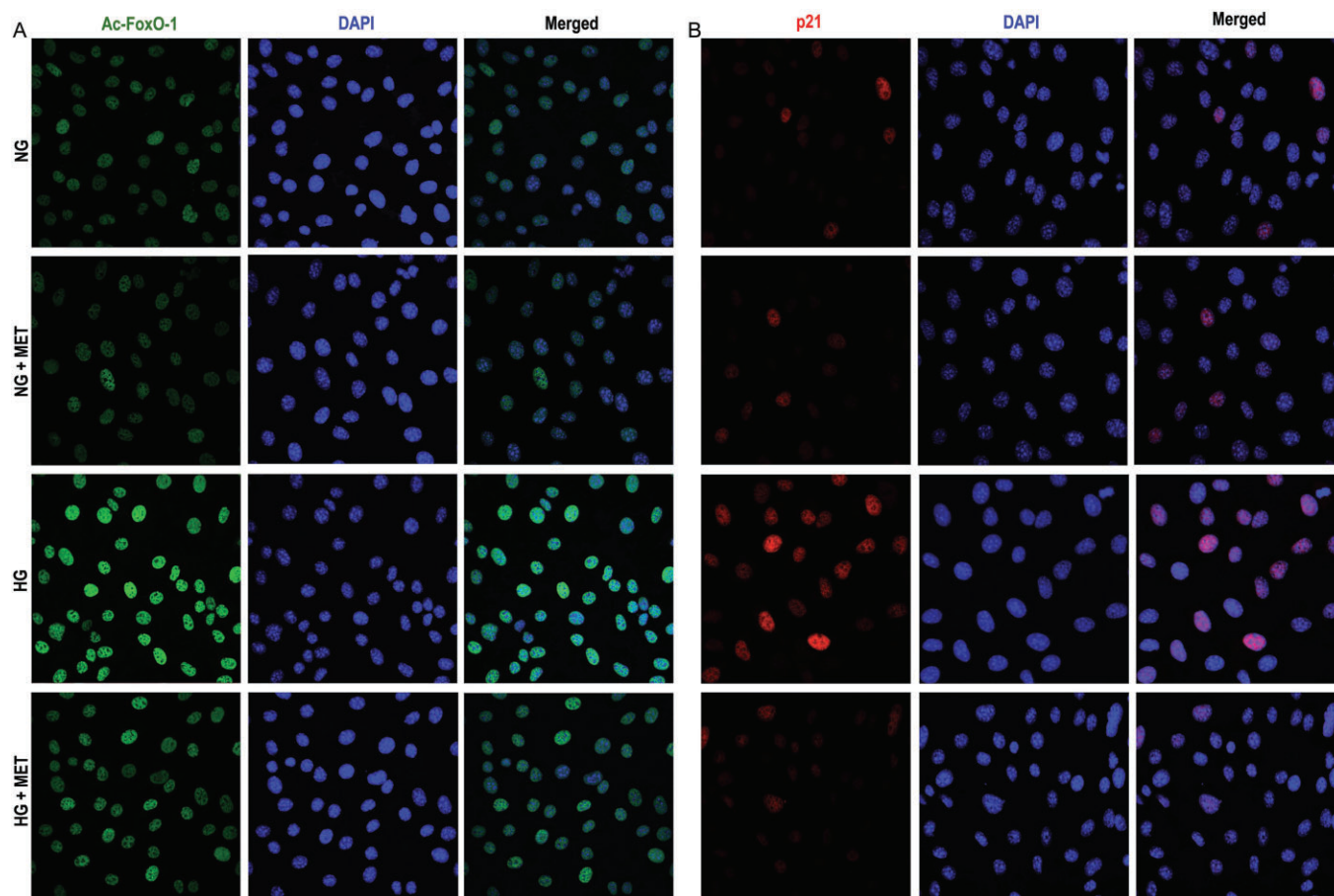


Figure 3

Effect of treatment with metformin during HG exposure on expression of Ac-FoxO-1 and p21 in MMECs. MMECs were cultured either in NG (11 mM) or HG (40 mM) media for 72 h in the presence and absence of metformin (50 μ M). Acetylated FoxO-1 (A) and p21 (B) levels were measured by immunofluorescence staining. The images are taken by 63 $\times$ magnification. A representative image from three separate experiments is illustrated.

In order to assess the cellular and molecular basis of the metformin-associated decrease in endothelial senescence, we examined SIRT1 expression and explored changes in the downstream signalling pathways for SIRT1 in MMECs treated with HG and metformin for 72 h. There was a significant reduction in SIRT1 and increase of p21 ($P < 0.05$; Figure 2E, G and H) as well as a subsequent increase in acetylation of FoxO-1 and p53 in MMECs maintained in HG, but these changes were prevented when the MMECs were also treated with metformin ($P < 0.05$; Figure 2F, J and K). Furthermore, there was also a significant decrease in anti-apoptotic Bcl-2 protein in HG, compared with MMECs in NG, but this decrease was prevented when the cells exposed to HG were treated with metformin ($P < 0.05$; Figure 2E and I). Similarly, the immunofluorescence studies showed that treatment with metformin attenuated FoxO-1 acetylation and p21 expression in MMECs that were exposed to HG (Figure 3A and B).

SMP-30 has been identified as an important protein marker of ageing. HG exposure also reduced SMP-30 protein expression in MMECs ($P < 0.05$; Figure 2L and M). Treatment with metformin increased SMP-30 protein in MMECs with HG, compared with MMECs that were not exposed to metformin.

Vascular-protective action of metformin is SIRT1 dependent

In order to ascertain whether the endothelial protective effect of metformin was SIRT1 dependent, the effect of metformin in SIRT1-silenced MMECs was investigated in NG and HG cell culture protocols. Under NG conditions, endothelial senescence (assessed by β -galactosidase activity) was increased following SIRT1 knockdown (Figure 4A and B) and persisted despite treatment with metformin. The immunoblotting data illustrated in Figure 4C–I also provide molecular evidence for the SIRT1-dependent action of metformin. Thus, there was increased expression of p21 (Figure 4C and F) as well as in FoxO-1 and p53 acetylation (Figure 4D, H and I) in SIRT1-silenced MMECs, compared with MMECs transfected with control (scrambled) siRNA. Furthermore, the knockdown of SIRT1 in MMECs significantly decreased the expression of Bcl-2 protein, compared with MMECs transfected with control siRNA (Figure 4C and G). SIRT1-silenced endothelial cells showed a significant decrease in SMP-30 protein expression (Figure 4J and K). In addition, the results from the immunofluorescence studies (Figure 5A and B) also revealed that SIRT1-silenced endothelial cells showed increased

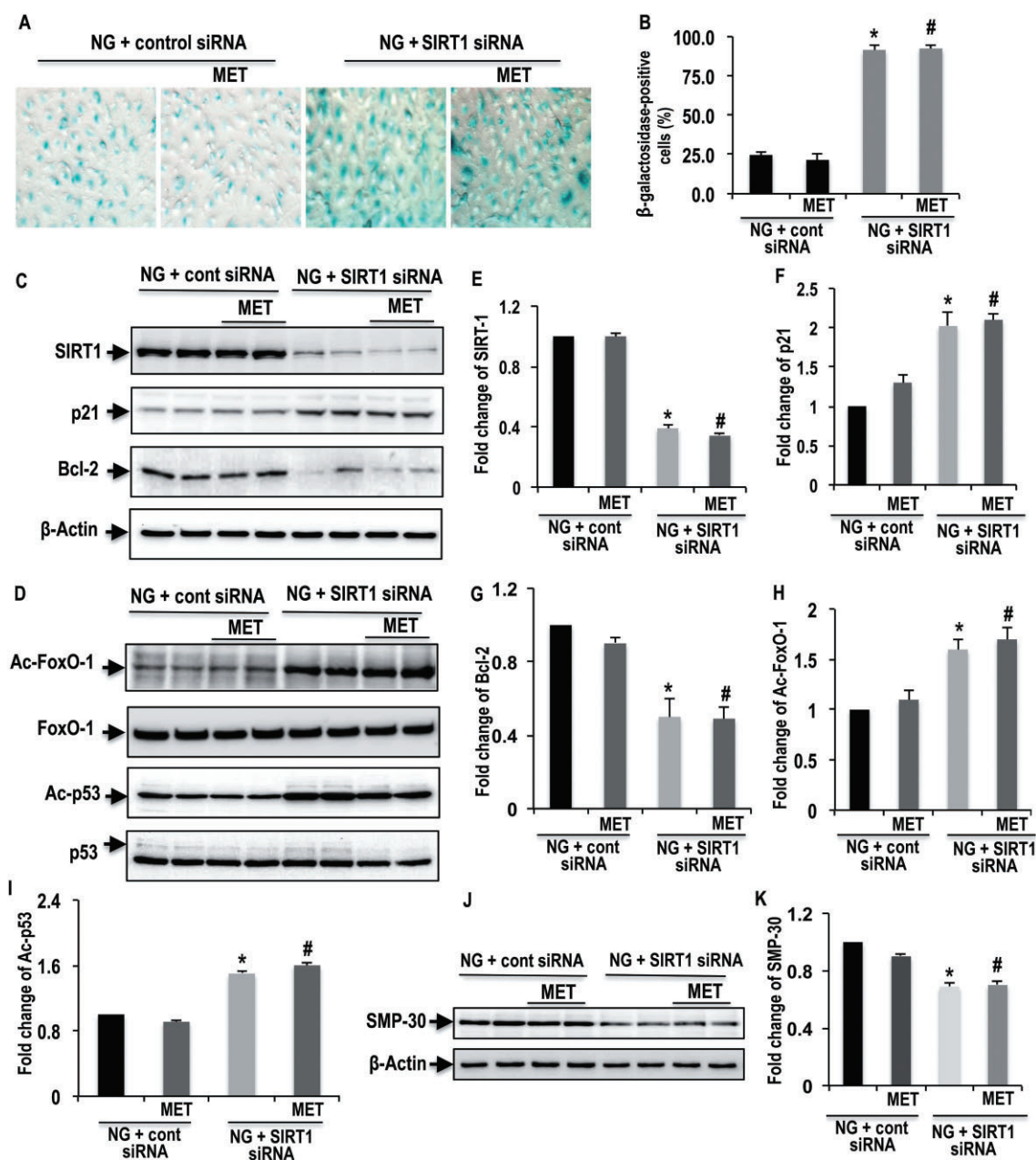


Figure 4

SIRT1 knockdown accelerates endothelial cell senescence. MMECs were transfected with either control siRNA or SIRT1 siRNA and exposed to NG (11 mM) in the presence or absence of metformin (MET). After 72 h of transfection, cells were fixed and stained for senescence-associated β -galactosidase activity (A; 20 $\times$ magnification). Histogram represents the percentage of senescence-associated β -galactosidase-positive cells (B). Values represent mean $\pm$ SEM. * P < 0.05, significantly different from control siRNA; # P < 0.05, significantly different from siRNA-transfected cells alone or with metformin. A representative image from three separate experiments is illustrated. Cell lysates were used for immunoblotting assessment of SIRT1, p21, Bcl-2, Ac-FoxO-1, FoxO-1, Ac-p53, p53 and SMP-30 levels (C, D and J). Results were normalized to controls, and histograms represent the relative intensity of SIRT1, p21, Bcl-2, Ac-FoxO-1, Ac-p53 and SMP-30 levels (E–I and K). Values represent mean $\pm$ SEM (n = 3–4 per group). * P < 0.05, significantly different from control siRNA; # P < 0.05, significantly different from control siRNA-transfected cells alone or with metformin.

FoxO-1 acetylation and expression of p21 protein, compared with MMECs transfected with control siRNA. However, metformin no longer modulated the expression of FoxO-1, p21, p53 and Bcl-2 in SIRT1-silenced endothelial cells.

To determine the SIRT1-dependent effect of metformin in MMECs exposed to HG, SIRT1 was silenced with SIRT1 siRNA

and then treated with HG and metformin. In control (scrambled) siRNA-transfected cells, endothelial senescence was decreased after metformin treatment, compared with MMECs exposed to HG, but not treated with metformin. However, in SIRT1-silenced cells, β -galactosidase activity remained elevated despite the presence of metformin (Figure 6A and B).

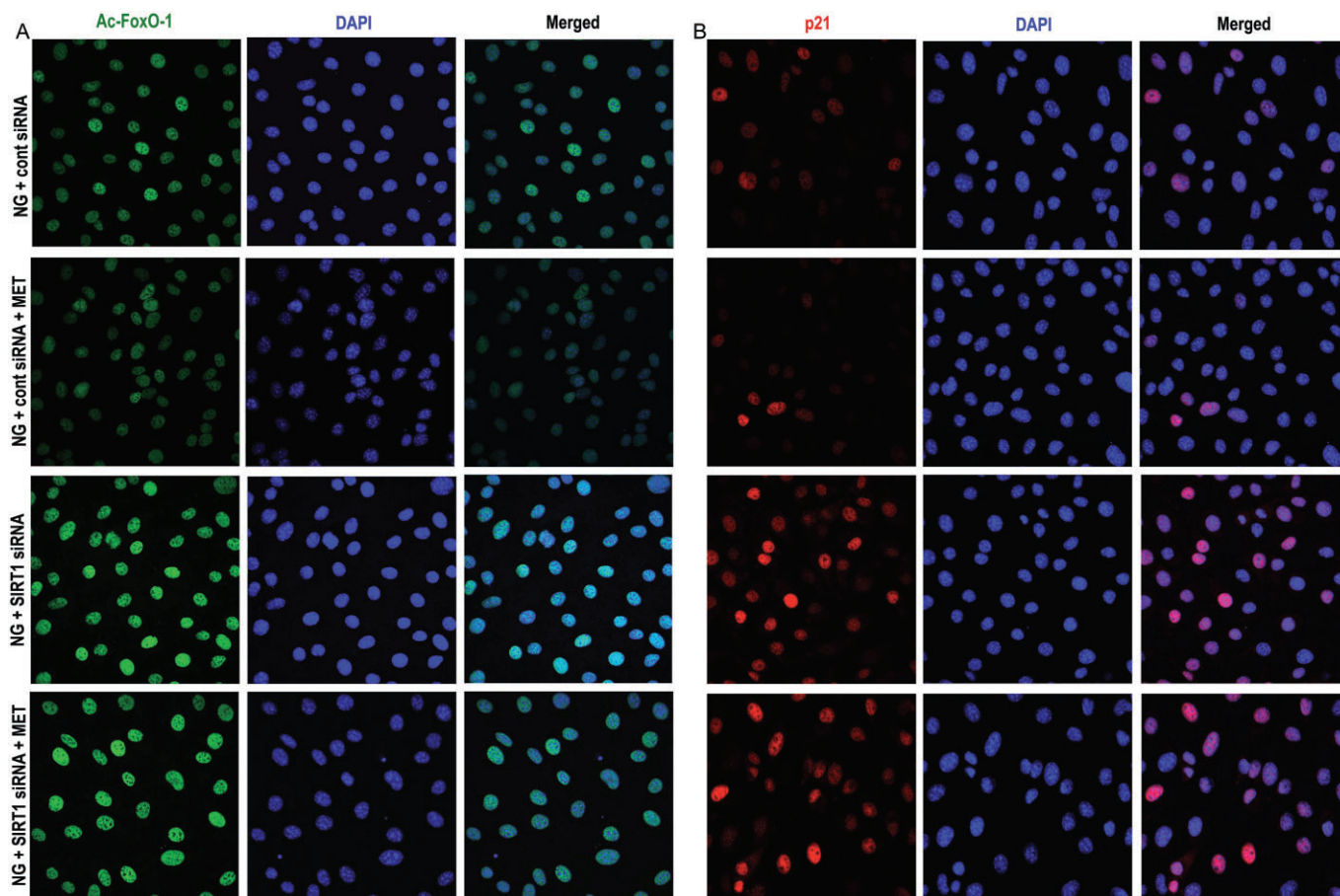


Figure 5

Effect of SIRT1 silencing on expression of Ac-FoxO-1 and p21 in MMECs. MMECs were transfected with either control siRNA or SIRT1 siRNA alone or with metformin (MET). Immunofluorescence staining was used to measure the level of protein expression for Ac-FoxO-1 (A) and p21 (B). The images were taken with a magnification of 63 $\times$. A representative image from three separate experiments is illustrated.

Likewise, immunoblotting studies also indicated that, in control siRNA-transfected cells, in HG, the expression of p21 (Figure 6C and F), acetylated FoxO-1 (Figure 6D and H) and p53 (Figure 6D and I) proteins were attenuated upon treatment of metformin with increased Bcl-2 expression, compared with HG alone. However, treatment with metformin did not reduce the expression of the Ac-FoxO-1, Ac-p53 and p21 proteins or increase Bcl-2 protein in SIRT1-silenced endothelial cells, particularly in HG condition indicating that metformin-associated reduction in HG-induced endothelial senescence was mediated through a SIRT1-dependent mechanism(s).

Discussion and conclusions

Metformin prevents hyperglycaemia-reduced SIRT1 expression in microvascular endothelial cells

Data from the current study support the hypothesis that incubation in HG media (as a model of hyperglycaemia) decreased in the expression of SIRT1 protein in microvascular endothelial cells, and that metformin, at a clinically relevant

concentration, reduced the effects of HG on endothelial function by reducing the generation of ROS and maintaining/restoring SIRT1 protein expression. Furthermore, the endothelial-protective effect of metformin was lost when the expression of SIRT1 was silenced, using a siRNA protocol. Metformin has been shown to improve endothelial function and protect the macro- and microvasculature in diabetes via mechanisms that appear to be independent of its hypoglycaemic actions (Mather *et al.*, 2001; Majithiya and Balaraman, 2006; Sena *et al.*, 2011). Thus, these data from microvascular endothelial cells suggest that SIRT1 is a key target for the endothelial-protective action of metformin. SIRT1 is highly expressed in the vasculature and has been shown to function as a deacetylase for a number of transcription factors including FoxO-1 and p53, and thus potentially regulates cell differentiation, senescence and survival in response to cellular stress (Brunet *et al.*, 2004; Ota *et al.*, 2007; 2008; Potente *et al.*, 2007). Arunachalam *et al.* (2010) have reported that oxidative stress-mediated down-regulation of SIRT1 resulted in eNOS acetylation and endothelial dysfunction. In addition, hyperglycaemia/ROS-mediated decrease in SIRT1 expression as well as the SIRT1 inhibitor, sirtinol, amplifies vascular senescence as shown by increased

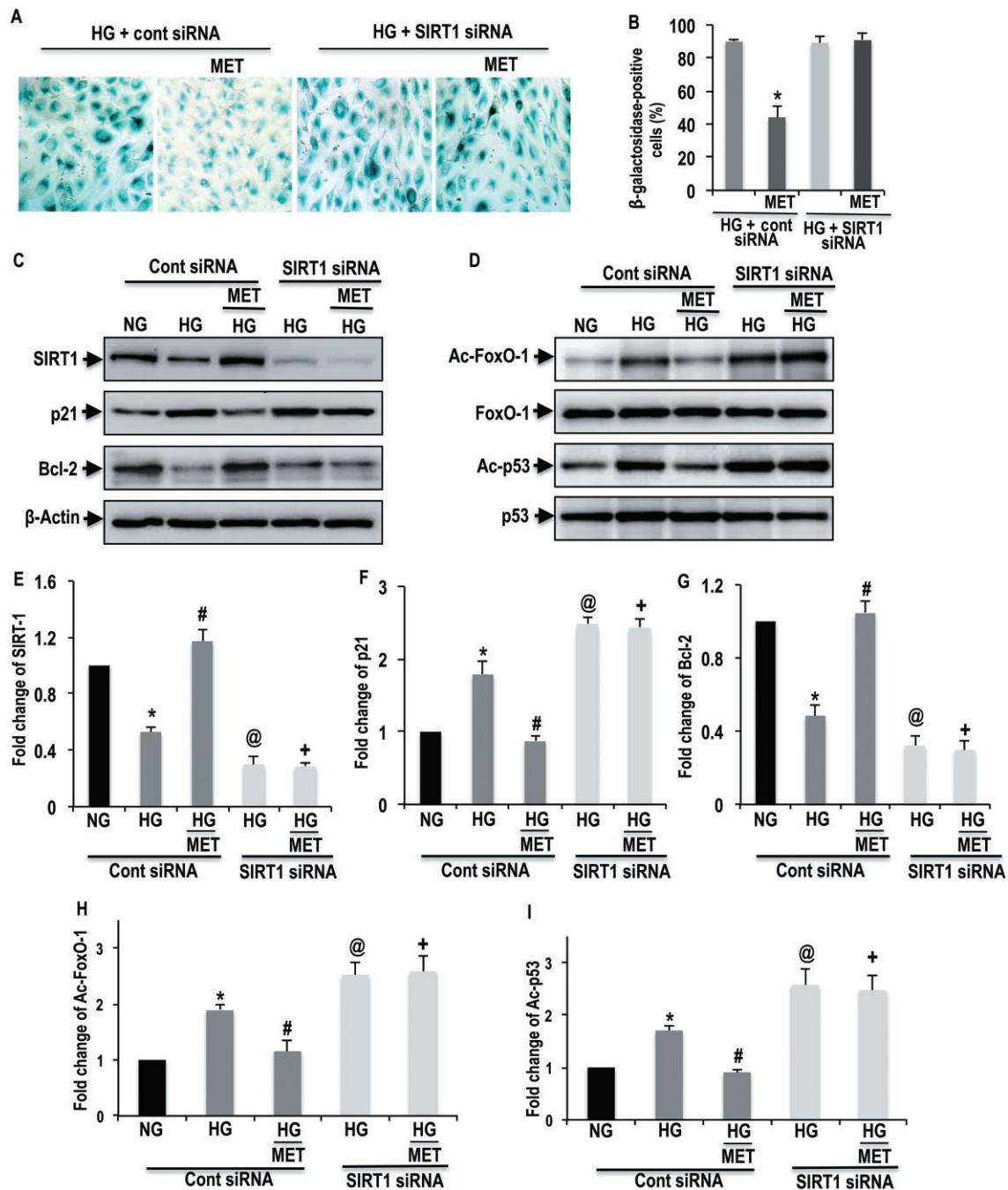


Figure 6

Effect of metformin treatment on HG-induced senescence in SIRT1-silenced endothelial cells. MMECs were transfected with either control siRNA or SIRT1 siRNA and exposed to HG (40 mM) in the presence or absence of metformin (MET). After 72 h of transfection, cells were fixed and stained for senescence-associated β-galactosidase activity (A; 20× magnification). Histogram represents the percentage of senescence-associated β-galactosidase-positive cells (B). Values represent mean ± SEM. **P* < 0.05, significantly different from control siRNA transfected cells. A representative image from three separate experiments is illustrated. Cell lysates were used for immunoblotting assessment of SIRT1, p21, Bcl-2, Ac-FoxO-1, FoxO-1, Ac-p53 and p53 levels (C, D). Results were normalized to controls, and histograms represent the relative intensity of SIRT1, p21, Bcl-2, Ac-FoxO-1 and Ac-p53 levels (E–I). Values represent mean ± SEM (*n* = 3–4 per group). **P* < 0.05, significantly different from control siRNA-transfected cells exposed to NG, #*P* < 0.05, significantly different from control siRNA-transfected cells exposed to HG alone; @*P* < 0.05, significantly different from control siRNA-transfected cells exposed to HG alone or with metformin.

β -galactosidase activity in HUVECs (Ota *et al.*, 2008; Orimo *et al.*, 2009). In the current study, the effects of HG were not mimicked by the osmotic control for glucose, mannitol, nor by the non-metabolizable glucose analogue, 3-OMG. Thus, endothelial cell uptake and metabolism of glucose are likely prerequisites for HG-induced endothelial dysfunction. In SIRT1-silenced endothelial cells, higher β -galactosidase activity was observed, and metformin treatment in these cells was no longer effective.

Hyperglycaemia-induced acetylation of FoxO-1 is attenuated by metformin

In this study, MMECs, cultured in HG, showed a significant decrease in SIRT1 protein expression and a parallel significant increase in FoxO-1 acetylation. This increase in FoxO-1 acetylation may result in the switching of FoxO-1 activity towards transcription of cell cycle arrest genes, which are associated with a HG-induced increase in oxidative stress. The presence of metformin significantly reduced the production of ROS in MMECs maintained in HG for 72 h, which, in turn, significantly increased SIRT1 followed by attenuation of Ac-FoxO-1 and Ac-p53 with a subsequent decrease in p21. FoxO transcription factors play a central role in endothelial cell proliferation and survival by regulating the genes involved in cell cycle arrest, DNA repair and apoptosis (Papanicolaou *et al.*, 2008). It has been reported that SIRT1 deacetylates FoxO-1 and regulates angiogenic function in endothelial cells and glucose deprivation-induced autophagy in cardiac muscle (Hariharan *et al.*, 2010). Furthermore, hydrogen peroxide-induced oxidative stress-mediated SIRT1 depletion accelerates endothelial senescence in a FoxO-1-dependent manner (Menghini *et al.*, 2009). HG also down-regulated human endothelial progenitor cell number as a result of a reduction in SIRT1 expression and associated increases in acetylated FoxO-1 (Balestrieri *et al.*, 2008). Our data also show that the presence of metformin did not significantly attenuate the FoxO-1 acetylation in SIRT1-silenced endothelial cells exposed to either NG or HG. Similarly, Potente *et al.* (2007) reported that SIRT1 knockdown in HUVECs increased FoxO-1 acetylation and thereby enhanced the transcriptional activity of FoxO-1 with diminished angiogenesis. The data from the current study thus support the hypothesis that metformin activates SIRT1 and negatively regulates FoxO-1-dependent cell cycle arrest and apoptotic gene transcription. To the best of our knowledge, this is the first study to link metformin-mediated activation of SIRT1 and FoxO-1 acetylation to the effects of hyperglycaemia on microvascular endothelial cells.

Metformin protects endothelial cells against hyperglycaemia-induced senescence and apoptosis

When MMECs were exposed to HG for 72 h, p53 acetylation increased and there was also an increase in the expression of p21 protein. This finding is in accordance with a previous study that reported hyperglycaemia-mediated SIRT1 depletion accelerated p53/p21-mediated endothelial senescence in macrovascular HUVECs (Orimo *et al.*, 2009). We therefore tested the hypothesis that metformin would reduce HG-induced p53/p21 activation in microvascular endothelial cells. Our data indicated that the presence of metformin in

HG-exposed MMECs significantly attenuated p53 acetylation and decreased the expression of p21 protein. Furthermore, even in the presence of metformin, SIRT1 silencing in MMECs exposed to either NG or HG increased p53 acetylation with a subsequent increase in p21 protein expression, thus also supporting our conclusion that the protective effect of metformin was SIRT1 dependent. These data also provide support for a link between SIRT1 and the regulation of p53/p21 in endothelial cells. Therefore, in addition to its hypoglycaemic action, metformin can also protect microvascular endothelial cells from glucose toxicity via a mechanism that presumably involves SIRT1-mediated deacetylation and the inhibition of p53/p21-dependent growth arrest. A comparable action of metformin via the AMPK-SIRT1 pathway has also been shown in hepatic HepG2 cells that have been exposed to HG (Nelson *et al.*, 2012). An increase in hyperglycaemia/oxidative stress-mediated p53 acetylation has also been reported to induce the transactivation of genes such as p21 (Orimo *et al.*, 2009) and pro-apoptotic Bcl-2 associated protein, Bax, in the human immortalized lymph node cell line, H1299 (Tang *et al.*, 2008), whereas deacetylation of p53 by SIRT1 inhibited ROS-mediated cellular senescence and apoptosis in HUVECs (Orimo *et al.*, 2009). Furthermore, deacetylation of p53 by SIRT1 inhibits the suppressive action of p53 on the transcription of anti-apoptotic Bcl-2 in tissues from rats that had been placed on a calorie-restricted diet (Cohen *et al.*, 2004).

Our study shows a significant decrease in the levels of the anti-apoptotic Bcl-2 protein and anti-ageing protein, SMP-30, in MMECs that had been cultured in HG for 72 h, but in the presence of metformin, the levels of expression of both Bcl-2 and SMP-30 proteins were maintained and the equivalent to those for MMECs kept in NG for 72 h. The decrease in SMP-30 expression in SIRT1-silenced cells also suggests a positive link between SIRT1 and the regulation of SMP-30 expression. Activation of SIRT1 in the vasculature has been reported to inhibit pro-apoptotic Bad and caspase-3 activation and apoptosis in cerebral endothelial cells isolated from rats and subsequently exposed to HG; the knockdown of SIRT1 in these cells exacerbated HG-induced apoptosis (Hou *et al.*, 2010). It has also been reported that endothelium-specific overexpression of SIRT1 significantly inhibits ox-LDL-induced apoptosis and improves endothelium-dependent vasodilatation (Zhang *et al.*, 2008).

Role of AMPK

Metformin, 100 μ M–10 mM, activates the AMPK/SIRT1 pathway and, via mitochondria complex 1, inhibits HG-induced apoptosis in human vascular endothelial cells (Detaille *et al.*, 2005). Metformin may also activate AMPK by activating its upstream kinase liver kinase B-1 (LKB-1; Shaw *et al.*, 2005). AMPK positively regulates SIRT1 expression by elevating the intracellular levels of its co-substrate NAD⁺ while SIRT1 activation leads to AMPK activation via deacetylation and activation of the AMPK, LKB-1 (Hou *et al.*, 2008; Canto *et al.*, 2009). Activation of AMPK by metformin has been shown to decrease intracellular ROS production in podocytes (Piwkowska *et al.*, 2010) and inhibit diabetes-induced renal hypertrophy (Lee *et al.*, 2007). In contrast, Zheng *et al.* (2012) have reported that in retinal endothelial cells, under hyperglycaemic conditions, metformin increases

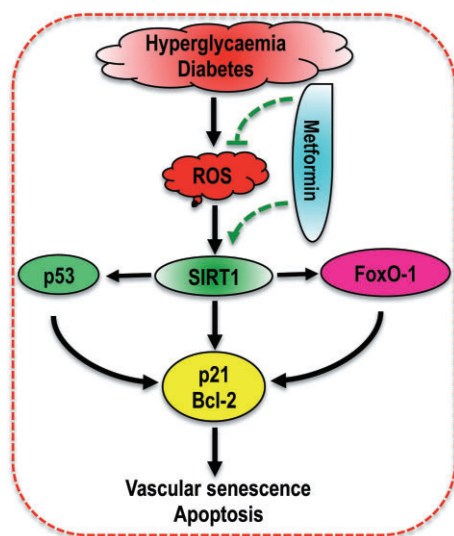


Figure 7

Metformin modulates hyperglycaemia-induced post-translational modification of SIRT1-mediated pathways in endothelial cells. Hyperglycaemia down-regulates expression of SIRT1 and thereby augments the FoxO-1 and p53-mediated endothelial cell senescence and apoptosis. Treatment with metformin up-regulates SIRT1 and attenuates hyperglycaemia-induced endothelial dysfunction.

SIRT1 level/activity directly or, in part, via the LKB-1/AMPK pathway and thereby inhibits the production of ROS, NF- κ B activation and Bax-induced apoptosis. In the current study metformin protected MMECs against the effects of HG; however, whether the effects of metformin are mediated via the activation of AMPK and/or directly via SIRT1 requires further investigation.

Metformin protects against hyperglycaemia-induced endothelial dysfunction

Collectively, our data, summarized in Figure 7, demonstrated that HG-induced oxidative stress reduced SIRT1 expression, concomitant with accelerated FoxO-1/p53-mediated senescence and apoptosis. The presence of metformin attenuated the effect of HG on SIRT1 expression and thereby reduced hyperglycaemia-induced senescence and apoptosis in microvascular endothelial cells. The results from the current study provide molecular insights into the cellular actions of this important oral hypoglycaemic drug, whereby metformin, at a concentration that is clinically relevant, can protect the mouse microvascular endothelium against the deleterious effects of glucose, at a concentration that is routinely observed in type 2 diabetic mice. The endothelial protective effect of metformin was exerted via a SIRT1-dependent molecular mechanism(s), thus suggesting new avenues of research for counteracting diabetes-associated CVD.

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Conflict of interest

None declared.

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