p90 RSK-1 associates with and inhibits neuronal nitric oxide synthase

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Evidence is presented that RSK1 (ribosomal S6 kinase 1), a downstream target of MAPK (mitogen-activated protein kinase), directly phosphorylates nNOS (neuronal nitric oxide synthase) on Ser^{847} in response to mitogens. The phosphorylation thus increases greatly following EGF (epidermal growth factor) treatment of rat pituitary tumour GH3 cells and is reduced by exposure to the MEK (MAPK/extracellular-signal-regulated kinase kinase) inhibitor PD98059. Furthermore, it is significantly enhanced by expression of wild-type RSK1 and antagonized by kinase-inactive RSK1 or specific reduction of endogenous RSK1. EGF treatment of HEK-293 (human embryonic kidney) cells, expressing RSK1 and nNOS, led to inhibition of NOS enzyme activity, associated with an increase in phosphorylation of nNOS at Ser^{847}, as is also the case in an *in vitro* assay. In addition, these phenomena were significantly blocked by treatment with the RSK inhibitor Ro31-8220. Cells expressing mutant nNOS (S847A) proved resistant to phosphorylation and decrease of NOS activity. Within minutes of adding EGF to transfected cells, RSK1 associated with nNOS and subsequently dissociated following more prolonged agonist stimulation. EGF-induced formation of the nNOS–RSK1 complex was significantly decreased by PD98059 treatment. Treatment with EGF further revealed phosphorylation of nNOS on Ser^{847} in rat hippocampal neurons and cerebellar granule cells. This EGF-induced phosphorylation was partially blocked by PD98059 and Ro31-8220. Together, these data provide substantial evidence that RSK1 associates with and phosphorylates nNOS on Ser^{847} following mitogen stimulation and suggest a novel role for RSK1 in the regulation of nitric oxide function in brain.

Key words: neuronal nitric oxide synthase, phosphorylation, pituitary tumour GH3 cell, ribosomal S6 kinase.

INTRODUCTION

The RSKs (p90 ribosomal S6 kinases) are a family of serine/threonine kinases that lie at the terminus of the Ras–ERK1/2 (extracellular-signal-regulated kinase 1/2)–MAPK (mitogen-activated protein kinase) cascade [1]. RSKs contain two functional protein kinase domains, and in mammals four expressed homologues (RSK1–RSK4) have been identified. Many RSK substrates have been identified, implicating involvement of RSK in a myriad of cellular processes. Cellular localization is thought to be important in this regard. Upon mitogen stimulation, RSK1 undergoes a rapid and transient localization to the plasma membranes, which requires ERK docking and phosphorylation [2]. RSK1 can then phosphorylate its substrates, which include the Na^+/H^+ exchanger NHE1 isoform at the plasma membrane, nuclear transcription factors and transcriptional co-activator proteins [1,3].

NO (nitric oxide), generated by NOSs (nitric oxide synthases), is important for both physiological processes and pathological alteration in mammals [4–6]. For example, synthesis by nNOS (neuronal NOS) has been implicated in the pathogenesis of brain injury from hypoxia/ischaemia [7]. Membrane localization of nNOS is mediated by a direct interaction with the postsynaptic protein, PSD-95 (postsynaptic density 95), which also associates with the NMDA (*N*-methyl-D-aspartate) receptor at excitatory synapses [8–10]. The binding of nNOS to the NMDA receptor is linked to Ca^{2+} influx [11], which in turn triggers cellular responses, such as activation of the Ras–ERK1/2 pathway [12,13].

RSK has broad substrate specificity *in vitro*, phosphorylating proteins and peptides at serine residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser or Arg-Arg-Xaa-Ser motifs [14,15]. Although there are numerous putative RSK phosphorylation consensus sequences in the nNOS molecule, it has not been previously established that nNOS itself is directly phosphorylated by RSK in cells. However, protein-kinase-dependent phosphorylation events in nNOS contribute to its regulation, involving AMPK (AMP-activated protein kinase) [16], PKC (protein kinase C) [17,18], cyclic-nucleotide-dependent protein kinases [19,20] and CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) [21,22]. Individual phosphorylated residues on nNOS have also been identified using site-specific antibodies [16,23–25]. Endothelial NOS is regulated by the Ras–ERK1/2 cascade [26], but the interrelationships of nNOS and Ras–ERK1/2 pathways are not well understood. It is well known that rat pituitary GH3 cells express constitutive nNOS and produce NO. In the present study, we therefore explored the role of EGF (epidermal growth factor) in modulating interactions between RSK1 and nNOS via the Ras–ERK1/2 pathway, and obtained evidence that nNOS is actually an RSK1 substrate in GH3, transfected and neuronal cells.

Abbreviations used: AICAR, 5-amino-4-imidazolecarboxamide riboside; AMPK, AMP-activated protein kinase; BAD, Bcl-2/Bcl-X_L-antagonist, causing cell death; CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; DIV, days *in vitro*; DTT, dithiothreitol; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; GH, growth hormone; HA, haemagglutinin; HEK-293, human embryonic kidney; IBMX, isobutylmethylxanthine; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MEM, minimum essential medium; NMDA, *N*-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NOS, nitric oxide synthase; PKC, protein kinase C; PRL, prolactin; PSD, postsynaptic density; pSer, phosphoserine; RNAi, RNA interference; RSK, ribosomal S6 kinase; siRNA, small interfering RNA; WT, wild-type.

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MATERIALS AND METHODS

Materials

The cDNA for rat brain nNOS and pMT2-rat-RSK2-WT, RSK2-K100R, a dominant-negative construct, were gifts from Dr Solomon H. Snyder (Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.) [27] and Dr Soren Impey (The Vollum Institute, Oregon Health Sciences University, Portland, OR, U.S.A.) respectively. RSK1 cDNA was cloned from a mouse brain cDNA library and then cloned into pME18s-FLAG vector [28]. The dominant-negative construct, RSK1-K94A, was subcloned into pME18s-FLAG vector. The plasmid pME18s-nNOS was generated as described previously [22]. Recombinant rat CaM (calmodulin) was expressed in *Escherichia coli* BL21 (DE3) using pET-CM, kindly provided by Dr Nobuhiro Hayashi (Fujita Health University, Toyoake, Japan) [29]. The rabbit polyclonal antibody, NP847, recognizing phosphorylation at Ser^{847} on nNOS, was prepared as described previously [23]. The rabbit anti-RSK1 and -RSK2 antibodies, activated RSK1 and RSK2 proteins, and pUSE-HA-rat-MEK1 (S218D/S222D) vector were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). A mouse anti-nNOS monoclonal antibody, anti-phospho-CaMKIIa at Thr^{286}, A23187, IBMX (isobutylmethylxanthine) and AICAR (5-amino-4-imidazolecarboxamide riboside) were obtained from Sigma (St Louis, MO, U.S.A.). The nNOS mutant, S847A (a mutant bearing alanine in place of Ser^{847}) was generated as described previously [23]. PD98059, Ro31-8220, LY294002, and wortmannin were purchased from Calbiochem and H89 was from Seikagaku. PMA was from Wako Chemical. The murine EGF and the mouse anti-HA (haemagglutinin) antibody were from Roche. L-[^{3}H]Arginine and ECL® (enhanced chemiluminescence) immunoblotting detection reagents were from Amersham Biosciences, and restriction enzymes and DNA-modifying enzymes were obtained from Takara Shuzo. Electrophoresis reagents were products of Bio-Rad. All other materials and reagents were of the highest quality available from commercial suppliers.

Cell culture, transfection and stimulation

GH3 cells were maintained in Ham's F10 medium (Sigma) supplemented with 15 % (v/v) horse serum and 2.5 % (v/v) foetal calf serum and subcultured in 6-cm-diameter dishes for 48 h, before the addition of activators or inhibitors. HEK-293 (human embryonic kidney) cells were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10 % foetal calf serum and subcultured for 24 h in 6-cm-diameter dishes. They were then transfected with pME18s-nNOS and RSK constructs using the Lipofectamine™ Plus method (Invitrogen Life Technologies). After 24–36 h of incubation, the cells were serum-starved for 18 h and stimulated with buffer alone or 100 ng/ml EGF for the time indicated.

Hippocampal neurons were prepared from embryonic day 18 Wistar rats (Nippon SLC) as described previously [30]. Cells were plated on poly(D-lysine) (Sigma)-treated dishes (six-well) and then maintained in neurobasal medium (Invitrogen Life Technologies) containing 2 % (v/v) foetal calf serum, 2 % B27 supplement (Invitrogen Life Technologies), 500 μM L-glutamine, 40 μg/ml streptomycin (Meiji) and 40 units/ml penicillin (Banyu). After 21 DIV (days *in vitro*), the cells were pre-incubated for 30 min with Hepes-buffered saline [20 mM Hepes (pH 7.4), 135 mM NaCl, 4 mM KCl, 1 mM Na_2HPO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM glucose] either with or without indicated inhibitors and stimulated with buffer alone or 100 ng/ml EGF for 10 min.

Cerebellar granule cells were obtained from 7–10-day-old Wistar rats as described previously [31]. Cells were plated on poly(ethyleneimine)-coated 100-mm-diameter culture dishes (Iwaki) and then maintained in MEM (minimum essential medium) containing 25 mM KCl with 5 % (v/v) foetal calf serum and 5 % (v/v) horse serum, 50 units/ml penicillin and 50 μg/ml streptomycin. After 17 DIV, the cells were pre-incubated for 60 min with MEM containing 25 mM KCl either with or without indicated inhibitors and stimulated with buffer alone or 100 ng/ml EGF for 10 min.

siRNA (small interfering RNA) transfection and stimulation

RSK1, RSK2 and non-specific control siRNA were purchased from Dharmacon Research. HEK-293 cells were seeded in six-well dishes. On the following day, cells were transfected with RSK1 (48 pmol) plus non-specific control (24 pmol), RSK2 (24 pmol) plus non-specific control (48 pmol), RSK1 (48 pmol) plus RSK2 (24 pmol), or non-specific control (72 pmol) siRNA using 24 μl of HiPerFect (Qiagen) according to the manufacturer's instructions. After 48 h, the cells were transfected with PME18s-nNOS (1 μg) using Fugene (Roche) and, approx. 24 h later, stimulated with EGF for 10 min.

Phosphorylation of recombinant nNOS *in vitro*

Recombinant rat nNOSs were expressed in *E. coli* and purified using 2′,5′-ADP–agarose (Sigma), as described previously [32]. Purified nNOS was incubated with or without activated RSK1/2 (10 ng) (Upstate Biotechnology) at 30 °C for the indicated periods in a solution containing 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT (dithiothreitol), 2 mM $CaCl_2$, 5 μM CaM, 200 μg/ml BSA, and either 200 μM [γ-^{32}P]ATP (1000 c.p.m./pmol) or 200 μM ATP for Western blotting. The reaction was terminated by the addition of SDS sample buffer. The samples were then subjected to SDS/7.5 % PAGE followed by Western blot analysis, autoradiography or quantification of ^{32}P incorporation into the nNOS by Cerenkov counting of the excised gels.

Preparation of lysates and immunoprecipitation

For preparation of lysates, cells or rat brain were homogenized by sonication (in Figures 1–5) or by incubation at 4 °C for 30 min (in Figures 7 and 8) with TNE buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 2 mM sodium pyrophosphate and 1 % Nonidet P40). After centrifugation at 15000 ***g*** for 15 min, 20 μl of anti-FLAG M2–agarose affinity gel (50 % slurry) (Sigma) was added to the supernatant, and the mixture was incubated for 1 h at 4 °C. For FLAG-tagged RSK1 chromatography, the FLAG immunoprecipitate was incubated with 5 ml of rat brain lysate for 1 h at 4 °C. After precipitation by centrifugation and removal of the supernatant, the resin was washed three times with 300 μl of TNE buffer and boiled with 50 μl of SDS sample buffer, and then analysed by SDS/PAGE followed by Western blot analyses. For immunoprecipitation of RSK1/2 from GH3 cells, lysates were pre-cleared by centrifugation with an excess of Protein G–Sepharose (Amersham Biosciences) and then incubated with 1 μg of purified pre-immune rabbit IgG, anti-RSK1 or RSK2 antibody for 1 h at 4 °C. Immune complexes were collected on 30 μl of Protein G–Sepharose (50 % slurry) and washed once with 400 μl of TNE buffer, twice with 400 μl of TNE buffer without Nonidet P40 and twice with 400 μl of kinase assay buffer (40 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 0.4 mM DTT, 1 mM EGTA and 10 mM ATP). The beads were used for an *in vitro* kinase assay

using purified nNOS as substrate at 30 °C for 10 min. Kinase reactions were stopped by the addition of SDS sample buffer and boiled for 2 min. Eluted proteins were analysed by SDS/PAGE and followed by Western blot analyses. For preparation of lysates from cultured hippocampal neurons, cells were sonicated with 0.2 ml of RIPA buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 50 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 % Nonidet P40, 0.5 % sodium deoxycholate and 0.1 % SDS). The lysates were collected by centrifugation at 15 000 ***g*** for 15 min. For preparation of lysates from cultured cerebellar granule cells, cells were sonicated in TNE buffer. After centrifugation at 15 000 ***g*** for 15 min, 20 μl of ADP–agarose gel (50 % slurry) (Sigma) was added to the supernatant, and the mixture was incubated for 1 h at 4 °C. After precipitation of the resin by centrifugation and removal of the supernatant, the resin was washed three times with 500 μl of TNE buffer, boiled with 50 ml of SDS sample buffer and then analysed by SDS/PAGE followed by Western blot analyses with the indicated antibodies.

NOS activities assay

Cell lysates were homogenized by passing them through 27-gauge syringe needles in lysis buffer (TNE without Nonidet P40) and were used as a source of nNOS enzyme. NOS activity was then determined by measuring the conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline as described previously [32,33].

Statistical analysis

The significance of variability between the results from each group and the corresponding control was determined using an unpaired Student's *t* test. The means $\pm$ S.E.M. were calculated. A value of $P < 0.05$ was considered statistically significant.

RESULTS

EGF-induced nNOS phosphorylation at Ser^{847} is mediated by an ERK1/2-activated kinase

Phosphorylation of nNOS at Ser^{847} has been detected *in vivo* in the brain [34–37]. We first set out to determine the effects of stimulation with various agonists on phosphorylation of Ser^{847} in GH3 cells using a phosphopeptide antiserum, NP847, specific to the pSer^{847} (phospho-Ser^{847}) nNOS form [23]. When serum-starved GH3 cells were treated with EGF, phosphorylation of Ser^{847} was observed, with a maximum stimulation at 10–30 min (Figures 1A and 1B). Calcium ionophore A23187-stimulated phosphorylation of Ser^{847} was also detected, but only very slightly (Figure 1A). Treatment of GH3 cells with 100 μM PD98059, a selective inhibitor of MEK (MAPK/ERK kinase), almost completely blocked EGF-induced phosphorylation of Ser^{847} (Figure 1C). This was not the case with the PI3K (phosphoinositide 3-kinase) inhibitors LY294002 and wortmannin. Co-expression of nNOS and a constitutively active form of MEK1 resulted in phosphorylation of Ser^{847} in HEK-293 cells (Figure 1D). As an approach to identifying kinases that may be involved in phosphorylation of Ser^{847} in EGF-stimulated cells, an *in vitro* kinase assay was employed using cell lysates as a source of kinases. Incubation of a purified nNOS with EGF-stimulated HEK-293 cell lysates in the presence of ATP revealed phosphorylation of Ser^{847} (Figure 2A), which was blocked by inclusion of RSK inhibitors, H89 and Ro31-8220 [38]. Furthermore, incubation of a purified nNOS with a constitutively active form of RSK1/2 resulted in phosphorylation of Ser^{847} (Figure 2B). Next, we kinetically analysed nNOS phosphorylation. A time-course experiment on the phosphorylation of nNOS (2 μg) by activated RSK1 (10 ng) in the presence of [γ-^{32}P]ATP revealed that nNOS was stoichiometrically ($\sim$1.0 mol of P_i incorporation into 1 mol of nNOS) phosphorylated under the conditions employed (Figure 2C, left-hand panel). It was observed that ^{32}P incorporation into nNOS-S847A was significantly lower than that into the WT (wild-type) nNOS, although weak residual phosphorylation was observed in the mutant (Figure 2C, right-hand panel).

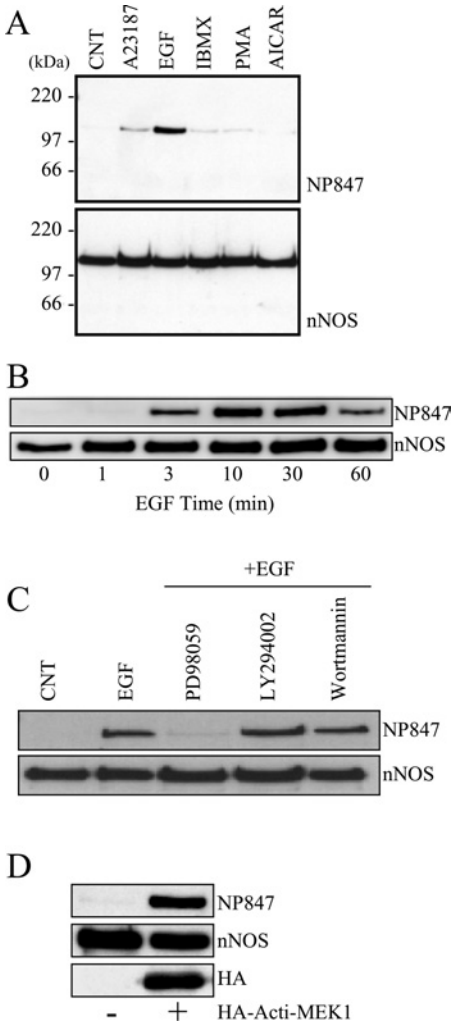

Figure 1 EGF-stimulated nNOS Ser^{847} phosphorylation in GH3 cells

(**A**) GH3 cells were incubated with buffer alone (CNT), A23187 (10 μM, 5 min), EGF (100 ng/ml, 5 min), IBMX (500 μM, 5 min), PMA (500 nM, 5 min) or AICAR (1 mM, 30 min) as indicated. nNOS was isolated by ADP–agarose precipitation, and its phosphorylation was monitored with anti-pSer^{847}-nNOS antibody (NP847). Immunoblot membranes were stripped of NP847 and reprobed with anti-nNOS antibody. Molecular masses are given in kDa. (**B**) GH3 cells were incubated with EGF (100 ng/ml) for 0, 1, 3, 10, 30 and 60 min, and phosphorylation of nNOS at Ser^{847} was monitored as described in (**A**). (**C**) GH3 cells were untreated or pre-treated with specific protein kinase inhibitors (PD98059, 100 μM; LY294002, 50 μM; wortmannin, 200 nM) as indicated for 1 h in Krebs–Ringer Hepes buffer, and then treated with buffer alone (CNT) or EGF (100 ng/ml) for 10 min. Phosphorylation of nNOS at Ser^{847} and total nNOS protein were monitored as described in (**A**). (**D**) HEK-293 cells were co-transfected with pME18s-nNOS (+) (1 μg) and a constitutively active form of pUSE-HA-tagged MEK1 (HA-Acti-MEK1) (1 μg) or empty vector (−) (1 μg). After 48 h, nNOS was isolated by ADP–agarose precipitation, and phosphorylation of nNOS was monitored with NP847. The immunoblot membrane was then stripped of NP847 and reprobed with anti-nNOS antibody. Overexpressed MEK1 was monitored using anti-HA antibody. The data are representative of at least two independent experiments.

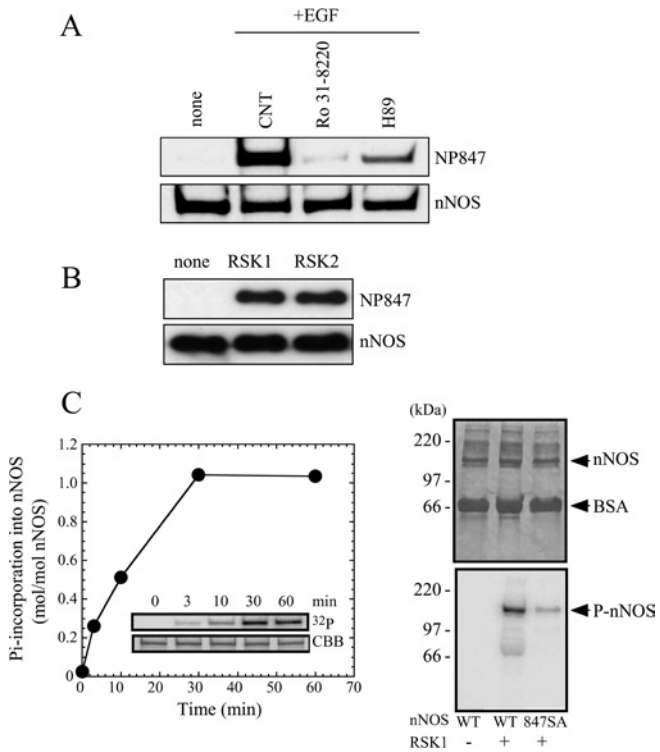

Figure 2 RSK1 and RSK2 phosphorylate nNOS directly at Ser^{847} *in vitro*

(**A**) HEK-293 cells were treated with buffer alone (none) or EGF (100 ng/ml) for 10 min. Lysates were incubated at 30 °C for 10 min with nNOS in a kinase reaction buffer in either the presence or the absence (CNT) of the indicated protein kinase inhibitor (Ro31-8220, 1 μM; H89, 10 μM). After terminating the reaction, phosphorylation of nNOS was monitored as described in Figure 1. (**B**) nNOS was incubated in a kinase reaction buffer with either RSK1 or RSK2 at 30 °C for 10 min. After terminating the reaction, phosphorylation of nNOS was monitored as described in Figure 1. The data are representative of at least two independent experiments. (**C**) Time-course experiment of nNOS phosphorylation. Recombinant nNOS (2 μg) was incubated with activated RSK1 (10 ng) in the presence of 200 μM [γ-^{32}P]ATP at 30 °C for the indicated period of time, in a solution containing 50 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 1 mM $CaCl_2$ and 5 μM CaM. Phosphorylated nNOS was subjected to SDS/7.5 % PAGE, followed by staining with CBB (Coomassie Brilliant Blue) (inset, lower panel) or autoradiography (inset, upper panel). ^{32}P incorporation into nNOS was quantified by Cerenkov counting of the excised gels. WT or S847A mutant (S847A) of purified nNOS (2 μg) was incubated with or without activated RSK1 (10 ng) at 30 °C for 10 min as described above. The reaction was terminated by the addition of SDS sample buffer. The samples were then subjected to SDS/7.5 % PAGE followed by either protein staining (upper right-hand panel) or autoradiography (lower right-hand panel). Molecular masses are given in kDa.

RSK1 is an ERK1/2-regulated kinase that can phosphorylate nNOS at Ser^{847} in response to EGF

Next we examined whether RSK could phosphorylate nNOS directly at Ser^{847} in EGF-stimulated GH3 cells. Solubilized GH3 cell lysates were immunoprecipitated with an antibody against RSK1 or RSK2, or with a control antibody, and immunoblots were analysed for RSK1 or RSK2 (Figure 3A). Incubation of a purified nNOS with the anti-RSK1 immunoprecipitates from EGF-stimulated cell lysates in the presence of ATP revealed phosphorylation of Ser^{847} (Figure 3B). The anti-RSK2 immunoprecipitates from EGF-stimulated cell lysates did phosphorylate Ser^{847}, but only very slightly.

In order to provide further evidence that RSK mediates EGF-induced nNOS Ser^{847} phosphorylation *in vivo*, we co-expressed RSKs with nNOS in HEK-293 cells and monitored Ser^{847} phosphorylation on stimulation with EGF. Overexpression of WT RSK1 led to a greatly enhanced phosphorylation of Ser^{847}, even in the absence of EGF stimulation. In contrast, overexpression

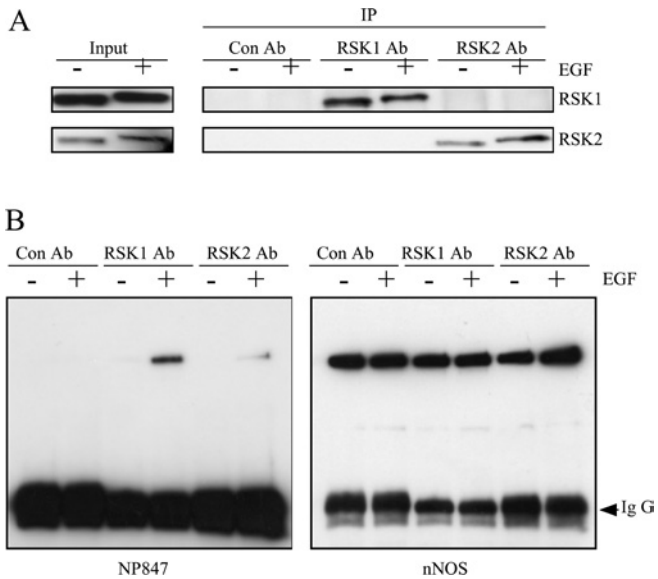

Figure 3 RSK1 phosphorylates nNOS at Ser^{847} in response to EGF in GH3 cells

(**A**) GH3 cells were treated with buffer alone (−) or EGF (100 ng/ml) for 10 min. Lysates were immunoprecipitated with an antibody (Ab) against RSK1 or RSK2, or a pre-immune antibody (Con). Identical samples were analysed by immunoblotting for RSK1 or RSK2. Input, 20 μg of lysates in each reaction. (**B**) Proportional amounts of each immunoprecipitate were incubated at 30 °C for 10 min with recombinant nNOS (2 μg) in a kinase reaction buffer. After terminating the reaction, phosphorylation of nNOS was monitored as described in Figure 1. The data are representative of three independent experiments.

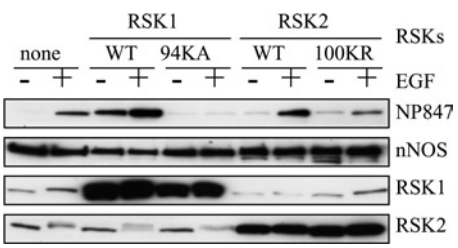

Figure 4 EGF-stimulated nNOS Ser^{847} phosphorylation is enhanced by WT RSK1 and antagonized by kinase-inactive RSK1

HEK-293 cells were co-transfected with pME18s-nNOS (1 μg) and 1 μg of pME18s-FLAG-RSK1-WT (WT), RSK1-K94A (94KA), pMT2-RSK2-WT (WT), RSK2-K100R (100KR) or empty vector (none). Cells were serum-starved for 18 h and stimulated with buffer alone (−) or 100 ng/ml EGF (+) for 10 min. Lysates were prepared, and overexpressed RSKs and pSer^{847} of nNOS were monitored by immunoblotting using anti-RSKs or anti-pSer^{847}-nNOS (NP847) antibody. The immunoblot membranes were then stripped of NP847 and reprobed with the anti-nNOS antibody. The data are representative of at least three independent experiments.

of RSK1-K94A revealed a dominant-negative effect on EGF-induced Ser^{847} phosphorylation (Figure 4), while WT RSK2 and RSK2-K100R had no remarkable effects. We examined the role of RSK in EGF-induced nNOS Ser^{847} phosphorylation directly using RNAi (RNA interference) techniques to inhibit endogenous RSKs. HEK-293 cells were transfected with control, RSK1, RSK2 or a combination of RSK1 and RSK2 siRNA oligonucleotides. At 48 h post-transfection, cells were additionally transfected with nNOS for 24 h, serum-starved first and then stimulated with EGF. Reduction of RSK1 protein levels by RNAi caused a substantial decrease in EGF-stimulated Ser^{847} phosphorylation (Figure 5). Expression of RSK1 and RSK2 siRNA constructs almost completely blocked EGF-stimulated Ser^{847} phosphorylation, indicating that RSK2 is also involved in the phosphorylation. It has

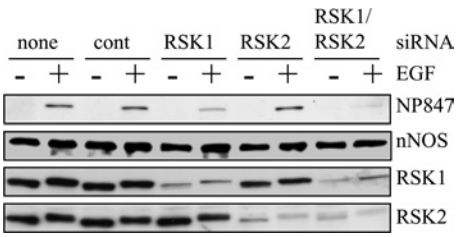

Figure 5 EGF-stimulated nNOS Ser^{847} phosphorylation is reduced by RSK siRNA

HEK-293 cells were transfected with RSK1, RSK2 or non-silencing control (cont) siRNA, or buffer alone (none). After 48 h, the cells were transfected with nNOS for 24 h, serum-starved first and then stimulated with buffer alone (−) or 100 ng/ml EGF (+) for 10 min. Lysates were prepared, and RSKs and pSer^{847} of nNOS were detected by immunoblotting using anti-RSKs and anti-pSer^{847}-nNOS (NP847) antibodies. The membranes were then stripped of NP847 and reprobed with nNOS antibody. The data are representative of at least three independent experiments.

been shown that phosphorylation of nNOS at Ser^{847} leads to a reduction in the enzyme activity in neuronal cells [22,36]. When HEK-293 cells were co-transfected with WT RSK1 and nNOS constructs, serum-starved and stimulated with EGF (with or without Ro31-8220 pre-treatment), 30 % decreased nNOS activity was observed, as determined from the rate of conversion of L-arginine into L-citrulline (Figure 6A). In addition, this EGF-induced attenuation of nNOS activity was completely Ro31-8220-sensitive. Co-transfection of nNOS-S847A with WT RSK1 antagonized EGF-induced decrease in nNOS activity. The activated RSK1-induced nNOS phosphorylation *in vitro* also resulted in a decrease in NOS enzyme activity to approx. 80 % as compared with that of the unphosphorylated enzyme (Figure 6B). Phosphorylation of nNOS-S847A by activated RSK1 appeared to increase NOS enzyme activity to 120 % as compared with that of the unphosphorylated enzyme.

EGF-regulated interactions of RSK1 with nNOS in cells

To test the physiological relevance of RSK1–nNOS in Ser^{847} phosphorylation signals, co-immunoprecipitation experiments were performed in cells treated with EGF. In resting HEK-293 cells expressing nNOS and FLAG-tagged RSK1, we found that co-immunoprecipitation of nNOS from solubilized cell lysates with anti-FLAG antibodies was faint or undetectable. EGF treatment led to an increase within 3 min; the heteromeric complex dissociated 10 min after the addition of EGF (Figure 7A). When solubilized rat brain lysates were applied to a column of RSK1 purified from the cells treated either with or without EGF, nNOS was eluted only on the presence of EGF (Figure 7B). We investigated the effects of PD98059 on the association of RSK1 with nNOS in response to EGF. We pre-treated HEK-293 cells expressing nNOS and FLAG-tagged RSK1 with PD98059 for 30 min, added EGF for 3 min before harvesting the cells, and then used the anti-FLAG antibody. In the absence of PD98059, the addition of EGF led to the association of RSK1 with nNOS, as shown in Figure 7(A). However, the addition of PD98059 completely blocked the ability of EGF to induce association of RSK1 with nNOS (Figure 8).

nNOS Ser^{847} phosphorylation by ERK1/2-regulated kinase in hippocampal neurons and cerebellar granule cells

To identify *in vivo* nNOS Ser^{847} phosphorylation by ERK1/2-regulated kinase, we investigated Ser^{847} phosphorylation signals in rat hippocampal neurons and rat cerebellar granule cells. Treatment of hippocampal neurons or cerebellar granule cells with

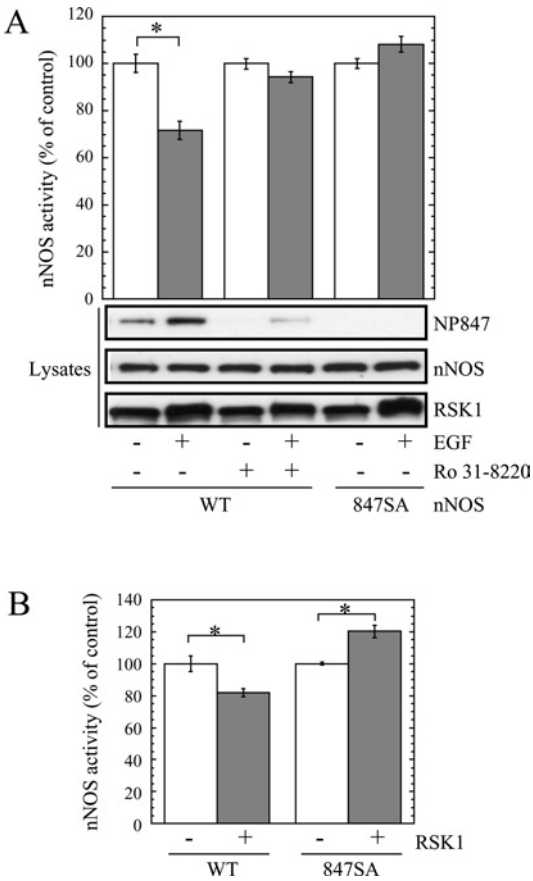

Figure 6 Effects of protein kinase inhibitors on EGF-stimulated nNOS activity

(**A**) HEK-293 cells were transfected with pME18s-nNOS (0.4 μg) or pME18s-nNOS (S847A) (0.4 μg) and pME18s-FLAG-RSK1 (2 μg). After 48 h, the cells were incubated with (+) or without (−) 1 μM Ro31-8220 for 30 min and stimulated with 100 ng/ml EGF for 5 min. Lysates were prepared, and RSKs and pSer^{847} of nNOS were detected by immunoblotting using anti-RSKs or anti-pSer^{847}-nNOS (NP847) antibody. The membranes were stripped and reprobed with anti-nNOS antibody (lower panels). Proportional amounts of lysates were subjected to NOS assay (upper panel). Data were normalized to the control value, defined as NOS activity for transfected cells without EGF treatment. (**B**) WT and S847A mutant (S847A) were incubated with or without an activated RSK1 at 30 °C for 30 min as in Figure 2(B). Equivalent amounts (100 nM) of unphosphorylated (open bar) and phosphorylated (closed bar) nNOSs were used for the NOS enzyme activity assay. The means ± S.E.M. for three experiments are shown. *$P < 0.05$.

100 ng/ml EGF increased the level of pSer^{847} nNOS (Figure 9). Both cells showed a faint or undetectable staining of RSK2 protein determined by Western blotting. Treatment with PD98059 or Ro31-8220 significantly blocked EGF-induced phosphorylation of Ser^{847}.

DISCUSSION

The major finding of the present study is that activated RSK1 associates with, directly phosphorylates and negatively regulates nNOS in the EGF-induced ERK1/2 cascade. EGF-induced association of RSK1 and nNOS was demonstrated by: (i) the ability of RSK1 to bind nNOS from brain lysates, and (ii) co-precipitation of transfected RSK1 and nNOS. nNOS appears to be targeted to membranes by binding to PSD-95/SAP90 (synapse-associated protein 90) or PSD-93 [39,40], and rapid and transient re-localization of RSK to the cell membrane has been observed upon mitogen stimulation [2]. It is known that both inactive and active RSKs can bind to some of the PDZ domain proteins because

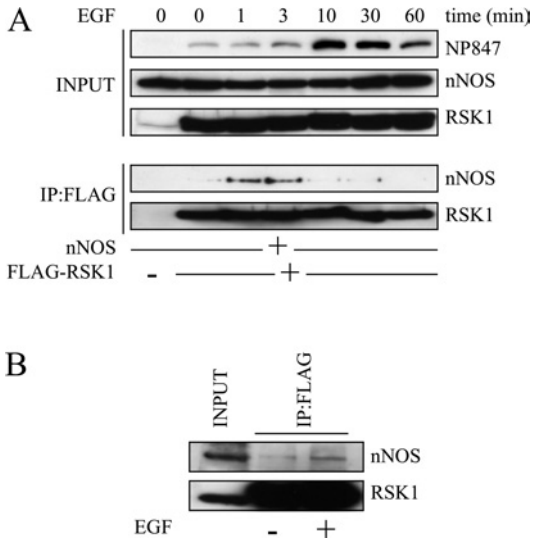

Figure 7 Effects of EGF on interactions of nNOS with RSK1

(**A**) HEK-293 cells were transfected with pME18s-nNOS (0.4 μg) alone or together with pME18s-FLAG-tagged RSK1 (2 μg). After 48 h, the cells were stimulated 100 ng/ml EGF (+) for the indicated time period. Lysates were prepared, and overexpressed RSK1 and pSer^{847} of nNOS were monitored by immunoblotting using anti-RSK1 or anti-pSer^{847}-nNOS (NP847) antibody. The membranes were stripped and reprobed with nNOS antibody (INPUT). Anti-FLAG immunoprecipitates from lysates of the cells were immunoblotted with anti-nNOS and anti-RSK1 antibodies (IP:FLAG). The data are representative of at least two independent experiments. (**B**) HEK-293 cells expressing FLAG-tagged RSK1 were stimulated with buffer alone (–) or 100 ng/ml EGF (+) for 3 min. Anti-FLAG immunoprecipitates from lysates of the cells were incubated with solubilized rat brain homogenates. After extensive washing, bound nNOS was eluted with SDS sample buffer and analysed by immunoblotting using nNOS antibody. The data are representative of at least two independent experiments. INPUT = 0.5 % of protein present in rat brain homogenates.

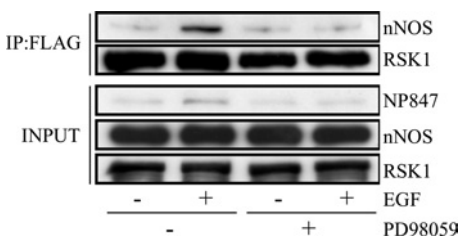

Figure 8 Effects of protein kinase inhibitors on EGF-induced interactions of nNOS with RSK1

HEK-293 cells were transfected with pME18s-nNOS (0.4 μg) together with pME18s-FLAG-RSK1 (2 μg). After 48 h, the cells were incubated with 100 μM PD98059 for 30 min and were stimulated with 100 ng/ml EGF for 3 min. Anti-FLAG immunoprecipitates from lysates of the cells were then immunoblotted with anti-nNOS and anti-RSK1 antibodies (IP:FLAG). Cell lysates were immunoblotted with anti-nNOS, anti-RSK1 or anti-pSer^{847}-nNOS (NP847) antibody (INPUT). INPUT = 5 % of protein present in each binding reaction. The data are representative of at least three independent experiments.

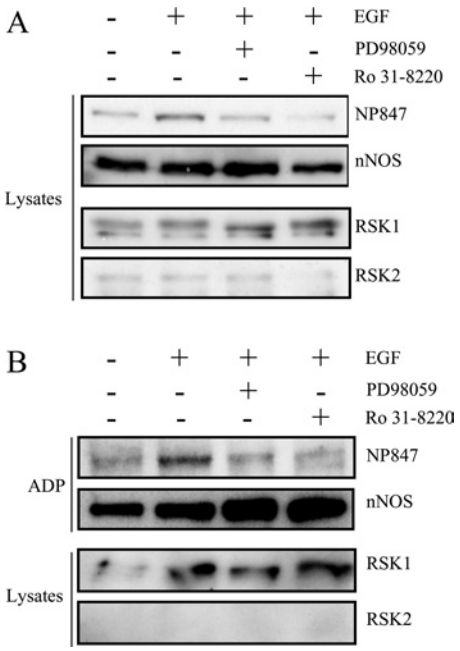

Figure 9 EGF-induced phosphorylation of nNOS at Ser^{847} in cultured hippocampal neurons and cerebellar granule cells

(**A**) Cultured hippocampal neurons at 21 DIV were untreated or pre-treated with 1 μM Ro31-8220 or 100 μM PD98059 for 30 min, and then treated with 100 ng/ml EGF A for 10 min. Cell lysates were immunoblotted with anti-nNOS, anti-pSer^{847}-nNOS (NP847), anti-RSK1 or anti-RSK2 antibody. (**B**) Cultured cerebellar granule cells at 17 DIV were treated as in (**A**). nNOS was isolated by ADP–agarose precipitation, and its phosphorylation was monitored with anti-pSer^{847}-nNOS (NP847) antibody. The immunoblot membranes were stripped of NP847 and reprobed with the anti-nNOS antibody. Cell lysates were immunoblotted with anti-RSK1 and anti-RSK2 antibodies. The data are representative of at least two independent experiments.

of their PDZ ligand (STTL) [41] and here an inhibitor of ERK1/2 signalling, PD98059, almost completely blocks the effect of EGF on the RSK1–nNOS complex (Figure 7). Although the exact mechanism of EGF-stimulated RSK1–nNOS association remains unclear, ERK appears to have a role in escorting RSK1 to the nNOS-containing complex.

Studies of nNOS Ser^{847} phosphorylation using neuronal cells have indicated that down-regulation of nNOS activity by CaMKII may represent an important component of the 'cross-talk' between Ca^{2+}- and NO-regulated signal transduction pathways [22,34,36]. It is also known that nNOS and CaMKII co-localize in rat brain [34]. Kinases responsible for Ser^{847} phosphorylation, other than CaMKII [24], have not been clearly established and, in the present study, PKA (protein kinase A), PKC and AMPK all failed to catalyse in GH3 cells under the conditions employed (Figure 1). *In vitro* phosphorylation of nNOS-S847A by activated RSK1 was significantly lower than that of WT enzyme (Figure 2C); however, it appeared to increase NOS enzyme activity, as compared with that of un-phosphorylated enzyme (Figure 6B). Although the results of the present study do not exclude potential involvement of other phosphorylation sites targeted by RSK1, only Ser^{847} appears to be necessary for the inactivation of nNOS by RSK1. RSK1 protein is present in brain, where RSK2 protein is reported to be conspicuous [42,43], consistent with our results (Figure 9). The content of RSK2 protein is also lower than that of RSK1 in GH3 cells (Figure 3). Our present results point to a function of the RSK1 isoform in the brain, i.e. regulation of nNOS enzyme activity. ERK1/2 signalling-induced Ser^{847} phosphorylation can be considered to be physiological, since it was observed in rat hippocampal neurons and rat cerebellar granule cells and was significantly blocked by PD98059 and Ro31-8220 (Figure 9).

What are the downstream physiological events controlled by RSK1-induced phosphorylation of nNOS at Ser^{847}? RSK-induced phosphorylation of BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death) at Ser^{112} suppresses BAD-mediated apoptosis in neurons [44] and attenuates cells death induced by β-amyloid in cultured hippocampal neurons [45]. We speculate that nNOS Ser^{847} phosphorylation limits the activity of the enzyme following an ERK1/2–RSK1 stimulus and prevents the accumulation of toxic levels of NO. Our observations suggest potential therapeutic value for activation of the ERK1/2–RSK1–nNOS pathway in protecting

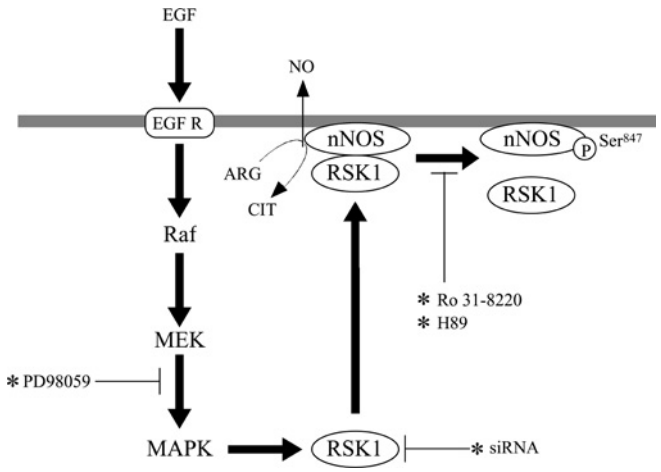

Figure 10 Model for the regulation of nNOS by RSK1 activated by EGF

NO production by nNOS is reduced through generation of pSer^{847}-nNOS by activated RSK1, resulting in alteration of downstream signalling. The asterisks indicate the reagents used in the present study. ARG, arginine; CIT, citrulline; EGF R, EGF receptor.

the aging brain against neurodegenerative diseases. Alternatively, nNOS Ser^{847} phosphorylation may regulate its association with RSK1. Preliminary data suggest, however, that the association ability of RSK1 with nNOS is not affected by phosphorylation using nNOS mutated at Ser^{847} to alanine or aspartate in transfected cells (results not shown). Long-term incubation of GH3 cells with EGF induces reciprocal changes in PRL (prolactin) and GH (growth hormone) production [46] and suppresses dopaminergic neurotoxin-mediated apoptosis [47]. Endogenous NO is known to regulate secretion of PRL and GH in GH3 cells [48–50] and an EGF-dependent ERK1/2–RSK1–nNOS cascade might be involved in such GH3-specific pathways.

In summary, we have demonstrated for the first time that RSK1 is an nNOS-associated protein and provided evidence of a functional role for RSK1–nNOS complexes *in vivo*. On the basis of the model shown in Figure 10, we propose that RSK1 phosphorylates nNOS directly, blocking the accumulation of toxic levels of NO. EGF stimulation induces an RSK1–nNOS complex form, although whether this is due to direct or indirect interaction is unclear. This allows phosphorylation of Ser^{847}. It has been shown that the movement of RSKs from cytoplasm to membrane is nNOS-independent [2,51]. In this context, the exact mechanisms underlying RSK1 association with nNOS are of obvious interest.

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