

# AMP-activated protein kinase phosphorylation of endothelial NO synthase

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**Abstract** The AMP-activated protein kinase (AMPK) in rat skeletal and cardiac muscle is activated by vigorous exercise and ischaemic stress. Under these conditions AMPK phosphorylates and inhibits acetyl-coenzyme A carboxylase causing increased oxidation of fatty acids. Here we show that AMPK co-immunoprecipitates with cardiac endothelial NO synthase (eNOS) and phosphorylates Ser-1177 in the presence of Ca<sup>2+</sup>-calmodulin (CaM) to activate eNOS both in vitro and during ischaemia in rat hearts. In the absence of Ca<sup>2+</sup>-calmodulin, AMPK also phosphorylates eNOS at Thr-495 in the CaM-binding sequence, resulting in inhibition of eNOS activity but Thr-495 phosphorylation is unchanged during ischaemia. Phosphorylation of eNOS by the AMPK in endothelial cells and myocytes provides a further regulatory link between metabolic stress and cardiovascular function.

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**Key words:** AMP-activated protein kinase; Endothelial NO synthase; Cardiac; Ischaemia

## 1. Introduction

The AMP-activated protein kinase (AMPK) isoforms  $\alpha 1$  and  $\alpha 2$  consist of a catalytic  $\alpha$  subunit [1,2] together with  $\beta$  and  $\gamma$  non-catalytic subunits [3–5]. AMPK is related to the *Saccharomyces cerevisiae* SNF1 protein kinase required for expression of glucose-repressed genes during nutritional stress [6]. AMPK phosphorylates a range of metabolic enzymes [7] and has recently been implicated in an increasing number of physiological functions including glucose transport [8] and regulation of transcription [9] (reviewed in [10]). The AMPK- $\alpha 2$  isoform is present in capillary endothelial cells in cardiac and skeletal muscle and the AMPK- $\alpha 1$  isoforms occurs in cardiac myocytes and vessels (results not shown). The presence of AMPK in endothelial cells prompted us to test the hypothesis that endothelial NO synthase (eNOS) may be an AMPK substrate using bacterially expressed eNOS.

## 2. Materials and methods

### 2.1. AMPK- $\alpha 1$ purification and assay

The AMPK was purified from rat liver and assayed using the procedures previously reported [11]. Immunoblotting of the isoforms of the AMPK was done with antibodies raised against the following peptides:  $\alpha 2$ (490–516) or  $\alpha 1$ (373–390).

### 2.2. Phosphorylation site sequencing

Phosphopeptides isolated from in-gel tryptic digests as described [12] were separated by reversed phase chromatography (Pharmacia SMART system). More than 98% of the radioactivity was recovered from the gel. The separated peptides were analysed using a linear Voyager DE (PerSeptive Biosystems) MALDI-TOF instrument in delayed extraction mode. Location of the phosphorylation site in peptide 'TQXFSLQER' was identified by [<sup>32</sup>P]phosphate release sequencing [12] (Fig. 1).

### 2.3. NO synthase assay

The eNOS activity was determined by measuring L-[<sup>3</sup>H]citrulline production [13]. The recombinant eNOS was co-expressed with calmodulin (CaM) [14]. In the absence of added EGTA, CaM dependence was observed at 0–100 nM added CaM. In order to investigate the changes in NOS activity with phosphorylation, in the absence and presence of Ca<sup>2+</sup>-CaM preincubation, EGTA buffering was used to achieve CaM dose-response curves in the range 0–1  $\mu$ M. Routinely, 7–15  $\mu$ M EGTA was added to make eNOS activity dependent upon added CaM. Where Ca<sup>2+</sup>-CaM was used in the phosphorylation reaction, prior to the eNOS assay, the samples were either diluted so that the added Ca<sup>2+</sup>-CaM was negligible or the indicated concentrations represent total final concentrations of added Ca<sup>2+</sup>-CaM. Cardiac eNOS was partially purified from 20 rat hearts homogenised in 80 ml of ice-cold buffer A (50 mM Tris-HCl buffer, pH 7.5 containing 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM NaF, 5 mM Na pyrophosphate, 10  $\mu$ g/ml trypsin inhibitor, 2  $\mu$ g/ml aprotinin, 1 mM benzamide, 1 mM PMSF, 10% glycerol and 1% Triton X-100). The homogenate was put on ice for 30 min and centrifuged at 16000  $\times g$  for 30 min. The supernatant was incubated with 2 ml of 2',5'-ADP Sepharose [15]. After 1 h incubation, the suspension was poured into a fritted column, washed with 20 ml of buffer A, 20 ml of buffer A with 0.5 M NaCl, and then with 20 ml of buffer B (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10% glycerol, 0.1% Triton X-100). eNOS was eluted with buffer B containing 2 mM NADPH then subjected to centrifugal ultrafiltration (Ultrafree-MC, Millipore) to remove NADPH. The isolated eNOS contained only a faint trace of nNOS $\mu$  detected by immunoblotting using an nNOS antibody that recognises nNOS $\mu$  from skeletal muscle [16].

### 2.4. Antiphosphopeptide-specific antibodies

Rabbit polyclonal antibodies raised against phosphopeptides based on the amino acid sequence of human eNOS RIRTQSpFSLQER (S-1177) and GITRKKTpFKEVANCV (T-495) were purified using the corresponding phosphopeptide affinity columns after pre-clearing with dephosphopeptide affinity columns. The specificity of the purified

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antibodies was evaluated by both enzyme immunoassay and immunoblotting, showing that they did not recognise dephospho-eNOS.

### 3. Results and discussion

Recombinant eNOS [17] was readily phosphorylated in the presence of AMPK and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  but not in the absence of AMPK. AMPK- $\alpha 1$  (Fig. 1, top left panel) and AMPK- $\alpha 2$  (not shown) isoforms phosphorylate eNOS. Following tryptic digestion of the phosphorylated recombinant eNOS revealed four phosphopeptides generated from three separate sites (Fig. 1, bottom panel, A, A', B, C) on the phosphopeptide map. Identification of phosphorylation sites by mass spectrometry and Edman sequencing [18] revealed Ser-1177 was the most prominent phosphorylation site (Fig. 1, bottom panel, A (TQSFSLQER), A' (IRTQSFSLQER)). A second site, Thr-495, was phosphorylated in the absence of  $\text{Ca}^{2+}$ -CaM or when EGTA was present (Fig. 1, bottom panel, B (KKTFKEVANAVK)). This residue is located in the CaM-binding sequence, TRKKT<sup>495</sup>FKEVANAVKISASLM, between the oxidase and reductase domains of eNOS [19]. Ser-101 was identified as a minor site with low stoichiometry (Fig. 1, bottom panel, C, CLGS<sup>101</sup>LVFPR). Synthetic peptides corresponding to Thr-495 and Ser-1177 phosphorylation sites were readily phosphorylated by AMPK: GTGIT-RKKT<sup>495</sup>FKEVANAVK,  $K_m$   $39 \pm 10$   $\mu\text{M}$  and  $V_{max}$   $6.7 \pm 0.6$   $\mu\text{mol/min/mg}$ ; RIRTQS<sup>1177</sup>FSLQERQLRG,  $K_m$   $54 \pm 6$   $\mu\text{M}$  and  $V_{max}$   $5.8 \pm 0.3$   $\mu\text{mol/min/mg}$ . The well characterised AMPK peptide substrate, SAMS is phosphorylated with

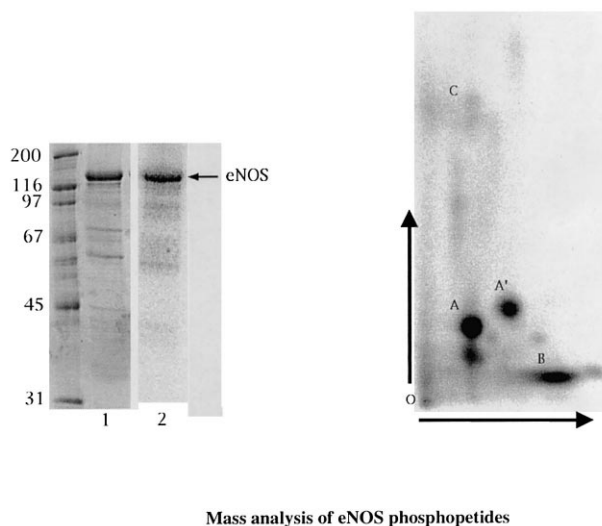


Fig. 1. Phosphorylation of recombinant eNOS with AMPK. Upper left panel: Recombinant eNOS (1  $\mu\text{M}$ ), co-expressed with CaM [14], was incubated with rat liver AMPK  $\alpha 1$  (10 nM) and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  for 20 min at 20°C. Coomassie-stained SDS-PAGE (lane 1) and autoradiograph (lane 2) are shown. Upper right panel:  $[\gamma\text{-}^{32}\text{P}]\text{tryptic}$  phosphopeptide map of eNOS from in-gel tryptic digests as previously described [12] with o marking the origin. Lower table: mass analysis of phosphopeptides; pc denotes pyridylethyl cysteine, \* denotes calculated mass of mono-phosphorylated peptide. Location of the phosphorylation site in peptide 'TQSFSLQER' was identified by  $[\gamma\text{-}^{32}\text{P}]\text{phosphate}$  release sequencing in the third cycle [12].

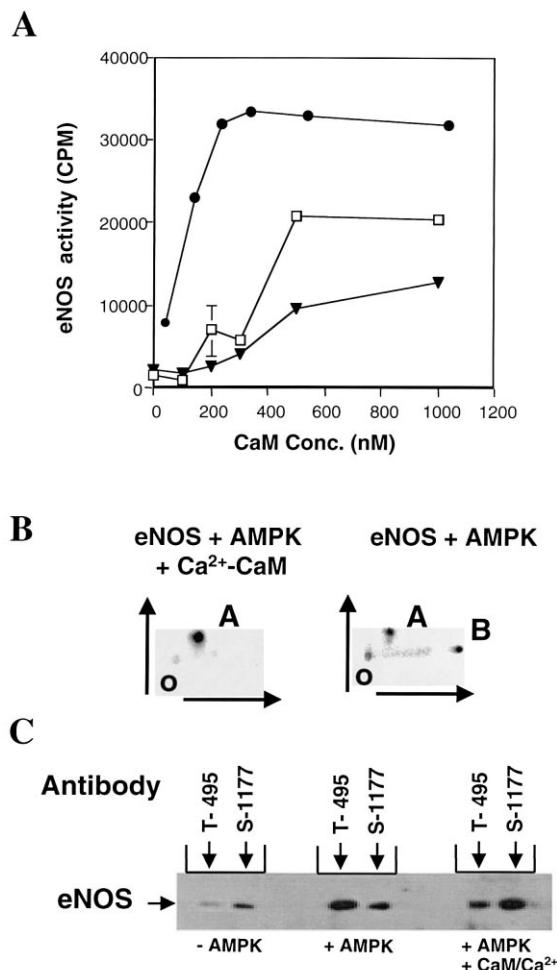


Fig. 2. Effect of phosphorylation of rat heart eNOS by AMPK with or without added  $\text{Ca}^{2+}$ -CaM. A: Rat heart eNOS (30 nM) purified by 2',5'-ADP-Sepharose affinity chromatography was phosphorylated by AMPK (1.3 nM) (20 min at 20°C) in the presence of 0.8  $\mu\text{M}$  CaM/3.2  $\mu\text{M}$   $\text{Ca}^{2+}$  (●), in the absence of  $\text{Ca}^{2+}$ -CaM (▼) and without AMPK (□). After phosphorylation, the samples were diluted, and eNOS activity was measured. B: Phosphopeptide maps of rat heart eNOS phosphorylated with and without added  $\text{Ca}^{2+}$ -CaM. The phosphorylation stoichiometry ranged from 0.3 to 0.5 mol/mol under these conditions. C: Immunoblotting of phosphorylated eNOS, eNOS phosphorylation as described in A. Polyclonal antibodies raised against synthetic phosphopeptides to the eNOS phosphorylation sites RIRTQSpFSLQER (labeled S-1177) and GITRKKTpFKEVANCV (labeled T-495) were used to detect phosphorylation of eNOS by immunoblotting.

a  $K_m$  of  $33 \pm 3$   $\mu\text{M}$  and  $V_{max}$  of  $8.1 \pm 1.5$   $\mu\text{mol/min/mg}$  [20,21]. Previous studies on the specificity of AMPK with synthetic peptides supported the concept that hydrophobic residues at the P-5 and the P+4 position were important [21]. While the Thr-495 site conforms to this arrangement, the Ser-1177 site has hydrophobic residues at P-4 and P+3 and yet it is a good substrate, suggesting that the AMPK may target a greater diversity of sequences than was previously recognised.

Unlike native eNOS, bacterial expressed enzyme is not post-translationally myristoylated or palmitoylated and for this reason it was important to test purified rat heart eNOS as a substrate for the AMPK. Phosphorylation of rat heart eNOS with AMPK also resulted in Thr-495 and Ser-1177

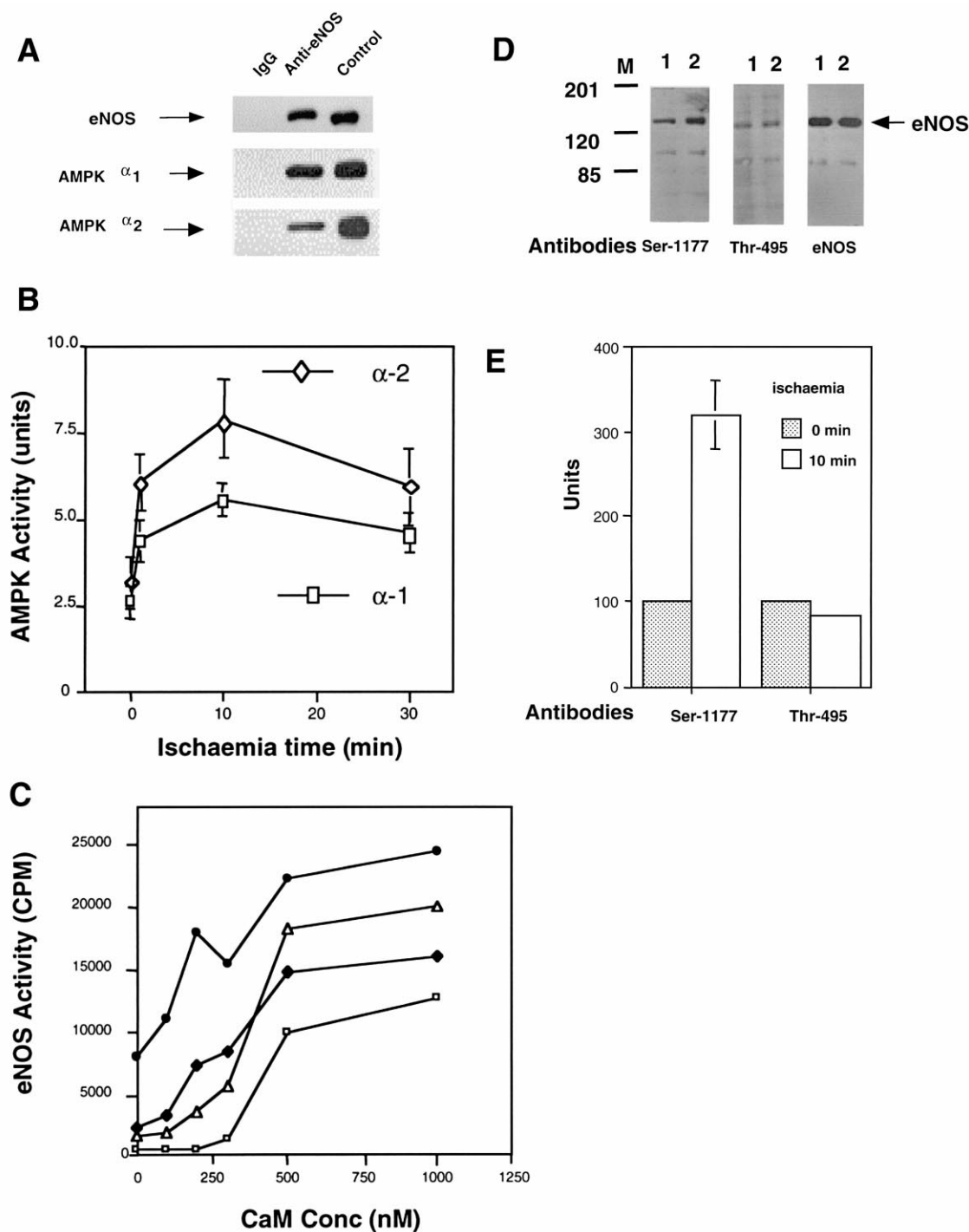


Fig. 3. Effect of ischaemia on the AMPK- $\alpha_1$  and  $\alpha_2$  activities and eNOS. A: Rat heart homogenates were immunoprecipitated with either an anti-mouse immunoglobulin (IgG, negative control) or an anti-eNOS monoclonal antibody (ECNOS, Trans.Lab.). Immunoprecipitates were immunoblotted for eNOS and AMPK- $\alpha_1$  and  $\alpha_2$ . An eNOS and AMPK positive controls are shown (Control). B: Langendorf isolated perfused rat hearts were subjected to ischaemia as described previously [23]. The AMPK- $\alpha_1$  and  $\alpha_2$  were immunoprecipitated and assayed using the SAMS peptide substrate. Results shown are means  $\pm$  S.E.M. for  $n = 5$ . C: The results show eNOS activities with full CaM-dose responses for a representative experiment. Ischaemia time point 0 min ( $\square$ ), 1 min ( $\blacklozenge$ ), 10 min ( $\bullet$ ) and 20 min ( $\triangle$ ). D, E: Effect of ischaemia on eNOS phosphorylation. Rat heart eNOS was isolated from homogenates run on SDS-PAGE and immunoblotted. Perfused hearts followed by 0 min (lane 1) or 10 min (lane 2) ischaemia as described above. Anti-eNOS antibody (eNOS-6c6, Zymed, eNOS (1–35)) and purified polyclonal antiphosphopeptide antibodies for Ser-1177 and Thr-495 were used. Quantitative densitometric data shown, mean  $\pm$  S.E.M.,  $n = 6$  experiments.

being phosphorylated with Ser-1177 phosphorylation more prominent in the presence of  $\text{Ca}^{2+}$ -CaM (Fig. 2A). Phosphorylation of rat heart eNOS Ser-1177 was associated with activation of eNOS. Phosphorylation shifted the dose-response

curve for CaM to the left (Fig. 2A). Activation of recombinant eNOS or bovine aortic endothelial cell eNOS incubated in the presence of  $\text{Ca}^{2+}$ -CaM was also observed with Ser-1177 phosphorylation (not shown). In order to ensure that the

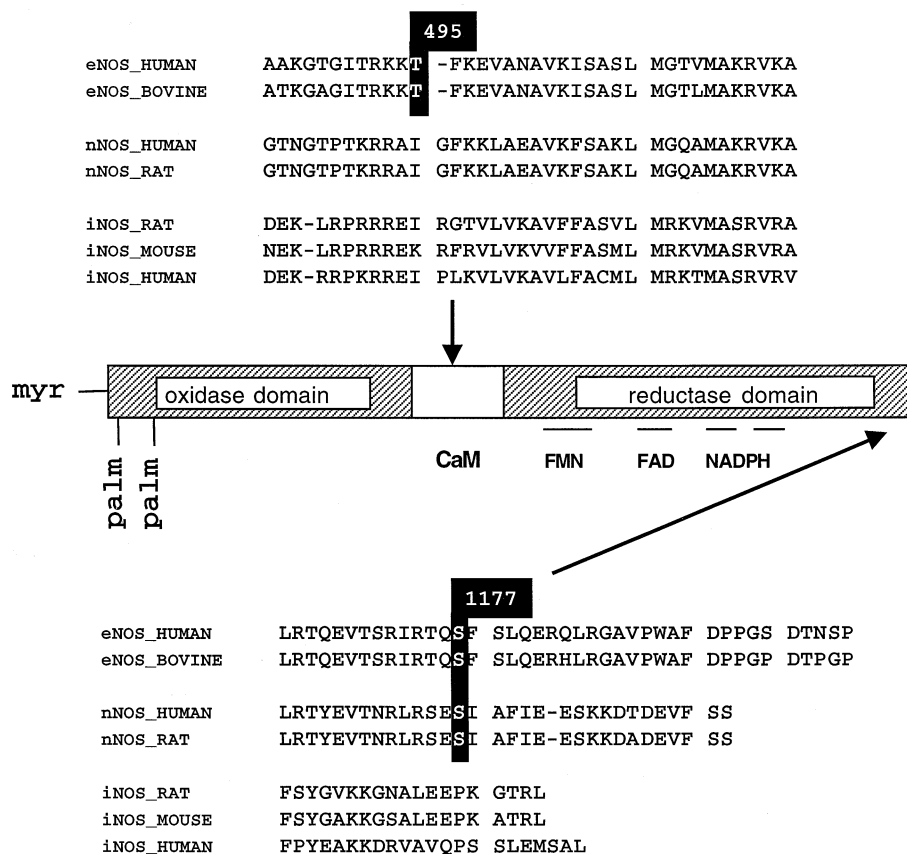


Fig. 4. Comparison of NO synthase phosphorylation site sequences. Schematic model of NO synthase showing sequences from the CaM-binding region (around Thr-495 phosphorylation site in eNOS) and for the COOH-terminal tails (around Ser-1177 phosphorylation site in eNOS).

presence of CaM in the phosphorylation reaction did not influence the subsequent eNOS assay, EGTA buffering was used to lower  $\text{Ca}^{2+}$  to ensure that the eNOS activity was dependent on added CaM to its assay. By inclusion of EGTA the eNOS CaM dependence was shifted from the low nM range to the high nM range. In the absence of added AMPK pre-incubation with or without CaM did not alter the activity of eNOS assayed using EGTA buffering. Using phosphopeptide mapping we found that activation of eNOS correlated with phosphorylation of Ser-1177 and not Thr-495 (Fig. 2B). Phosphorylation without added  $\text{Ca}^{2+}$ -CaM enhanced Thr-495 phosphorylation, suppressed Ser-1177 phosphorylation, and reduced eNOS activity (Fig. 2A,B). Phosphopeptide-specific antibodies to these sites were used in immunoblots following phosphorylation of rat heart eNOS (Fig. 2C) and these gave similar results to the phosphopeptide maps. Isolated rat heart eNOS had detectable phospho-Thr-495 and -Ser-1177. Previously it has been shown that phosphorylation of synthetic peptides containing Thr-495 by protein kinase C inhibits their CaM binding [22] and the inhibition of eNOS following phosphorylation at this site is consistent with reducing CaM binding.

In rat heart extracts both the  $\alpha 1$  and  $\alpha 2$  AMPK isoforms co-immunoprecipitated with eNOS (Fig. 3A) but not with a control antibody showing that eNOS in both capillary endothelial cells and myocytes [13] is associated with the AMPK, supporting the idea that the AMPK and eNOS co-localise in heart. AMPK is a multisubstrate enzyme and we expect only a portion of the enzyme to be associated with eNOS. Previously

it was reported that cardiac AMPK is activated during ischaemia [23], and we find both  $\alpha 1$  and  $\alpha 2$  isoforms activated 2- and 3-fold respectively up to 10 min then decline (Fig. 3B), indicating that AMPK derived from both capillary endothelial cells and cardiac myocytes is activated. During ischaemia there was an approximately 2-fold increase in eNOS activity by 10 min and a shift in the CaM dose response (Fig. 3C) mimicking the effect of eNOS phosphorylation by AMPK in vitro. Using the antiphosphopeptide antibodies to Ser-1177 and Thr-495 phosphorylation sites we observed that phosphorylation of Ser-1177 was increased approximately 3-fold with ischaemia (Fig. 3D,E) but there was no detectable change in the Thr-495 phosphorylation under these conditions. These results support the concept that activation of the AMPK during ischaemia directly correlates with the activation of eNOS. The presence of nNOS $\mu$  has been reported for heart but we could detect only trace nNOS $\mu$  immunoreactivity in NOS purified from hearts using ADP-Sepharose chromatography. For this reason we do not consider that nNOS $\mu$  is contributing to the increased activity observed with ischaemia.

Our results support the concept that eNOS is autoinhibited by its COOH-terminal tail, and phosphorylation at Ser-1177 reverses this inhibition to further activate the enzyme in concert with  $\text{Ca}^{2+}$ -CaM. There is a high level of similarity between eNOS and nNOS in their COOH-terminal tails whereas iNOS is distinct (Fig. 4). In contrast to eNOS and nNOS, the CaM-binding properties of iNOS are characterised by a low  $\text{Ca}^{2+}$ -dependence. By using chimeras of iNOS and nNOS it was found that the COOH terminal domain is important in

conferring the lower  $\text{Ca}^{2+}$ -dependence of iNOS [24] and it may be consistent with iNOS not having the same form of autoinhibition by its COOH terminal tail. Further, both iNOS and nNOS lack a phosphorylatable residue equivalent to Thr-495 and therefore would not have their CaM binding properties altered by phosphorylation in this region.

Previous studies have shown that eNOS may be phosphorylated both in vitro and in vivo but the function and precise sites of phosphorylation have not been fully characterised (reviewed in [25]). Thus far eNOS is the only example of an enzyme activated by AMPK. Phosphorylation alters eNOS activity in concert with the  $\text{Ca}^{2+}$ -CaM regulation by inhibiting activity with low calcium and increasing it with high calcium. During ischaemia only the Ser-1177 site phosphorylation is increased and not the Thr-495 site. The mammalian AMPK provides an interesting means of integrating metabolic stress signals within endothelial cells and myocytes to control eNOS and thereby potentially modulating the local circulatory system to improve nutrient supply and suppress mechanical activity of the muscle.

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