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Piceatannol is more effective than resveratrol in restoring endothelial cell dimethylarginine dimethylaminohydrolase expression and activity after high-glucose oxidative stress

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Abstract

Glucose-induced oxidative stress is involved in endothelial dysfunction. Dimethylarginine dimethylaminohydrolase (DDAH) and arginase are regulators of the endothelial NO synthase (eNOS). This study aimed to compare the effect of two polyphenolic antioxidants, resveratrol and piceatannol, on DDAH and arginase pathways in bovine aortic endothelial cells under 25 mM glucose for 24 h. DDAH activity and expression were decreased in these cells as compared to control cells, whereas arginase activity was unchanged. DDAH inhibition led to intracellular accumulation of asymmetric dimethylarginine (ADMA), a natural inhibitor of eNOS. Under these conditions, cell pre-treatment with resveratrol (0.1–10 μM) restored basal DDAH activity and ADMA level with a dose-dependent effect. Piceatannol acted as resveratrol on DDAH pathway but at 10-fold lower concentrations. Resveratrol and piceatannol restored DDAH activity even in the presence of splitomicin, a specific inhibitor of Sirtuin 1. These results suggest potential therapeutic intervention targeting resveratrol or piceatannol administration to improve endothelial dysfunction.

Keywords: *Piceatannol, resveratrol, ADMA, oxidative stress, DDAH, endothelial dysfunction*

Abbreviations: *ADMA, asymmetric dimethylarginine; BAEC, bovine aortic endothelial cells; DDAH, dimethylarginine dimethylaminohydrolase; BIAM, Biotine-conjugated iodoactamine; DMEM, Dulbecco's modified Eagle medium; FCS, foetal calf serum; GLC, Glucose;* H_2*DCF-DA, dichlorodihydrofluoresceine diacetate; ISPF, 1-phenyl-1,2-propanedione-2-oxime or* α*-nitrosopropiophenone; MAN, Mannose;* $^{\bullet}$*NO, nitric oxide; PIC, piceatannol; PS, penicillin streptomycin; RES, resveratrol; ROS, radical oxygen species; SOD, superoxide dismutase.*

Introduction

Oxidative stress occurring in response to hyperglycaemia is involved in the onset and development of cardiovascular complications of diabetes mellitus such as atherosclerosis [1,2]. Indeed, chronic hyperglycaemia induces an over-production of reactive oxygen species (ROS), which is involved in endothelial dysfunction [3]. Mechanism of diabetes-induced endothelial dysfunction includes the increased oxidative degradation of $^{\bullet}$NO and a reduced $^{\bullet}$NO biosynthesis caused by an endogenous competitive inhibitor of $^{\bullet}$NO synthase (NOS), i.e. asymmetric dimethylarginine (ADMA) [4]. This amino acid arises from the post-translational methylation and hydrolysis of proteins. ADMA is

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currently considered a marker of cell stress and, consequently, a potential novel pharmacological target [5] secondary to growing evidence of its contribution to endothelial dysfunction [6]. Under physiological conditions, ADMA is actively catabolized into citrulline and methylamines by the action of dimethylarginine dimethylaminohydrolase (DDAH). There are two isoforms of DDAH, i.e. DDAH I and DDAH II. DDAH I is the major form expressed in neuronal tissues, whereas DDAH II is essentially expressed in endothelial tissues [7]. This enzyme appears to be highly sensitive to oxidative stress, an effect that is likely responsible for ADMA accumulation in cells [8].

Arginase, which plays an important role for endothelial •NO bioavailability, is a binuclear manganese metalloenzyme that catalyses hydrolysis of L-arginine (i.e. NOS substrate) into L-ornithine and urea. Two distinct genetic arginase isoforms have been identified. Several reports have highlighted the relationship between an increased arginase activity and the development of endothelial dysfunction [9,10].

Resveratrol or 3, 4', 5-trans-trihydroxystilbene (RES) is a phytoestrogen found in red wine that could have a protective effect against cardiovascular diseases [11]. A study showed a vasodilator effect of this natural compound in rat aorta in addition to its possible action on the L-arginine-NO-cGMP pathway [12]. RES structural analogues such as piceatannol or 3, 3', 4', 5-trans-tetrahydroxystilbene (PIC) have been studied. PIC, which is a metabolite of RES via cyt.P450 1A2 [13], has been reported to exhibit a higher antioxidant effect than resveratrol, partly due to the presence of an additional hydroxyl group [14,15]. RES and PIC effects on •NO pathway are still misunderstood. Recently, a study reported the effect of a non-hydroxylated derivative of RES (BTM-0512) on the DDAH/ADMA pathway via Sirtuin 1 activation [16].

Since both pathways are involved in the bioavailability of •NO, this study is aimed at assessing the potential RES and PIC antioxidant effects through a modulation of DDAH and arginase activities in bovine aortic endothelial cells (BAEC). The effects of RES and PIC were evaluated in BAEC stimulated by high glucose levels (25 mM) for 24 h in order to mimic the pathophysiological stimulus of the diabetic states.

Materials and methods

Reagents

Dulbecco's modified Eagle medium (DMEM), foetal calf serum (FCS), penicillin streptomycin, glutamine, ADMA, PMSF, aprotinin, leupeptin, L-citrulline and splitomicin were obtained from Sigma (St Louis, MO). RES was obtained from Alexis (San Diego, CA) and PIC from A.G. Scientific, Inc (San Diego, CA). Goat antibody raised against 273–285 residues of DDAH isoform 2 and peroxydase-conjugated anti-goat IgG were obtained from Calbiochem (USA).

Cell culture

Bovine aortic endothelial cells (BAEC) were kindly provided by Dr A. Negre-Salvayre (INSERM U858, Toulouse, France). Cells were grown at confluence in DMEM supplemented with 10% FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin, at 37°C under a 5% CO_2 humidified atmosphere. Cells used in this study were between the sixth and tenth passages. Viability was assessed by the neutral red assay; cell viability higher than 95% was constantly required for performing experiments.

Cell treatment

For all experiments, cells were pre-incubated overnight with RES (0.1–10 μM) or PIC (0.01–1 μM), then treated by glucose-supplemented medium (25 mM or 5 mM) and 20 mM mannose-supplemented medium was used as osmotic control. Incubations were performed in the presence of RES (0.1–10 μM) or PIC (0.01–1 μM) for 24 h. Control cells were not treated with glucose-supplemented medium. For the Sirtuin 1 pathway exploration, cells were treated 1 h before high glucose incubation with 50 μM splitomicin.

Determination of thiol concentration

Determination of thiol levels was based on the reaction of thiols with Ellman's reagent 5, 5'–dithiobis (2-nitrobenzoic acid), DTNB [17]. A 50 μL sample was mixed with 1 mL 0.1 M Tris and 10 mM EDTA pH 8.2, constituting the blank. The reaction was then assessed at 412 nm (UVIKON). The addition of 40 μL of 10 mM DTNB in methanol triggered the reaction; absorbance at 412 nm was measured after stable colour formation (1–3 min). The concentration of thiol groups was calculated using a molar extinction coefficient of 13 600 M^{-1} cm^{-1}. Thiol concentration was expressed as μM.

DDAH activity

Cell fractionation. Cells treated as described above were collected and washed three times in ice-cold PBS and re-suspended in a 50 mM Tris-HCl buffer pH 7.4 containing EDTA (1 mM), EGTA (2.5 mM), Triton (1%), phenylmethylsulphonyl fluorid (PMSF, 1 mM), leupeptin (10 μg/mL) and aprotinin (10 μg/mL) (lysis buffer). Cells were then lysed by sonication (3 × 10 s at 80 W, Brand Sonicator). Homomogenates were centrifuged at 6000 g for 10 min to remove nuclei and unbroken cells. All preparation steps were performed at 4°C. Total protein concentration was determined using the Bio-Rad protein assay.

DDAH activity assay. DDAH activity was measured by a spectrophotometric method as previously reported [18] with a slight modification. This method is based

on the determination of L-citrulline formation. Ten microlitres of cell homogenate (100 µg expressed as total proteins) were pre-incubated for 5 min in 45 µL of 0.1 M Na/K-P buffer, pH 6.2. The enzymatic reaction was started by a rapid addition of 5 µL of 80 mM ADMA (Sigma) and then incubated for 30 min at 37°C in a water bath. The reaction was stopped with 200 µL of the colour developing reagent (COLDER) and the concentration of L-citrulline was determined by reading sample absorbance at 540 nm in a plate reader Multiskan AX (Thermo).

DDAH 2 and arginase 2 Western Blot analyses

Cell homogenates (50 µg expressed as total proteins) were analysed on a 12% SDS-polyacrylamide electrophoresis gel then transferred to a nitrocellulose membrane (Biorad). The membrane was first incubated with a blocking buffer TTBS (2 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 5% non-fat dry milk, pH 7.5) for 1 h at room temperature followed by a 1.5 h incubation period in the presence of either goat antibody raised against 273–285 residues of DDAH isoform 2 (Calbiochem) or goat antibody raised against arginase 2 (Tebu-bio) (dilution: 1/1500 in TTBS). Peroxidase-conjugated anti-goat IgG (Calbiochem) was used for detection at a dilution of 1/10 000 and the immunoreactive proteins were visualized by chemiluminescence.

Immunoprecipitation of biotine-conjugated iodoactamine (BIAM)-labelled DDAH 2 in BAEC

The BIAM-labelled DDAH 2 detection procedure was based on the fact that proteins containing cysteine residues were labelled with BIAM, whereas oxidized cysteines were not susceptible to BIAM labelling. After treatment (as previously described), cells were rinsed in PBS and then exposed to 1 mL oxygen-free lysis buffer (50 mM Bis-Tris-HCl (pH 6.5), 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, leupeptine (1 µg/mL), aprotinin (1 µg/mL)) containing 20 µM BIAM. After incubation for 30 min at 37°C in the dark, the labelling reaction was stopped by adding 1 mM iodoacetamine. DDAH 2 was precipitated with the goat antibody anti-DDAH 2 and Protein G-Sephadex (TEBU, France). DDAH 2 labelled with BIAM was detected with HRP-conjugated streptavidin and ECL-Plus [19].

ADMA measurement

Cells were treated as previously described and ADMA concentration was determined by reversed-phase HPLC with fluorescence detection after derivatization with ortho-phtalaldialdehyde (OPA) reagent in cell homogenates as previously described [20].

Arginase activity assay

Arginase activity was evaluated by a spectrophotometric method as previously reported [21]. Fifty microlitres of cell homogenate (500 µg expressed as total proteins) were incubated with 50 µL of 10 mM $MnCl_2$, 50 mM Tris-HCl, pH 7.5 and the enzyme was then activated for 10 min at 55°C. Arginine hydrolysis was initiated by addition of 25 µL of 0.5 M arginine, pH 9.7, to a 25 µL-aliquot of the previously activated lysate. Incubation was performed at 37°C for 60 min and the reaction stopped by the addition of 400 µL of an acid mixture containing H_2SO_4, H_3PO_4 and H_2O (1:3:7, v/v). The urea formed was quantified at 540 nm after addition of 25 µL 9% 1-phenyl-1,2-propanedione-2-oxime or α-nitrosopropiophenone (ISPF) (dissolved in 100% ethanol) and heating at 100°C for 45 min. After 10 min in the dark, the absorbance at 540 nm was determined in a microplate reader on 200 µL-aliquots in a 96-well micro culture plate. A calibration curve was established using increasing amounts of urea (from 1.5 to 30 µg). In the case to 100 µL of urea solution at the appropriate concentration, 400 µL of the acid mixture and 25 µL of ISPF were added and the procedure was followed as described above.

Statistical analysis

The results are expressed as the mean ± SEM of at least three different cultures. For all experiments, each condition was measured in a triplicate manner. Cells used in this study were between the sixth and tenth passages. Statistical significance was determined by the non-parametric Mann-Whitney test. p-values < 0.05 were considered statistically significant.

Results

Cell viability

Cell viability was assessed by the neutral red assay. Neither RES, PIC or MAN at the different concentrations studied, nor high glucose conditions used for experiments resulted in a cell viability $< 95\%$.

Concentration of thiol groups

Concentration of thiol groups was significantly lower in cells treated for 24 h with 25 mM glucose, as compared to control cells treated with 5 mM glucose (Figures 1A and B). RES restored intracellular thiol content with a dose-dependent effect. Similarly, PIC restored this thiol content with a dose-dependent effect, but at 10-fold lower concentrations than RES.

RES (10 µM), PIC (1 µM) and MAN (20 mM) had no effect on thiol groups, as compared to control cells (Figures 1A and B).

DDAH activity

Initially, cells were treated with 25 mM glucose for 4 h, but DDAH activity was not affected (data not shown). Treatment of cells for 24 h with 25 mM glucose reduced DDAH activity in cell lysates to 20% of control activity (Figure 2A). Mannose treatment (20 mM) did not induce any change in DDAH activity when compared to controls incubated with 5 mM glucose (Figures 2A and B). RES restored DDAH activity of cells activated by high glucose with a dose-dependent effect. Cells treated with 10 μM RES exhibited a non-significantly different DDAH activity as compared to controls (Figure 2A). Similarly, PIC restored DDAH activity with a dose-dependent effect under the same conditions at a concentration of 1 μM, which was lower than RES, but with a restored DDAH activity 80% of the control activity (Figure 2B). RES (10 μM), PIC (1 μM) and MAN (20 mM) had no effect on DDAH activity, as compared to control cells (Figures 2A and B). Moreover, both RES 10 μM and PIC 1 μM were able to restore DDAH activity in the presence of splitomicin.

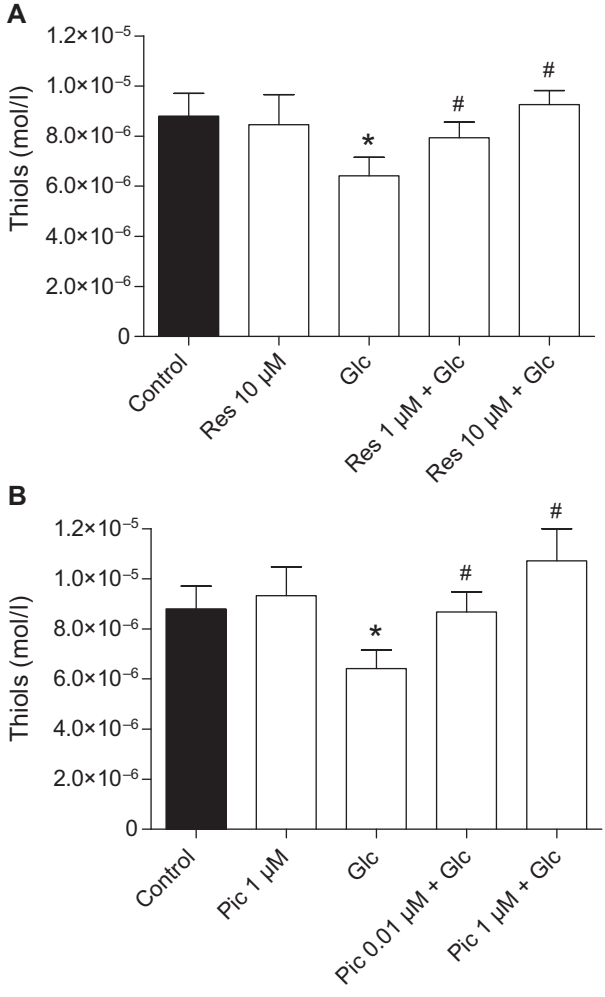

Figure 1. Intracellular thiol concentration after treatment of BAEC with 25 mM glucose and increasing doses of resveratrol (A) or piceatannol (B). The concentration of thiol groups was calculated using a molar extinction coefficient of 13 600 M^{-1} cm^{-1}. Thiols were expressed as μM. $^{*}p < 0.05$ vs Control; $^{\#}p < 0.05$ vs Glc. Data are means ± SEM from five independent experiments. Glc: glucose 25 mM; Man: mannose; Control: glucose 5 mM.

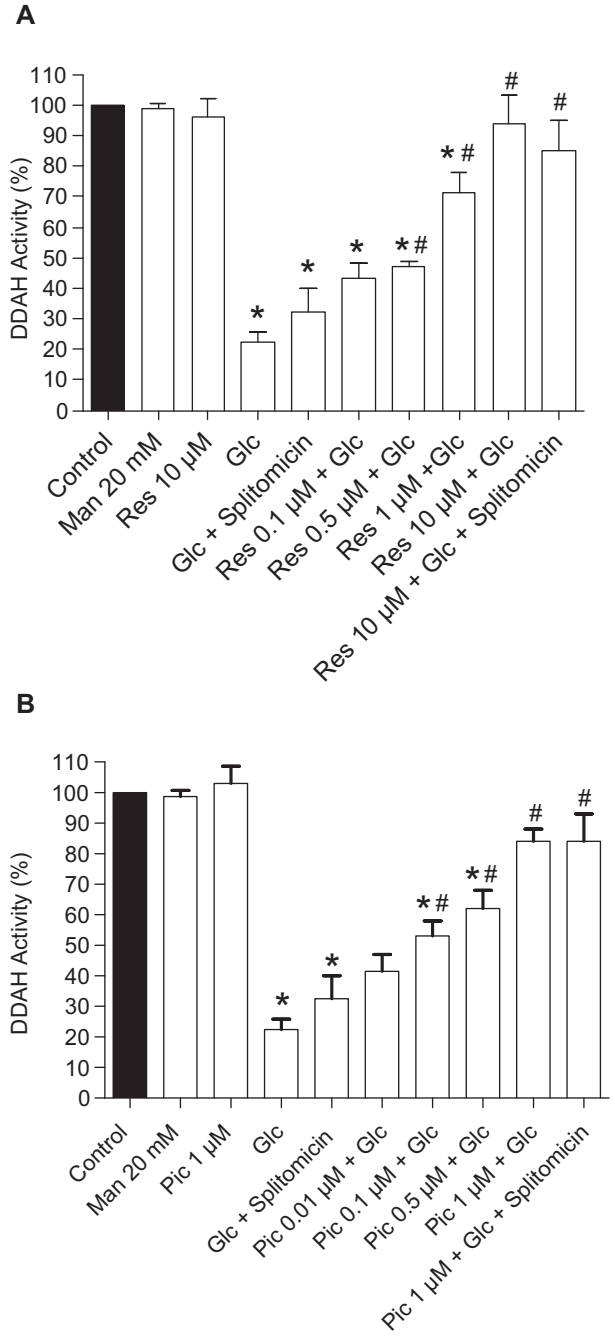

Figure 2. Dose-dependent effects of resveratrol on DDAH activity. Cells were incubated in DMEM (1% FCS, 1% PS, 1% glutamine) with 25 mM glucose and increasing doses of resveratrol (0.1–10 μM) (A) or piceatannol (0.01–1 μM) (B). For Sirtuin 1 study, cells were incubated in the presence of 50 μM splitomicin 1 h before high glucose incubation. Values are expressed in percentage of DDAH activity in control cells (not treated with glucose-supplemented medium). $^{*}p < 0.05$ vs Control; $^{\#}p < 0.05$ vs Glc. Data are means ± SEM from five independent experiments. Glc: glucose 25 mM; Man: mannose; Control: glucose 5 mM.

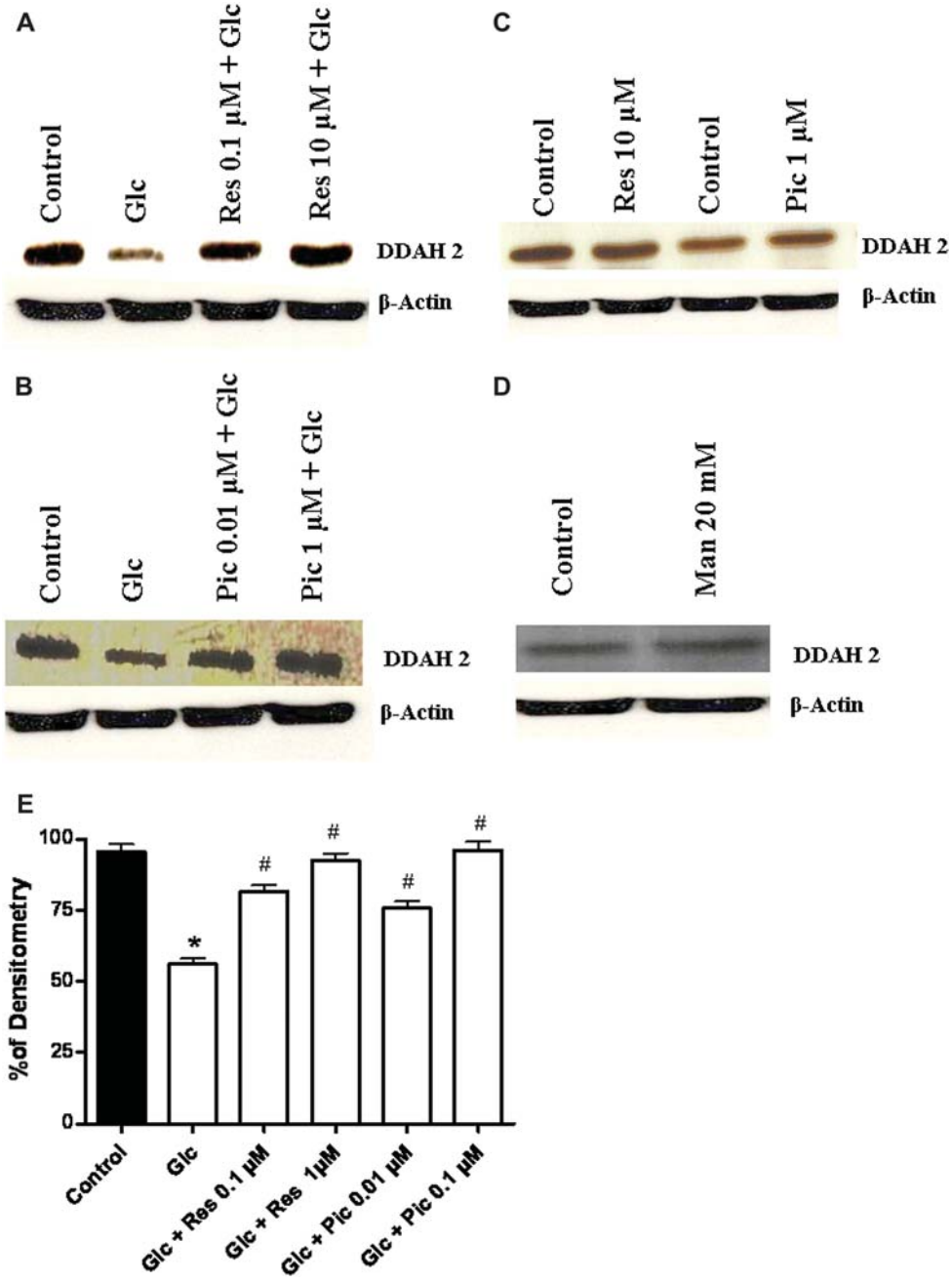

Figure 3. Immunoblot analysis of unstimulated (control) and stimulated BAEC incubated with resveratrol (A and C) or piceatannol (B and C) or mannose (D). Total cellular proteins were analysed for DDAH 2. The blot was further incubated with the anti-actin antibody and the obtained signal was used to normalize the protein load. Quantitative intracellular DDAH 2 expression levels were evaluated using NIH Image J software and expressed as arbitrary densitometric units (mean ± SD). $^{*}p < 0.05$ vs Control; $^{\#}p < 0.05$ vs Glc (E). Data are means ± SD from five independent experiments. Glc: glucose 25 mM; Control: glucose 5 mM.

DDAH 2 expression

Western blot analyses showed that treatment of cells with 25 mM glucose for 24 h resulted in a significant inhibition of DDAH 2 expression (Figures 3A and B). The simultaneous incubation of cells with high glucose level and RES (Figure 3A) or PIC (Figure 3B) inhibited the decrease in DDAH 2 expression. Conversely, incubation of 5 mM glucose-treated BAEC with RES (10 µM), PIC (1 µM) or MAN (20 mM) did not modify DDAH 2 expression. The whole results regarding DDAH 2 expression are reported in Figure 3C.

Oxidative modification of DDAH 2

The selective BIAM labelling procedure was used as a probe to monitor the oxidation status of cysteine at the site of DDAH 2 catalysis. Blotting confirmed that the free cysteinyl group of DDAH2 was targeted by ROS when cells were incubated with high glucose concentration (Figure 4). In these conditions, RES and PIC

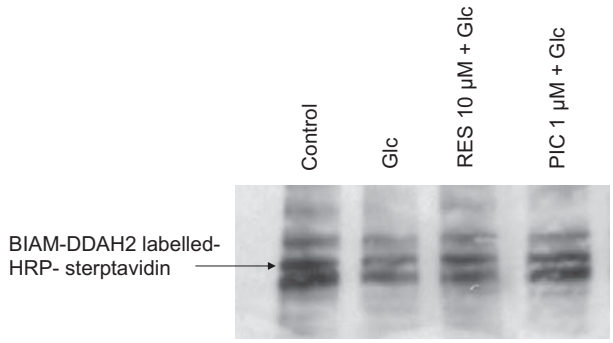

Figure 4. BIAM-labelled DDAH 2 detection of unstimulated (control) and stimulated BAEC incubated with resveratrol (10 µM) or piceatannol (1 µM). DDAH 2 was precipitated with the goat antibody anti-DDAH 2 and Protein G-Sephadex (TEBU, France). DDAH 2 labelled with BIAM was detected with HRP-conjugated streptavidin and ECL-Plus. Glc: glucose 25 mM, Control: glucose 5 mM.

protected cysteinyl group of DDAH2 against oxidation damage (Figure 4). However, there was no change in cysteinyl group oxidation status when cells were incubated with 20 mM mannose (Figure 4).

ADMA concentration

Treatment of cells with 25 mM glucose for 24 h increased ADMA concentration (+80% compared to controls) in cell lysates. Mannose treatment (20 mM) did not increase ADMA level in cells when compared to cells treated with 5 mM glucose (Figures 5A and B). These results are in agreement with the reduced DDAH activity observed under the same conditions. RES restored the intra-cellular concentration of ADMA in a dose-dependent manner (Figure 5A). Cells treated with 10 µM RES contained a non-significantly different ADMA level, as compared to controls. Similarly, PIC restored intracellular ADMA level with a dose-dependent effect as observed with DDAH activity, making it efficient at a 10-fold lower concentration than RES (Figure 5B). Indeed, 1 µM PIC was sufficient to completely restore intracellular basal ADMA concentration. RES (10 µM), PIC (1 µM) and MAN (20 mM) had no effect on intracellular ADMA level in control cells (Figures 5A and B).

Arginase activity

Treatment of cells with 25 mM glucose for 24 h had no effect on arginase activity (Figures 6A and B), as compared to control cells. Similarly, we failed to show any significant effect of RES (Figure 6A) or PIC (Figure 6B) on arginase activity in 25 mM glucose-treated cells, nor in control cells.

Arginase 2 expression

Treatment of cells with 25 mM glucose for 24 h had no effect on arginase 2 expression as compared to control cells (Figure 7). Similarly, we failed to show any significant effect of RES or PIC on arginase 2 expression in 25 mM glucose-treated cells (Figure 7).

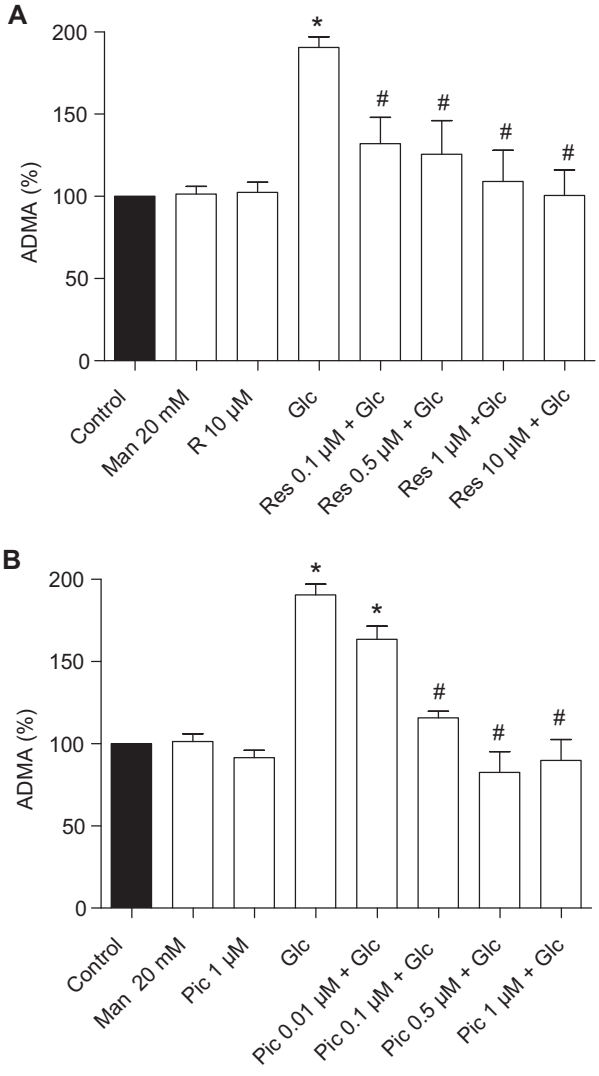

Figure 5. Effect of resveratrol (A) or piceatannol (B) on intracellular ADMA concentration. Cells were incubated in DMEM (1% FCS, 1% PS, 1% Glutamine) with 25 mM glucose and increasing doses of resveratrol (0.1–10 µM) (A) or piceatannol (0.01–1 µM) (B). Values are expressed in percentage of ADMA concentration in control cells (not treated with glucose-supplemented medium). Data are means ± SEM from five independent experiments. $^{*}p < 0.05$ vs Control; $^{\#}p < 0.05$ vs Glc ($n = 5$). Glc: glucose 25 mM; Man: mannose; Control: glucose 5 mM.

Discussion

The present study reports a significant decrease of DDAH activity and expression in BAEC cultured under conditions of high glucose concentrations (25 mM for 24 h). Under these experimental conditions, resveratrol and piceatannol restored DDAH activity in a dose-dependent manner.

Numerous studies demonstrated that oxidative stress contributes to the development and progress of cardiovascular diseases [22]. Over-production and/or

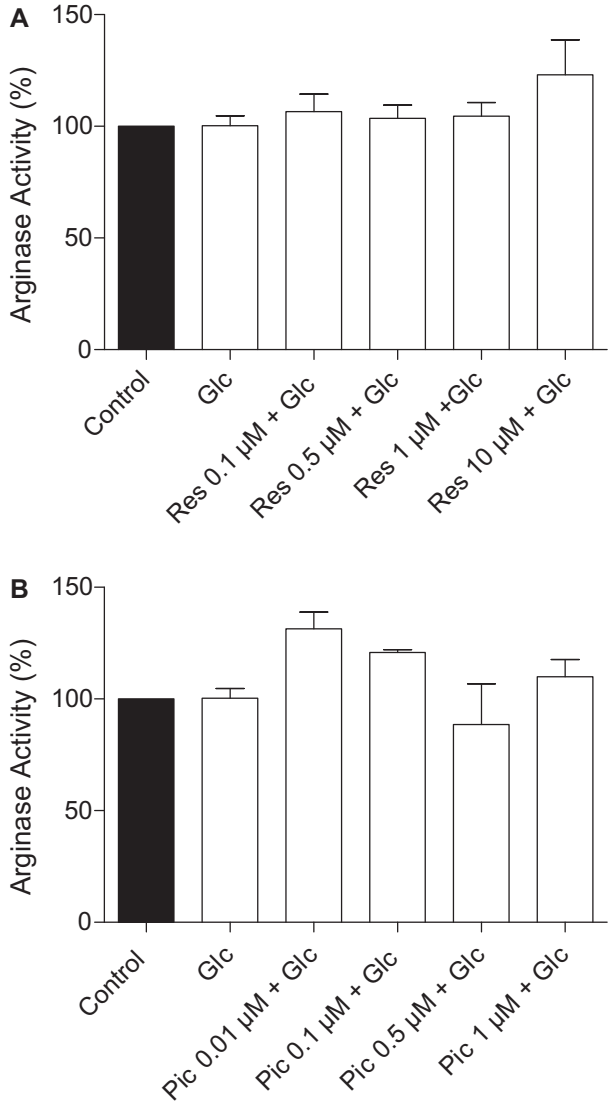

Figure 6. Arginase activity measurement after treatment with 25 mM glucose and increasing doses of resveratrol (A) or piceatannol (B). Cells were incubated in DMEM (1% FCS, 1% PS, 1% glutamine) with 25 mM glucose and increasing doses of resveratrol (0.1–10 µM) or piceatannol (0.01–1 µM). Values are expressed in percentage of arginase activity in control cells (not treated with glucose-supplemented medium). Data are means ± SEM from five independent experiments. Glc: glucose 25 mM; Control: glucose 5 mM.

insufficient removal of ROS result in endothelial dysfunction. Mechanism of endothelial dysfunction includes both decreased $^{\bullet}$NO availability and altered eNOS expression [23]. Moreover, eNOS activity is regulated by endogenous inhibitors, particularly ADMA, which is metabolized by DDAH. Therefore, DDAH activity is determinant for ADMA concentration [8] and its deregulation could play an important role in increasing ADMA concentration. A reduced DDAH activity has been reported in hypercholesterolaemia [24], hyperglycaemia, diabetes mellitus [25] and pathological conditions resulting in the generation of excessive ROS levels in endothelial cells. Indeed, the presence of a cysteine residue (Cys-249) in the active site of DDAH makes the enzyme sensitive to S-nitrosylation and to oxidation by ROS such as superoxide radicals, thereby leading to a decreased DDAH activity [26]. Recent studies confirmed the ROS-sensitive regulation of DDAH. As an example, incubation of endothelial cells in the presence of high glucose concentrations, conditions that mimic the oxidative stress observed in diabetes, increased ADMA level; this phenomenon was inhibited by the antioxidant polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), which catalyses the dismutation of superoxide radicals [25]. Consequently, modulation of DDAH activity seems to be a key step in ADMA production and its deregulation by oxidative stress appears to play a central role in $^{\bullet}$NO endothelial production.

Sorrenti et al. [27] showed results close to ours in HIAE-101 (human iliac artery endothelial cells) cultured under high glucose (25 mM) conditions for 5 days, that is a decrease of DDAH activity and expression. The mechanisms regulating DDAH 2 expression are still that DDAH genes are redox-sensible, so that high glucose responses in endothelial cells might result in activation and repression of overlapping signal transduction cascades involving oxidative stress. We observed increased ADMA levels in cells treated with 25 mM glucose, conditions resulting in an oxidative stress evidenced by decreased intracellular thiol concentration; this oxidative stress led to DDAH inhibition. Another study showed that treatment of bovine retinal capillary endothelial cells with high glucose (30 mM) for 48 h resulted in increased ADMA levels [29]. Besides, several reports have linked an increased arginase activity with the development of endothelial dysfunction. The mechanism involved in the up-regulation of arginase is not known, but enhanced formation of ROS may also influence arginase activity, since potential redox-sensitive responsive elements have been identified in the arginase I promoter region [30]. Only one study showed that bovine coronary endothelial cells treated with high glucose concentrations exhibited high ROS levels and an increase in arginase activity and expression [31]. In our BAEC model, we failed to show any effect of high glucose concentrations on arginase expression and activity. Similarly, RES (1–10 µM) and PIC (0.1–1 µM) had no effect on arginase expression and activity under the same experimental conditions. These results are not in agreement with those of Ishizaka et al. [32]. However, these authors used endothelial cells produced in a rabbit diabetic model (submitted to 13 weeks of hyperglycaemia). Such difference in the experimental model could explain these discrepancies results.

There is compelling evidence that RES can act on the atherosclerotic process by affecting the major participants in the atherosclerotic diseases by its anti-inflammatory and anti-thrombotic effects [33]. RES also exhibits antioxidant properties by its ability to scavenge ROS [34] and to induce cell enzymatic defense like superoxide dismutase (SOD) [35]. Moreover, Klinge et al. [36] showed that RES could up-regulate the constitutive

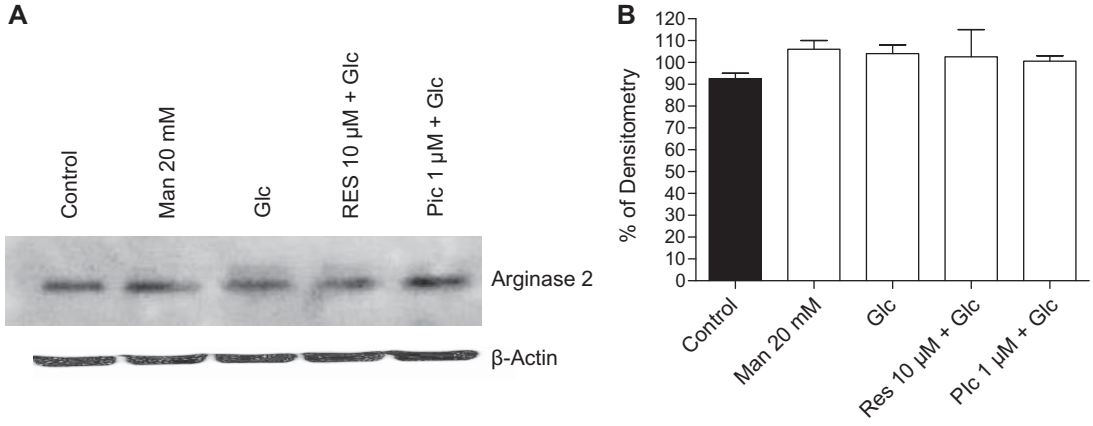

Figure 7. Immunoblot analysis of unstimulated (control) and stimulated BAEC incubated with resveratrol or piceatannol or mannose. Total cellular proteins were analysed for arginase 2. The blot was further incubated with the anti-actin antibody and the obtained signal was used to normalize the protein load. Quantitative intracellular arginase 2 expression levels were evaluated using NIH Image J software and expressed as arbitrary densitometric units (mean ± SD).

eNOS activity [36]. A recent study showed the inhibitory effect of the BTM-0512 RES derivative on high glucose-induced endothelial cell senescence, this effect could involve DDAH/ADMA pathway [16]. These authors suggested that the BTM-0512 effect on DDAH/ADMA pathway could be explained by the activation of Sirtuin 1.

Like RES, PIC has antioxidant effects. Lorenz et al. [37] showed that PIC was found to be a more effective scavenger than RES towards 2,2-diphenyl-1-picrylhydrazyl (DDP)-derived radical. Moreover, PIC has been shown to be anti-inflammatory via a selective inhibition of COX-2 [38]. It exhibited anti-cancerous properties [39], which could be explained because PIC is an antagonist of the aryl hydrocarbon receptor (AhR) and a selective oestrogen receptor modulator (SERM) [40,41]. Another study showed that PIC was more protective than RES towards DNA damage induced by hydrogen peroxide in leukaemia cells [42].

This study aimed at determining the effect of RES and PIC on DDAH activity. We showed that RES restored DDAH activity with a dose-dependent effect in cells stimulated by high glucose concentrations, concomitantly with an inhibition of the glucose-induced increase in intracellular ADMA levels. This could be related to the antioxidant effect of RES and above all PIC, which was evidenced in our study by the restoration of intracellular thiols under our conditions of glucose-induced oxidative stress. A recent study reported similar observation with another antioxidant, i.e. the polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) [34]. Our results showed that PIC was more efficient than RES in restoring DDAH activity and an ADMA intracellular level similar to controls, in BAEC incubated in the presence of high glucose concentrations. This result could be compatible with the presence of an additional hydroxyl group on PIC as compared with RES, which increases its ROS scavenging capacity [43]. In an *in vitro* cell model, RES and PIC could exhibit a direct scavenging effect, but also an indirect effect, by protecting cysteinyl groups in proteins. To assess the latter effect, we performed BIAM analyses. The results indicated that RES and PIC protected the cysteinyl group of the catabolic site of DDAH 2 enzyme from oxidation damage.

As Yuan et al. [16], we used splitomicin, a specific inhibitor of Sirt 1 (50 µM 1 h before high glucose incubation), but we did not observe a SIRT 1-mediated effect of RES or PIC. Indeed, RES and PIC restored DDAH activity of cells activated by high glucose with a dose-dependent effect even in the presence of splitomicin. Yuan et al. [16] did not assess the effects of SIRT 1 on RES, only BTM-0512 (a non-hydroxylated derivative of RES) was tested. The difference in the chemical structures of these two compounds could explain the absence of effect on SIRT 1 pathway. This result is of particular interest, because RES and PIC could modulate the DDAH/ADMA pathway by another metabolic pathway than BTM-0512 did.

In conclusion, inhibition of both DDAH activity and expression by glucose-induced oxidative stress results in ADMA accumulation in BAEC, which could participate to the endothelial dysfunction observed in diabetes status. RES (10 µM) and PIC (1 µM) restored DDAH activity and expression under these conditions of oxidative stress. These results may suggest potential therapeutic intervention targeting RES or PIC administration to improve endothelial dysfunction.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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