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Folic acid modulates eNOS activity via effects on posttranslational modifications and protein–protein interactions ☆

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ABSTRACT

Folic acid enhances endothelial function and improves outcome in primary prevention of cardiovascular disease. The exact intracellular signalling mechanisms involved remain elusive and were therefore the subject of this study. Particular focus was placed on folic acid-induced changes in posttranslational modifications of endothelial nitric oxide synthase (eNOS). Cultured endothelial cells were exposed to folic acid in the absence or presence of phosphatidylinositol-3' kinase/Akt (PI3K/Akt) inhibitors. The phosphorylation status of eNOS was determined via western blotting. The activities of eNOS and PI3K/Akt were evaluated. The interaction of eNOS with caveolin-1, Heat-Shock Protein 90 and calmodulin was studied using co-immunoprecipitation. Intracellular localisation of eNOS was investigated using sucrose gradient centrifugation and confocal microscopy. Folic acid promoted eNOS dephosphorylation at negative regulatory sites, and increased phosphorylation at positive regulatory sites. Modulation of phosphorylation status was concomitant with increased cGMP concentrations, and PI3K/Akt activity. Inhibition of PI3K/Akt revealed specific roles for this kinase pathway in folic acid-mediated eNOS phosphorylation. Regulatory protein and eNOS protein associations were altered in favour of a positive regulatory effect in the absence of bulk changes in intracellular eNOS localisation. Folic acid-mediated eNOS activation involves the modulation of eNOS phosphorylation status at multiple residues and positive changes in important protein–protein interactions. Such intracellular mechanisms may in part explain improvements in clinical vascular outcome following folic acid treatment.

1. Introduction

Controversy exists as to the role of folic acid in the prevention/treatment of cardiovascular disease. However, our own studies have shown six weeks of daily folic acid (5 mg) supplementation in patients with coronary artery disease to improve endothelial function, independently of homocysteine lowering (Doshi et al., 2001, 2002; Moat et al., 2006b). Further *in vitro* investigations demonstrate that the folic acid-mediated improvement in endothelial function involves increased endothelial nitric oxide (NO) synthase (eNOS) dimerisation (Moat et al., 2006b), independently of tetrahydrobiopterin levels or an antioxidant effect (Moat et al., 2006a).

Numerous other studies have shown folic acid to produce significant improvements in endothelial function (Bellamy et al., 1999; Chambers et al., 2000; Shirodaria et al., 2007), however, secondary prevention studies have found no beneficial effect of folic acid on clinical outcomes in patients with established cardiovascular disease (Albert et al., 2008; Bonaa et al., 2006; Ebbing et al., 2008; Hankey et al., 2010; Lonn et al., 2006; Toole et al., 2004). This suggests that the endothelium is perhaps "beyond repair" in the latter populations while optimised endothelial function is crucial in preventing a first ever vascular event. An understanding of the mechanism(s) by which folic acid improves endothelial function is therefore essential.

A major contributor to cardiovascular health is the bioavailability of eNOS-derived NO. A complex series of post-translational events tightly regulates eNOS function (Rafikov et al., 2011), in particular, changes in phosphorylation at multiple serine (Ser) and threonine (Thr) residues (Mount et al., 2007). Negative regulation occurs through increased eNOS phosphorylation at Ser114/6 (Li et al., 2007) and Thr495/7 (Fleming et al., 2001), while positive regulation occurs through increased phosphorylation at Ser633/5 and Ser1177/9 (Boo et al., 2003; Dimmeler et al., 1999; Fulton et al., 1999; McCabe et al., 2000). Such changes are thought to be mediated via the actions of several serine/threonine kinases, in

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particular phosphatidylinositol-3 kinase/Akt (PI3K/Akt) (Mount et al., 2007).

Further control is provided by interactions of eNOS with other proteins (Fulton et al., 2001; Kone et al., 2003) such as that with caveolin-1 within plasmalemmal caveolae (Feron et al., 1996; Shaul et al., 1996). In this location caveolin-1 acts as an eNOS inhibitor, preventing inappropriate release of NO under "resting" conditions (Ju et al., 1997). Upon endothelial cell activation eNOS interacts with the calcium/calmodulin (Ca^{2+}/calmodulin) complex and dissociates from caveolin-1 (Michel et al., 1997a). A subsequent interaction between eNOS and Heat-Shock Protein 90 (HSP90) is thought to further increase eNOS activity by facilitating Ca^{2+}/calmodulin-associated dissociation from caveolin-1 (Gratton et al., 2000). Given the above, it would seem that the precise intracellular location of eNOS is also imperative with regard to achieving optimal enzyme activity. Indeed, that targeting eNOS to intracellular membrane domains is necessary for stimulated NO release (Sakoda et al., 1995; Sessa et al., 1995; Sullivan and Pollock, 2003), and Ser1179-phosphorylated eNOS becomes specifically localised to Golgi and plasma membrane pools of active enzyme (Fulton et al., 2002) supports this notion.

As such, determining the effects of folic acid on eNOS phosphorylation, protein–protein interactions and intracellular location/distribution is vital in understanding the mechanisms by which folate-mediated cardioprotection may arise.

2. Materials and methods

2.1. Cell culture

Porcine aortic endothelial cells were isolated as previously described (Moat et al., 2006b). Cells were grown to 80% confluence in Medium 199 (Life Technologies, Paisley, UK) containing 10% (*v*/*v*) foetal calf serum and 1% (*w*/*v*) penicillin/streptomycin in Primaria-coated flasks (Promega, Southampton, UK) before subculture at a ratio of 1:4. Confluent cells were serum starved overnight and before exposure to appropriate agents. All experiments were performed in serum-free medium.

2.2. Cell incubations

To investigate the time-dependent effects of folic acid on eNOS phosphorylation porcine aortic endothelial cells were incubated with either vehicle (serum-free medium, Control) or 5 μM folic acid (concentration based on a previous study, Moat et al., 2006b) for 1, 2, 4, 8, 16 or 24 h. The concentration-dependent effects of folic acid on eNOS phosphorylation were subsequently investigated by incubation of cells with either vehicle (Control) or 0.5, 5 or 50 μM folic acid (concentration range based on a previous study, Moat et al., 2006b) for 24 h.

In other experiments, and 30 min before addition of either vehicle or folic acid (5 μM, 24 h), some cultures received cell permeable PI3K/Akt inhibitors: wortmannin (100 nM) or LY294002 (10 μm).

Following each experiment cells were washed twice in ice-cold sterile 0.9% (*w*/*v*) saline then scraped into the appropriate lysis buffer.

2.3. Western blotting

Porcine aortic endothelial cells were lysed on ice for 30 min in modified radioimmunoprecipitation assay buffer (150 mM sodium chloride, 1% *v*/*v* Triton-X 100, 0.5% *w*/*v* deoxycholic acid, 0.1% w/v SDS, 50 mM Tris, pH 8.0) containing Complete Protease Inhibitor Cocktail (Roche, Welwyn Garden City, UK) and Halt™ Phosphatase Inhibitor Cocktail (Fisher Scientific Ltd, Loughborough, UK). Following centrifugation (16,000 g at 4 °C for 15 min) supernatant total protein content was determined by Coomassie protein assay. Reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to separate 20 μg of protein per well into a 7.5% gel, followed by transfer onto Hybond P membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) using methanol-based wet transfer at 4 °C for 2 h. Subsequently membranes were blocked overnight in 1% (*w*/*v*) non-fat milk/Tris-buffered saline Tween for eNOS or phospho-eNOS detection. For β-actin, caveolin-1 and calmodulin 5% (*w*/*v*) non-fat milk/Tris-buffered saline Tween was used. For HSP90 detection blocking was not applied. Phosphorylated proteins were first detected in membranes using rabbit anti-human phospho-eNOS Ser114/6, rabbit anti-bovine phospho-eNOS Ser633/5, rabbit anti-human phospho-eNOS Thr495/7 (all Millipore Ltd, Oxfordshire, UK), mouse anti-human phospho-eNOS Ser1177/9 (BD, Oxford, UK) and rabbit anti-human phospho-Akt Ser473 (Abcam, Cambridge, UK) primary antibodies. As appropriate membranes were then stripped and reprobed for total eNOS or Akt followed by detection of β-actin (using mouse anti-human eNOS (BD, Oxford, UK), rabbit anti-mouse Akt (New England Biolabs, Hitchin, UK) and goat anti-human β-actin (Santa Cruz Biotechnology, Heidelberg, Germany) primary antibodies). Alternatively the total protein levels of soluble guanylate cyclase, PI3K, caveolin-1, HSP90 or calmodulin were determined using rabbit anti-human soluble guanylate cyclase (Abcam, Cambridge, UK), PI3K, caveolin-1 (both Santa Cruz Biotechnology, Heidelberg, Germany), HSP90 and calmodulin (both Abcam, Cambridge, UK), and then membranes stripped and reprobed for β-actin (as above). Secondary HRP-conjugated antibody and enhanced chemiluminescence (Santa Cruz Biotechnology, Heidelberg, Germany) were used to visualise immunoreactive protein bands on photographic film (GE Healthcare Life Sciences, Buckinghamshire, UK).

Photographic films were scanned using a GS700 Densitometer and quantification achieved using Quantity One software (both Biorad, Hemel Hempstead, UK). Total eNOS, Akt, soluble guanylate cyclase, PI3K, caveolin-1, HSP90 and calmodulin were normalised to β-actin and then as a ratio to control in the absence of folic acid as appropriate. Phosphorylation of eNOS or Akt was expressed as the ratio of phosphorylated/total protein optical density (ODu) and then as a ratio to control in the absence of folic acid as appropriate.

2.4. Activity of eNOS

Porcine aortic endothelial cell cGMP content was measured as an index of eNOS activity. Following appropriate incubations in a concentration range of folic acid with some being performed in the presence of the eNOS inhibitor N^G-nitro-L-arginine-methyl ester (L-NAME, 100 μM) cells were stimulated with calcium ionophore A23187 (10 μM) for 30 min. In additional experiments cells were incubated with the PI3K and PKA inhibitors in the absence and presence of 5 μM folic acid as described above.

At appropriate end points cells were scraped into 0.1 M hydrochloric acid and cGMP concentration determined by commercial immuno-based assay (Abcam, Cambridge, UK) according to manufacturer instructions. Total lysate protein concentrations were measured by Coomassie protein assay, and cGMP concentration normalised.

2.5. Co-immunoprecipitation

Porcine aortic endothelial cells were treated as described previously before scraping into immunoprecipitation lysis buffer provided in the Classic IP kit (Thermo Fisher Scientific, Rockford, IL, USA). Co-immunoprecipitation was performed as per kit instructions using rabbit anti-human eNOS primary antibody to couple eNOS in lysates to protein A/G agarose beads.

2.6. Cellular fractionation

To investigate the cellular location of eNOS following folic acid stimulation confluent, serum starved and appropriately treated porcine aortic endothelial cells (T75 flask) were scraped into 0.5 ml 500 mM sodium carbonate, pH 11. Lysates were Dounce homogenised and sonicated (3×20 s bursts at 30% maximum power). For a final sample volume of 2 ml, lysates from $4 \times$ T75 flasks were pooled. To adjust the lysate sucrose concentration to 42.5% (w/v) sucrose, 2 ml 85% (w/v) sucrose prepared in 2-(N-morpholino)ethanesulfonic acid (MES)-buffered saline (25 mM MES, pH 6.5, 0.15 M sodium chloride) was added to the lysate in an ultracentrifuge tube. As previously described (Fulton et al., 2002), a discontinuous sucrose gradient (5–30% w/v) was then formed above the lysate by adding 5 ml 30% (w/v) sucrose then 6 ml 5% (w/v) sucrose in MES-buffered saline containing 250 mM sodium carbonate followed by centrifugation at 125,755 g for 24 h using an SW32 Ti rotor (Beckman Coulter (UK) Ltd, High Wycombe). Following this procedure, the 5–30% sucrose interface is characterised by the presence of cholesterol-rich microdomains (lipid rafts and caveolae), larger soluble proteins and heavy membranes remain at the bottom of the gradient. Gradient fractions were collected from the top of the gradient (1 ml per fraction) and the presence of eNOS, eNOS Ser1179p and caveolin-1 (antibodies as above) analysed using western blotting as described above.

2.7. Confocal microscopy

Porcine aortic endothelial cells were isolated and cultured as previously described and plated onto sterile coverslips. Cells were then treated with either vehicle or folic acid (5 μM) as appropriate for 24 h before being fixed with para-formaldehyde (2% w/v in 0.1 M Tris-buffered saline, pH 7.4 for 15 min). Subsequently cells were washed (3×5 min in phosphate-buffered saline) before exposure to blocking solution (phosphate-buffered saline containing 5% v/v donkey serum, 1% w/v bovine serum albumin and 0.1% v/v triton X-100) for 1 h. Primary antibody (or blocking solution alone for negative controls) was then added as appropriate and the fixed endothelial cells left to react overnight at 4 °C. Following removal of excess antibody by 3×15 min washes in phosphate-buffered saline, fixed endothelial cells were then incubated with an appropriate fluorescence-conjugated secondary antibody for 2 h. After further phosphate-buffered saline washes (3×15 min) coverslips were mounted on slides and kept in the dark. Fluorescent labelling was visualised using a Leica SP2 confocal microscope and images captured using Leica software.

2.8. Statistical analysis

Experimental data is representative of $n \geq 3$ independent experiments expressed as mean ± standard error of the mean. Analysis of variance was used for multiple comparisons, followed by appropriate post-hoc analysis. Differences were considered significant at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. Folic acid and changes in total protein

Total protein levels of eNOS (Fig. 1A and E) and β-actin and (Fig. 1B and E) were unaffected by exposure to 5 μM folic acid from 1 to 24 h or by exposure to a concentration range of folic acid (0.5–50 μM) for 24 h. Total soluble guanylate cyclase (Fig. 2B), PI3K (Fig. 3B) and Akt (Fig. 3B) protein levels were similarly unaltered following exposure to a concentration range of folic acid (0.5–50 μM) for 24 h. Exposure to PI3K/Akt inhibitors wortmannin and LY294002 in the absence and presence of 5 μM folic acid over 24 h did not result in changes in total β-actin or eNOS (Fig. 3A). Before co-immunoprecipitation with eNOS protein, and after exposure to a concentration range of folic acid (0.5–50 μM) for 24 h, total cellular levels of β-actin, eNOS, and the eNOS regulatory proteins HSP90, caveolin-1 and calmodulin were not altered (Fig. 4A). This indicates that incubations did not alter the transcription/translation of these proteins per se.

3.2. Folic acid and modulation of eNOS phosphorylation

Phosphorylation of eNOS at the positive regulatory residue Ser1179 was increased after exposure to 5 μM folic acid for 24 h (Fig. 1C). No change in phosphorylation was observed at the 1, 2, 4, 8 or 16 h time points (Fig. 1C). Similarly decreased phosphorylation at the negative regulatory site Thr497 was also observed post-5 μM folic acid treatment (Fig. 1D). Interestingly this site would seem to be more sensitive to the effects of folic acid, the change being significant at both the 16 and 24 h time points. The 24 h time point was subsequently used for experiments investigating the effects of a concentration range of folic acid on eNOS phosphorylation.

Following 24 h exposure of porcine aortic endothelial cells to increasing concentrations of folic acid, phosphorylation of eNOS at the positive regulatory sites Ser635 and Ser1179 was augmented compared to controls (Fig. 1E). Sensitivity was greatest at Ser1179, with significant effects first observed at 0.5 μM (Fig. 1E) compared to 5 μM for Ser635 (Fig. 1E). The biggest changes in phosphorylation at these sites were also strikingly different as evidenced by a 300–400% increase at Ser1179 compared with only 100–150% at Ser635.

In the same experiments decreased phosphorylation of eNOS at the negative regulatory sites Ser116 and Thr497 was also observed (Fig. 1E). As in the temporal experiment, sensitivity was greatest at Thr497, with significant effects first observed at 0.5 μM folic acid compared to 5 μM for Ser116 (Fig. 1E). The maximum decreases in phosphorylation were similar at both sites.

3.3. Folic acid and eNOS activity: effects of protein kinase inhibition

Exposure of untreated porcine aortic endothelial cells to calcium ionophore A23187 produced an approximate 10-fold increase in cGMP (an index of eNOS activation) above baseline values (Fig. 2A). In the presence of folic acid significant concentration-dependent increases in both basal and A23187-stimulated endothelial cells cGMP levels were observed (Fig. 2A). That eNOS-derived NO mediates the elevation in cGMP is confirmed by the inhibitory action of L-NAME on these responses (Fig. 2A).

The presence of PI3K/Akt inhibition prevented A23187-stimulated increases in cGMP in both control (0.30 ± 0.17 and 0.21 ± 0.06 fmol/mg protein respectively for wortmannin and LY294002 cf 1140.41 ± 174.49 fmol/mg protein in their absence) and folic acid-treated cells (0.30 ± 0.17 and 0.21 ± 0.06 fmol/mg protein respectively for wortmannin and LY294002 cf 4624.10 ± 417.01 fmol/mg protein in their absence).

3.4. Folic acid and eNOS phosphorylation: effects of PI3K/Akt inhibition

The PI3K/Akt inhibitors wortmannin and LY294002 mediated a significant increase in phosphorylation at Ser116 (Fig. 3A) and significant decreases at Thr497, Ser635 and Ser1179 (Fig. 3A). In the presence of such inhibition folic acid was unable to promote the changes in phosphorylation at Ser116, Thr497, Ser635 and Ser1179 described above.

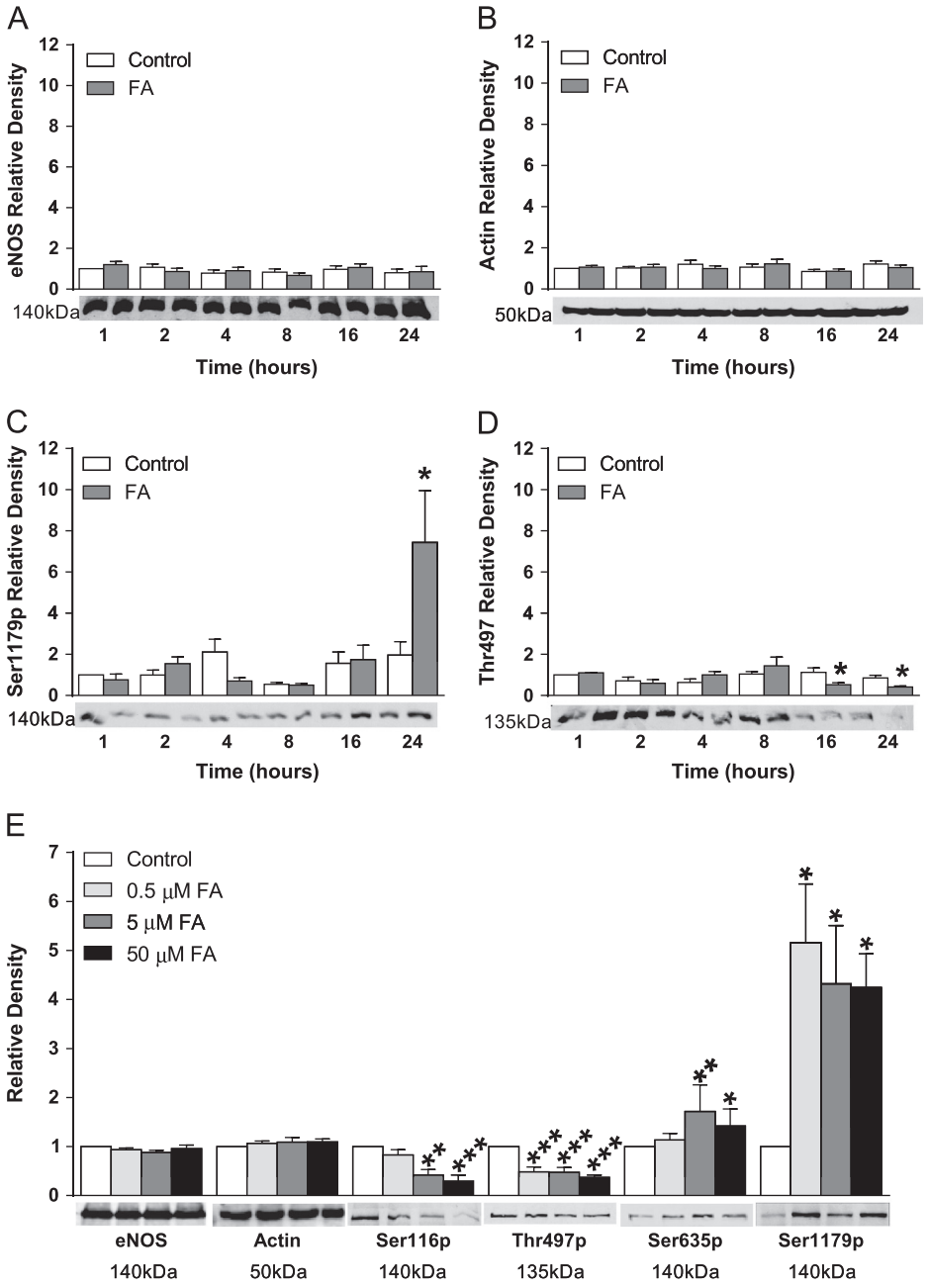

Fig. 1. Folic acid modulates eNOS phosphorylation status. Western blotting and densitometric analysis of porcine aortic endothelial cell lysates was used to assess the effects of folic acid (FA, 5 μM) incubation over 1–24 h (A–D), and a concentration range of folic acid (0–50 μM) for 24 h (E) on total eNOS, β-actin and eNOS Ser116p, Thr497p, Ser635p and Ser1179p (eNOS was normalised to β-actin levels and Ser116p, Thr497p, Ser635p and Ser1179p normalised to total eNOS). Appropriate representative blots are shown. Molecular weight of proteins given in kDa. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ cf the appropriate "Control".

3.5. Folic acid and PI3K/Akt activity

Exposure of porcine aortic endothelial cells to increasing concentrations of folic acid (0.5–50 μM) for 24 h had no effect on total levels of PI3K and Akt (Fig. 3B). A significant increase in Ser473-phosphorylated Akt (pAkt, an indicator of PI3K/Akt activity) was evident in the presence of the highest concentration of folic acid (Fig. 3B).

3.6. Regulatory protein association with eNOS

Folic acid treatment did not alter total eNOS, caveolin-1, HSP90 or calmodulin protein levels (Fig. 4A). However, co-immunoprecipitation revealed a concentration-dependent decrease in the interaction of eNOS with caveolin-1 (Fig. 4B) and an increase in that of eNOS with HSP90 (Fig. 4B). Folic acid had no effect on the interaction of eNOS and calmodulin (Fig. 4B).

3.7. Cellular localisation

Following discontinuous sucrose gradient ultracentrifugation, western blotting was used to identify proteins in the various cell fractions. Confirmation of adequate cellular separation was provided by the presence of the cytoskeletal protein β-actin in fractions 12–14 (soluble proteins and heavy membranes) (Fig. 5B) and the plasma membrane caveolae protein caveolin-1 in fractions 6–9 (buoyant cholesterol-rich light membranes) (Fig. 5D). Western blotting for both total eNOS and eNOS Ser1179p revealed that

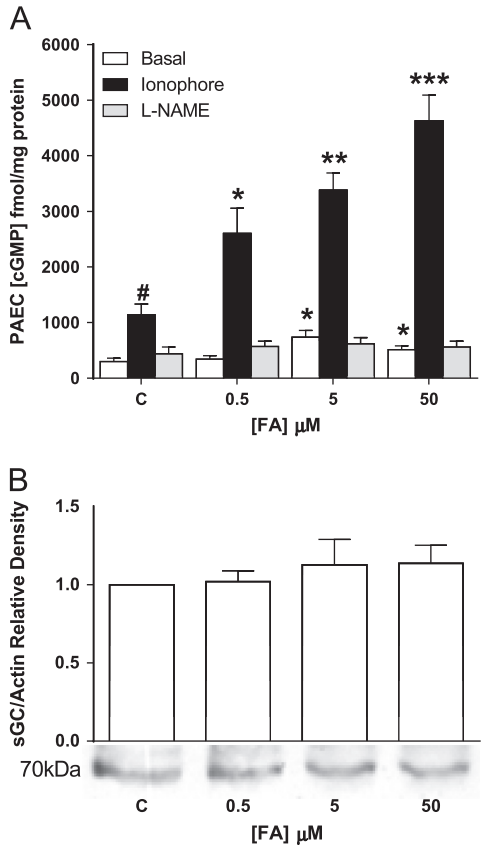

Fig. 2. Folic acid modulates eNOS phosphorylation activity. Activity of eNOS post folic acid incubation (0–50 μM, 24 h) was assessed indirectly via cGMP assay in unstimulated (Basal) porcine aortic endothelial cells (PAEC) and following calcium ionophore A23187 (Ionophore) stimulation in the absence or presence of L-NAME (A). Western blotting and densitometric analysis of PAEC lysates was used to assess the effect of folic acid (FA, 0–50 μM, 24 h) incubation on total soluble guanylate cyclase protein (sGC) (B). Appropriate representative blots are shown. Molecular weight of proteins given in kDa. $\#P < 0.01$ cf basal; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ cf Ionophore in the absence of folic acid (C).

these proteins reside in discrete intracellular locations corresponding to both light membrane and heavy membrane fractions (Fig. 5A and C respectively). No obvious change in the distribution of total eNOS protein or eNOS Ser1179p was found following stimulation with 5 μM folic acid (Fig. 5A and C respectively).

Confocal microscopy was used to confirm the subcellular location of porcine aortic endothelial cell eNOS in the absence and presence of folic acid (0.5, 5 or 50 μM for 24 h). Interestingly eNOS protein was found localised to both the perinuclear region in a "punctate vesicular pattern" (similar to that previously described (Fulton et al., 2002) and most likely representing a Golgi localised enzyme pool (Sowa et al., 1999)) and the periphery (most likely to representing a caveolae-localised enzyme pool) (Fig. 5E). Exposure to folic acid did not alter this distribution pattern (Fig. 5F–H).

4. Discussion

The data presented in this study provides a novel mechanism for folic acid-mediated eNOS activation. In the absence of a change in total eNOS protein, folic acid promotes decreased phosphorylation of eNOS at negative regulatory sites (Ser116 and Thr497), increased phosphorylation at positive regulatory sites (Ser635 and Ser1179) and augmentation of both basal and stimulated eNOS activity. The data identifies a role for the PI3K/Akt pathway in maintaining basal eNOS phosphorylation and mediating these folic acid-induced changes. Shifts in specific posttranslational protein–protein interactions that favour increased eNOS activity following folic acid exposure are also described. Importantly, decreased phosphorylation of Thr497, increased phosphorylation of Ser1179 and increased eNOS activity were observed at a folic acid concentration (0.5 μM) easily attainable following clinical supplementation (Doshi et al., 2002).

Folic acid initially received significant attention as a therapeutic agent in the amelioration of vascular disease due to its role in lowering plasma homocysteine. Though folic acid doses ranging from 400 μg to 5 mg/day produce similar (~25% decrease) homocysteine-lowering effects (Perrier et al., 2009), supplementary levels of up to 2.5 mg/daily have largely failed to improve endothelial function in patients with established cardiovascular disease (Bonaa et al., 2006; Carlsson et al., 2004; Durga et al., 2005; Dusitanond et al., 2005; Klerk et al., 2005; Lonn et al., 2006; Toole et al., 2004). Higher folic acid doses of 5 mg/day or greater are effective in improving cardiovascular outcome independently of an effect on homocysteine (Bellamy et al., 1999; Chambers et al., 2000; Doshi et al., 2001, 2002; Moat et al., 2006b). With regard to underlying mechanisms of action, folate-mediated peroxynitrite radical scavenging, decreased superoxide generation (Antoniades et al., 2006) and increased eNOS dimerisation (Moat et al., 2006b) have been suggested. More recently folic acid-induced PI3K/Akt-mediated activation of eNOS via phosphorylation at Ser1179 has been suggested (Seto et al., 2010). Data presented in the current study confirms this observation and demonstrates that folic acid affects three other eNOS phosphoregulatory sites in a concentration-dependent manner. While the clinical implications of these findings remain to be explored, it is possible that the folic acid-mediated effects on eNOS activity described herein may underlie the beneficial role of folic acid in specific cardiovascular disease conditions such as abdominal aortic aneurysm (Gao et al., 2012).

Human oral supplementation of 5 mg folic acid produces an acute rise in plasma folate concentration in excess of 0.5 μM (Doshi et al., 2002), the lowest concentration used in the present study. While no effect on eNOS phosphorylation at Ser116 was observed at this concentration, 5 μM and 50 μM folic acid promoted a significant decrease. Although some *in vitro* studies have shown that Ser116 dephosphorylation has no effect on eNOS activation (Boo et al., 2003), others have demonstrated increased endothelial NO release (Li et al., 2007). The latter corroborates the present study indicating a role for Ser116 dephosphorylation in folic acid-mediated increases in eNOS activity. Moreover it is possible that clinical doses of folic acid below 5 mg/day are not sufficient to improve endothelial function via this mechanism. However, such an assumption is based on a 24 h *in vitro* incubation and may be due to a lack of acute sensitivity. Continuous daily supplementation may produce different effects *in vivo*.

While others have shown protein kinase C (Monti et al., 2010) and cyclin-dependent kinase 5 (Cho et al., 2010) to be involved in regulation of eNOS Ser116 phosphorylation, to our knowledge this is the first study to demonstrate increased Ser116 phosphorylation following PI3K/Akt inhibition. The data clearly demonstrates an increase in baseline phosphorylation at this epitope in the presence of both wortmannin and LY294002 and that such inhibition prevents the folic acid-mediated decrease in phosphorylation described above. Since basal and stimulated porcine aortic endothelial cell cGMP production, and the effects of folic acid thereon, are also inhibited by wortmannin and LY294002, it is possible that a folic acid/PI3K/Akt/eNOS pSer116 axis exists. As such this putative pathway provides a novel avenue of investigation with regard to eNOS control mechanisms.

With regard to eNOS Thr497, significant decreases in phosphorylation were observed across the folic acid concentration range. Interestingly, PI3K/Akt inhibition was also accompanied by similar Thr497 dephosphorylation. While the latter implies that

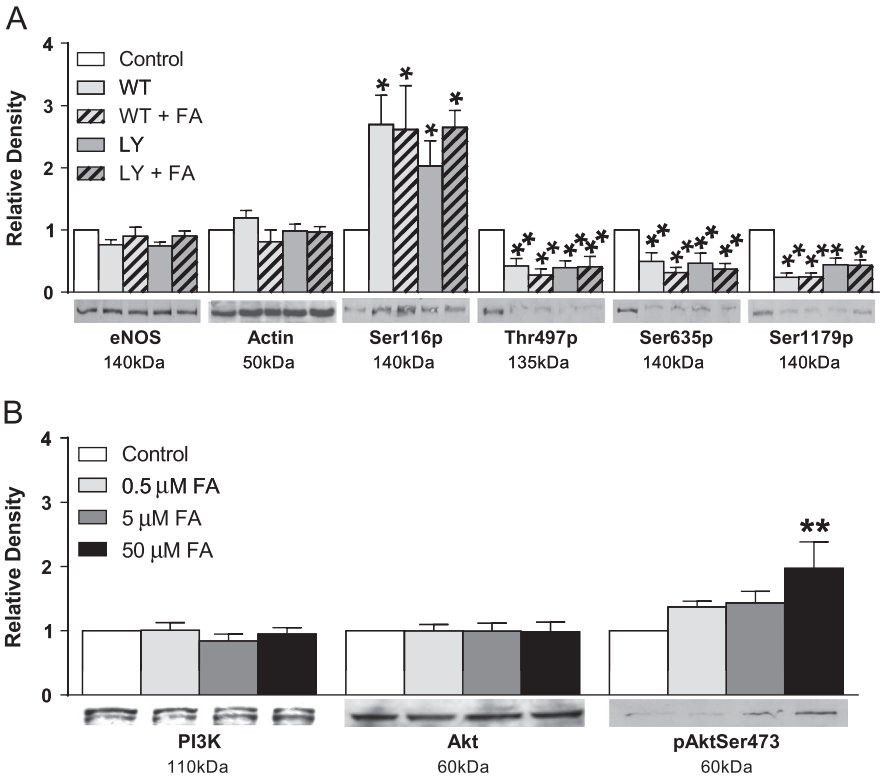

Fig. 3. Folic acid alters eNOS phosphorylation and kinase activity. Western blotting and densitometric analysis of porcine aortic endothelial cell lysates was used to assess the effects of PI3K/Akt inhibition with either wortmannin (WM) or LY294,002 (LY) on total eNOS, β-actin and eNOS Ser116p, Thr497p, Ser635p and Ser1179p in the absence (Control) or presence (+FA) of folic acid (FA, 5 μM, 24 h) (eNOS was normalised β-actin levels and Ser116p, Thr497p, Ser635p and Ser1179p normalised to total eNOS) (A). The same methodology was used to investigate the effects of folic acid incubation (0–50 μM, 24 h) on total PI3K, Akt and pAktSer473 (B). Appropriate representative blots are shown. Molecular weight of proteins given in kDa. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ cf the appropriate "Control".

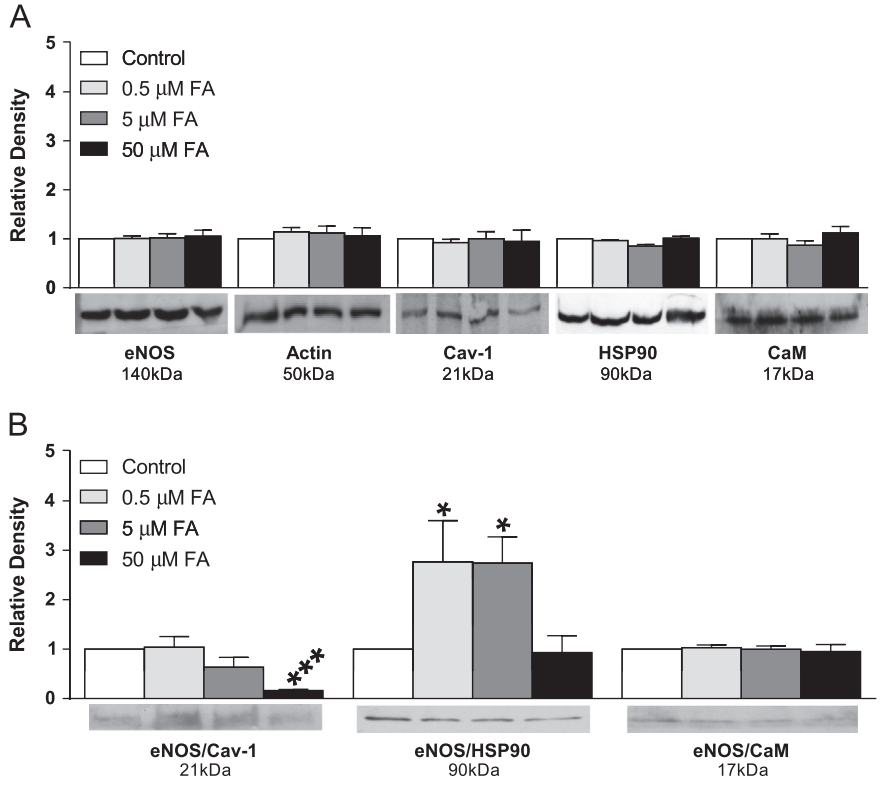

Fig. 4. Folic acid alters the interaction of eNOS with regulatory proteins. Western blotting and densitometric analysis of porcine aortic endothelial cell lysates was used to assess the effects of folic acid (FA, 0–50 μM, 24 h) on total protein levels of eNOS, β-actin and the associated regulatory proteins caveolin-1 (Cav-1), HSP90 and calmodulin (CaM) (A). Co-immunoprecipitation of eNOS with associated regulatory proteins in these cell lysates, followed by the same analysis, was used to investigate the effects of folic acid on eNOS/Cav-1, eNOS/HSP90 and eNOS/CaM associations (B). Appropriate representative blots are shown. Molecular weight of proteins given in kDa. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ cf the appropriate "Control".

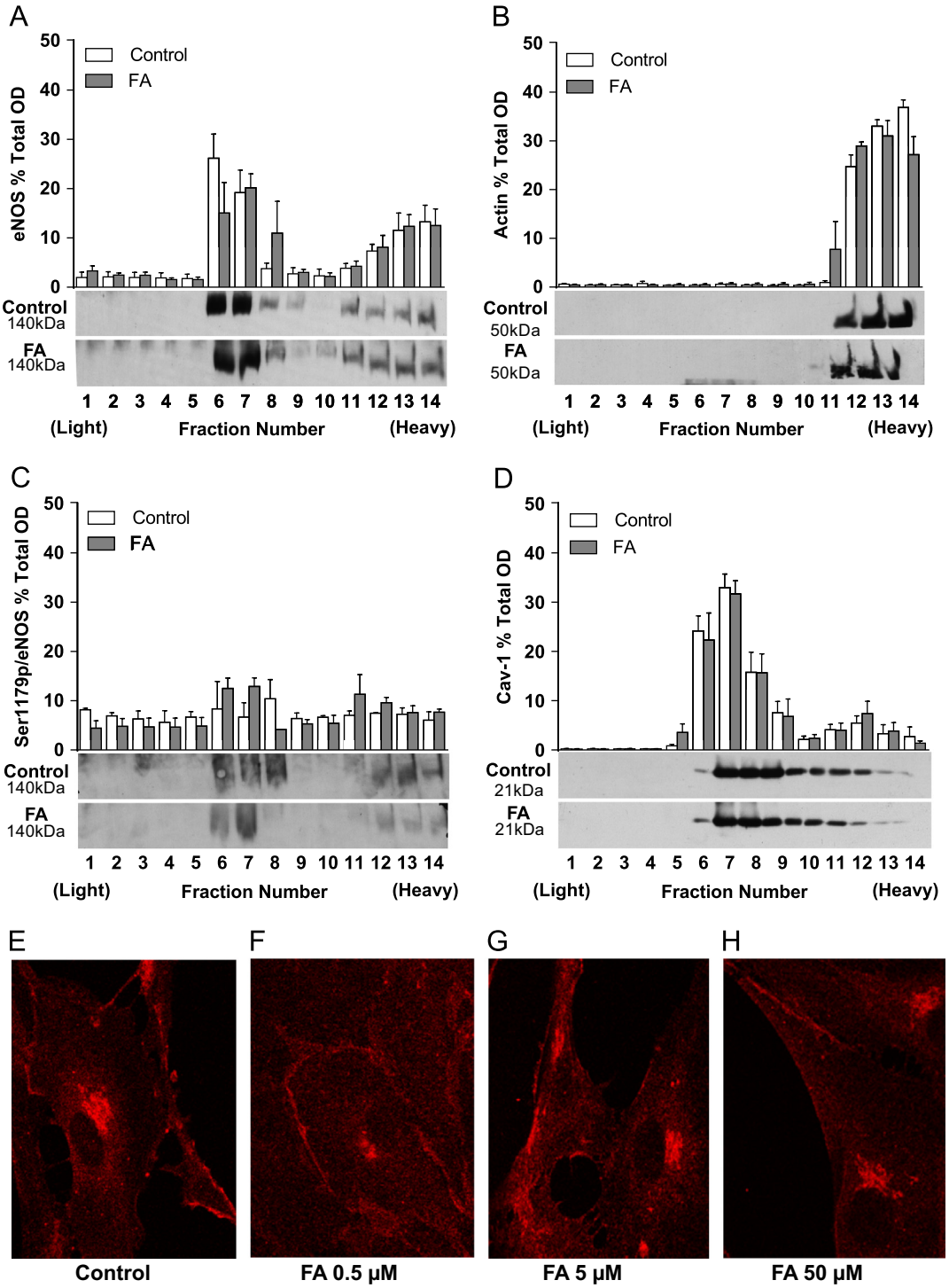

Fig. 5. Subcellular location of eNOS and eNOS pSer1179. After incubation of porcine aortic endothelial cells with folic acid (FA, 0 and 5 μM, 24 h) lysates were subject to discontinuous sucrose gradient ultracentrifugation to separate cholesterol-rich microdomains before western blotting for total eNOS (A), β-actin (B), Ser1179p/eNOS (C) and caveolin-1 (Cav-1) (D). Densitometric quantification was expressed as percentage relative protein across the sucrose gradient. Appropriate representative blots are shown. Molecular weight of proteins given in kDa. Values represent data from a minimum of two independent experiments. Confocal microscopy was used to confirm the subcellular location of porcine aortic endothelial cell eNOS (red) in the absence and presence of folic acid (FA, 0.5–50 μM, 24 h) (E–H). The images show that eNOS protein was found localised to both the perinuclear region (probable Golgi pool) and the cell membrane (probable caveolar pool). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PI3K/Akt is involved in sustaining basal levels of Thr497 phosphorylation, it also suggests that the effect of folic acid at this residue is PI3K/Akt independent. This indicates an important divergence with regard to folic acid and its role in moderating eNOS activity. Further in depth studies will be required to address the mechanisms and biochemical mediators involved.

Phosphorylation of eNOS at Ser635 is described as one of the most important points of regulation of basal NO production and in this respect is independent of phosphorylation at other sites (Bauer et al., 2003) and/or intracellular calcium release (Boo et al., 2003). Data presented here shows folic acid to promote increased Ser635 phosphorylation while PI3K/Akt inhibition does

the opposite, and indeed prevents the folic acid-mediated changes at this residue. This suggests the presence of a folic acid/PI3K/Akt/eNOS pSer635 axis. However, that only the highest folic acid concentrations produce minor increases in phosphorylation, whereas dephosphorylation occurs across the concentration range, questions the relevance of such a pathway. Interestingly previous studies have indicated that PI3K/Akt does not directly phosphorylate Ser635 (Michell et al., 2002), rather PI3K/Akt-dependent mechanisms may involve interactions with other signalling pathways or cooperative phosphorylation mechanisms (Zhang and Hintze, 2006). While the latter requires further study, that Ser635 phosphorylation is involved in basal eNOS regulation indicates that changes at this residue may play an important role in optimising normal endothelial function. Though a lack of effect of 0.5 μM folic acid may again question *in vivo* relevance, the same argument as to continuous daily supplementation described above would apply.

Phosphorylation at Ser1179 is suggested to play the most significant role in modifying eNOS activity (Lane and Gross, 2002; McCabe et al., 2000). The data presented here confirms that folic acid positively regulates Ser1179 phosphorylation and increased eNOS activity at all concentrations used (0.5–50 μM). Indeed it is evident that Ser1179 is the most "folic acid-sensitive" residue amongst those investigated with maximum phosphorylation levels following folic acid exposure around 3–4 fold greater than controls.

A significant role for Akt in mediating eNOS phosphorylation at Ser1179 has been widely described. It is therefore not surprising that PI3K/Akt inhibition in the experiments described here is accompanied by a decrease in phosphorylation at this epitope. Moreover, that this inhibition prevents any folic acid-induced increase in Ser1179 phosphorylation suggests the existence of a further folic acid/PI3K/Akt/eNOS pSer1179 axis. Given the "folic acid-sensitivity" of eNOS Ser1179, such an axis would have far reaching effects on eNOS activity.

The data presented here also demonstrates significant increases in both basal and calcium ionophore-induced cGMP production following exposure to folic acid in the absence of increased soluble guanylate cyclase expression. Furthermore, that L-NAME inhibits folic acid-mediated increases in both basal and ionophore-induced cGMP production would indeed imply that these measurements reflect changes in eNOS activity. Importantly, our previous studies would seem to rule out a role for folic acid-mediated stabilisation/regeneration of the eNOS co-factor tetrahydrobiopterin in this process (Moat et al., 2006a). Moreover, while it cannot be completely ruled out, a direct antioxidant role for folic acid is also highly unlikely (Moat et al. 2006a). That notwithstanding, given the negative effect of PI3K/Akt inhibition on porcine aortic endothelial cell cGMP production presented here, the folic acid/PI3K/Akt/eNOS phosphorylation axes described above provide important and novel pathways that may underlie the positive effects of folic acid in improving endothelial function.

Data from the pharmacological inhibitory experiments described here provide significant evidence of a definitive role for PI3K/Akt in mediating the effects of folic acid. However, with regard PI3K/Akt activity, significant increases in the Ser473-phosphorylated epitope of Akt were evident only in the presence of the highest concentration of folic acid. This anomaly is most likely caused by the sensitivity of our assay system (the western blots do indicate the presence of Ser473pAkt at lower folic acid concentrations) and should not detract from the importance of the other observations presented.

Undoubtedly the aforementioned changes in eNOS phosphorylation have a significant effect on enzyme activity. However, as mentioned above, there are other mechanisms of eNOS regulation, in particular the interaction with other proteins (Fulton et al., 2001; Kone et al., 2003) and the effects of subcellular localization (Shaul, 2002). It is clear from the present studies that folic acid promotes a significant increase/decrease in the interaction of eNOS with HSP90/caveolin-1 respectively, while that with calmodulin is unaffected. However, the lack of effect of 50 μM folic acid on the eNOS/HSP90 interaction is puzzling. It is possible that this very high concentration promotes more rapid eNOS/HSP90 interactions and that these are missed by our 24 h "snap-shot". Without significant further experiments, out of the scope of the present study, it is impossible to confirm this. However, given the other significant effects mediated by 50 μM folic acid, an HSP90 independent mechanism at this concentration seems unlikely.

Importantly the changes in protein–protein interactions described here are not a consequence of alterations in transcription/translation and occur in the absence of an obvious change in the subcellular distribution of eNOS protein. Given that several other stimuli are well reported to have similar effects on the HSP90/eNOS and caveolin-1/eNOS interactions, thus increasing NO synthesis (Brouet et al., 2001; Forstermann et al., 1991; Garcia-Cardena et al., 1998; Michel et al., 1997b; Takahashi and Mendelsohn, 2003) suggests that this action of folic acid could also play a significant role in improving endothelial function in the studies described above.

5. Conclusion

The present study demonstrates that folic acid modulates eNOS activity via both positive and negative phosphorylation events that involve important intracellular protein kinase-dependent signalling axes. Furthermore, folic acid also influences changes in important eNOS protein interactions which would ultimately lead to increased enzyme activity. Notably these data would implicate folic acid in the optimisation of normal endothelial function and the possible prevention of an initial, and deleterious, cardiovascular event.

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