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90-kDa ribosomal S6 kinase 1 is inhibited by *S*-glutathionylation of its active-site cysteine residue during oxidative stress

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ABSTRACT

Previously, we reported that p90-RSK1 phosphorylates neuronal nitric oxide synthase (nNOS) at Ser847 in cells treated with mitogens, leading to the inhibition of NOS activity. Here, we show RSK1 Cys223 glutathionylation limits the activity of the enzyme following an oxidative stimulus and attenuates the nNOS phosphorylation. Treatment of RSK1 with diamide/glutathione results in inactivation of the enzyme in vitro. Mutagenesis studies confirmed that *S*-glutathionylation of Cys223 is both necessary and sufficient for this inhibition of RSK1. In transfected cells expressing RSK1 and nNOS, treatment with diamide caused a decrease in EGF-induced phosphorylation of nNOS at Ser847. Cells expressing mutant RSK1 (C223S) proved resistant in this regard. Thus, RSK1 Cys223 glutathionylation may contribute to regulate the levels of NO in the brain.

1. Introduction

The 90-kDa ribosomal S6 kinases (RSKs) are a family of serine/threonine kinases that lie downstream of the Ras-mitogen-activated protein kinase (MAPK) cascade [1]. RSKs contain two functional protein kinase domains, and in mammals four expressed homologues (RSK1–4) have been identified. RSKs are activated by extracellular-signal-regulated kinase 1/2 (ERK1/2) [2] and 3-phosphoinositide-dependent protein kinase 1 (PDK1) [3] by sequential phosphorylations in the C-terminal kinase domain (CTKD) at Thr573 and N-terminal kinase domain (NTKD) at Ser 221 [1], respectively. When activated, RSK promotes the phosphorylation of many cytosolic and nuclear targets that regulate diverse cellular processes, including cell growth, proliferation, survival and motility [4].

Nitric oxide (NO) is a ubiquitous gaseous biologically active messenger [5]. Physiological functions of NO in the central nervous system include regulation of neurotransmission, neuroprotection and neurotoxicity [6]. NO is produced by neuronal NO synthase (nNOS) in nervous tissue in both the central and peripheral nervous system. It was shown that epidermal growth factor (EGF) induces inactivation of nNOS through phosphorylation of Ser847 by RSK1, leading to the reduction of NO levels [7]. In addition to NO, reactive oxygen species (ROS) are constantly produced by a number of normal cellular events, with a major source being aerobic respiration. Excessive ROS can induce oxidative damage in cell constituents and promote a number of degenerative diseases and aging [8]. Meanwhile, ROS are not only injurious to cell survival but also essential to cell signaling and regulation [9].

Studies have demonstrated that ROS can mediate the activation of the MAPK pathways [10]. A number of cellular stimuli that induce ROS production can activate MAPK pathways in multiple cell types [10,11]. Moreover, direct exposure of cells to exogenous hydrogen peroxide (H_2O_2) mimics oxidative stress and leads to activation of MAPK pathways [12,13]. The mechanism(s) by which ROS can regulate the MAPK pathways, however, is not well defined. Because ROS can alter protein structure and function by modifying critical amino acid residues of proteins [9], the oxidative modification of signaling proteins by ROS may be one of the plausible mechanisms for the activation of MAPK pathways. However, the precise molecular target(s) of ROS is unknown.

For the present paper, we investigated molecular mechanisms of oxidant dependent regulation of RSK1 in cells and demonstrated *S*-glutathionylation-mediated inactivation of the enzyme.

Abbreviations: DTT, dithiothreitol; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; GSH, glutathione; H_2O_2, hydrogen peroxide; HEK293, human embryonic kidney; MAPK, mitogen-activated protein kinase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; PDK1, 3-phosphoinositide-dependent protein kinase 1; PSSG, protein *S*-glutathionylation; RSK, ribosomal S6 kinase; WT, wild-type

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2. Materials and methods

2.1. Materials

RSK1 cDNA was cloned from a mouse brain cDNA library and then cloned into pME18s-FLAG vector. The rabbit polyclonal antibody, NP847, recognizing phosphorylation at Ser847 on nNOS, was prepared as described previously in [14]. Anti-nNOS polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit anti-RSK1 antibody was obtained from Sigma (St. Louis, MO, USA). Monoclonal antibody recognizing glutathionylated proteins (anti-protein *S*-glutathionylation (anti-PSSG) Ab) was obtained from Virogen (Watertown, MA, USA). Antibodies against phosphor-ERK1/2 and ERK1/2 were odtained from Cell Signaling Technology (Danvers, MA, USA). The human EGF was from Roche. Diamide was purchased from Sigma. [γ-^{32}P] ATP (6000 Ci/mmol) and ECL prime (enhanced chemiluminescence) immunoblotting detection reagents were from Perkin–Elmer (Waltham, MA, USA) and GE Healthcare (Piscataway, NJ, USA), respectively. All other materials and reagents were of the highest quality available from commercial suppliers.

2.2. Cell culture, transfection and stimulation

Human embryonic kidney (HEK293) cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum and sub-cultured for 24 h in 6-cm dishes. They were then transfected with the pME18s-FLAG-RSK1 construct (1 μg) using Lipofectamine LTX with Plus Reagent (Life Technologies, Inc.). After 24–36 h incubation, the cells were serum starved for 18 h and stimulated with buffer alone, 1 mM diamide for 15 min or 100 ng/ml EGF for 10 min.

2.3. Preparation of lysates and immunoprecipitation

For preparation of lysates, cells were homogenized by sonication in TNE buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.1 mM EDTA, 0.1 mM EGTA, and 1% Nonidet P40). After centrifugation at 15 000×*g* for 15 min, the supernatant was added to 10 μl of anti-FLAG M2-agarose affinity gel (50% slurry) (Sigma), and the mixture was incubated for 1 h at 4 °C. After precipitation of the resin by centrifugation and removal of the supernatant, resin was washed twice with 500 μl of TNE buffer. RSK1 was eluted with 3× FLAG Peptide (Sigma) from the beads. An aliquot of supernatant was also applied for SDS–PAGE followed by Western blot analyses or for an in vitro kinase assay. For the determination of glutathionylated RSK1, all the procedures were performed without any reducing reagents.

2.4. Inactivation of RSK1 by diamide and GSH

Purified RSK1 was incubated at 25 °C for 10 min in 250 mM HEPES (pH 7.5) and 0.5 mM EDTA with increasing amounts of diamide (0–1 mM), in the presence of 100 μM glutathione (GSH). An aliquot of reaction mixture was then removed and analyzed for activity. Kinase activity was measured at 30 °C for 10 min in 40 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 10 μM [γ-^{32}P] ATP, 50 μM S6 Kinase/Rsk Substrate Peptide 1 (Synthetic peptide (RRRLSSLRA) corresponding to amino acids 231–239 of human 40S ribosomal protein S6) (Upstate), and RSK1 in a final volume of 25 μl. ^{32}P incorporation was determined by spotting 20-μl aliquots onto Whatman P-81 phosphocellulose paper followed by washing in 75 mM phosphoric acid [15]. For reversal with glutaredoxin (AbFrontier, Seoul, Korea), following incubation with diamide and GSH, human recombinant glutaredoxin-1 (0.04 mg/ml) and more GSH (500 μM) were added.

2.5. Construction of plasmids

The plasmids pME18s-nNOS was generated as described previously in [16]. The RSK1 mutants, C223S, (the mutant featuring replacement of Cys223 with Ser) were generated with pME18s-RSK1 plasmid DNA and a QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The nucleotide sequences of each mutant were confirmed.

2.6. Phosphorylation of recombinant nNOS in vitro

Recombinant rat nNOS was expressed in *Escherichia coli* and purified using 2′,5′-ADP-agarose (Sigma), as described previously in [17]. Purified nNOS (0.6 μg) was incubated with pretreated RSK1 at 30 °C for 10 min in a solution containing 40 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 1 mM ATP for Western blotting. The reaction was terminated by the addition of SDS sample buffer. The samples were then subjected to SDS–PAGE followed by Western blot analysis with the indicated antibody.

2.7. Statistical analysis

All results are represented as means ± S.E. of at least three determinations. The statistical evaluation was performed using one-way ANOVA test. We considered $P < 0.05$ to be statistical significant.

3. Results

3.1. RSK1 is inactivated by treatment with diamide and reduced GSH

In initial experiments we determined the effects of the thiol-specific oxidant diamide with reduced GSH on RSK1 enzyme activity. For full activation, RSK1 requires phosphorylation of a crucial activation loop Thr221 by PDK1 [3]. To obtain activated RSK1, we expressed FLAG-RSK1 in HEK293 cells, treated with EGF (100 ng/ml, 10 min) and purified with FLAG-agarose gel. Purified RSK1 was incubated with GSH (100 μM) and diamide (100 μM) for 10 min, and the activity of RSK1 was measured using a synthetic peptide, S6 peptide, as a substrate. There was significant loss in the enzyme activity. Addition of dithiothreitol (DTT) (20 mM) almost completely reversed this inhibition (Fig. 1A). When purified RSK1 was incubated with diamide (>100 μM) in the presence of GSH (100 μM), there was a synergistic effect with rapid and near complete inhibition occurring within 10 min (Fig. 1B).

3.2. RSK1 is modified by S-glutathionylation, resulting in enzyme inhibition

To determine whether glutathionylated RSK1 could be generated by reaction of GSH with diamide, purified activated RSK1 was incubated either with or without GSH and diamide for 10 min, and then immunoblotted with a monoclonal antibody against GSH protein adducts and anti-RSK1 antibody in the non-reduced condition. Note that the band at ~120 kDa of RSK1 was additionally observed by treatment with GSH and diamide besides ~90 kDa of the enzyme (Fig. 2A). This treatment did induce RSK1 glutathionylation (Fig. 2A). Although *S*-glutathionylated RSK1 was evident in GSH/diamide-treated enzyme, we questioned whether this modification is sufficient to significantly dampen RSK1 activity. The enzyme thioltransferase (also known as glutaredoxin: Grx) selectively dethiolates *S*-glutathionylated protein by a

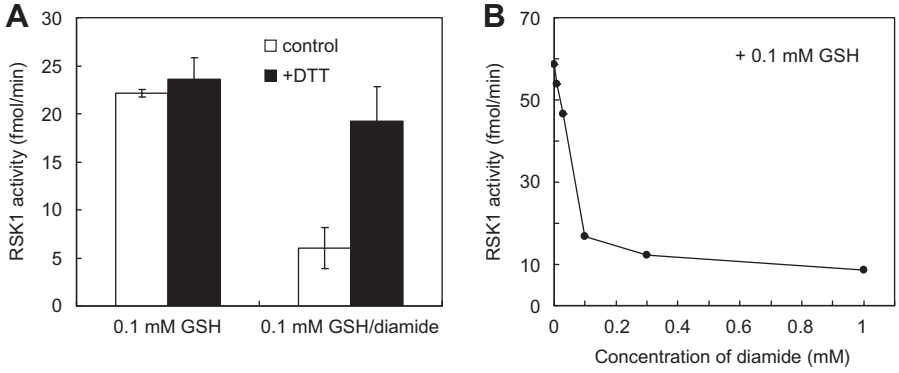

Fig. 1. RSK1 is inactivated by diamide/GSH. (A) Purified RSK1 was incubated (10 min at 25 °C) with 100 μM GSH and 100 μM diamide. Samples were then assayed for 10 min using 50 μM S6 Kinase/Rsk Substrate Peptide 1 and 10 μM [γ-^{32}P] ATP. For the DTT sample, an additional incubation with 20 mM DTT (10 min at 25 °C) was performed prior to assaying. Values are means ± S.E. of three independent experiments are shown. (B) Purified RSK1 was incubated (10 min at 25 °C) with the indicated amounts of diamide (0.01–1 mM) in the presence of 100 μM GSH. The enzyme activity of RSK1 was measured as described in panel A. The means ± S.E. of three independent experiments are shown.

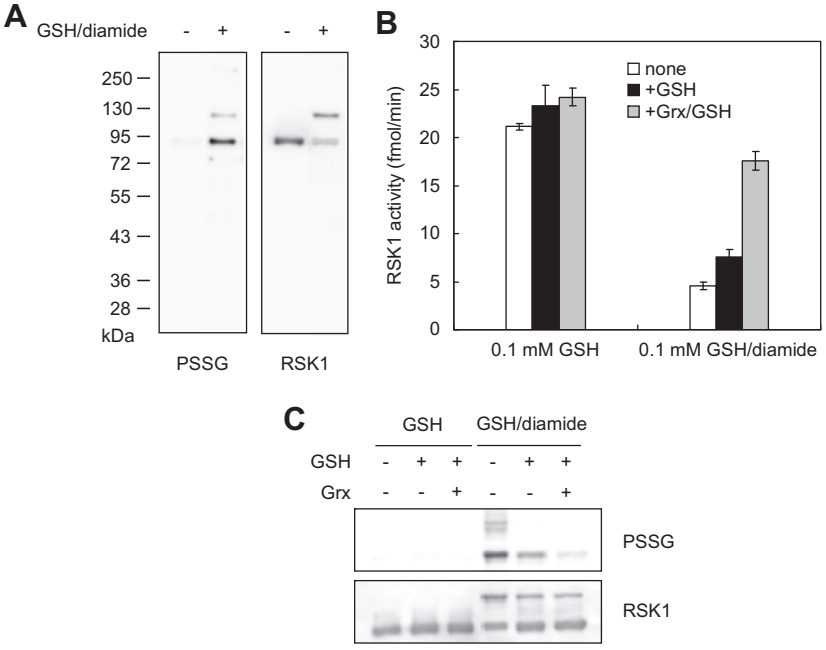

Fig. 2. RSK1 is rapidly and nearly completely inhibited upon being *S*-glutathionylated. (A) Purified RSK1 was incubated with 100 μM GSH and 100 μM diamide and sample was subjected to Western blotting with anti-GSH (PSSG) and RSK1 (RSK1) antibodies under the non-reducing condition. (B) Purified RSK1 was incubated with 100 μM GSH and 100 μM diamide as indicated. After the addition of buffer alone, 500 μM GSH, 3.3 μM glutaredoxin plus 500 μM GSH for 15 min at 25 °C, the enzyme activity of RSK1 was measured. The means ± S.E. of three independent experiments are shown. (C) Each sample was subjected to Western blotting with anti-GSH (PSSG) and RSK1 (RSK1) antibodies under non-reducing conditions.

GSH-dependent mechanism [18]. We therefore investigated whether thioltransferase catalysis could reverse GSH/diamide-potentiated RSK1 inactivation. When purified activated RSK1 was incubated with GSH (100 μM) and diamide (100 μM) for 10 min, its enzyme activity was inhibited. Treatment of Grx (3.3 μM) and GSH (500 μM) with the glutathioylated RSK1 resulted in a substantial reactivation of kinase activity (Fig. 2B). We next examined whether Grx and GSH reverse kinase inhibition by removal of GSH from RSK1. RSK1 was inactivated with GSH and diamide and then was analyzed by immunoblotting with a monoclonal antibody against GSH protein adducts. The glutathioylated RSK1 with the molecular weights of ~90 and ~120 kDa was decreased by the treatment with Grx and GSH (Fig. 2B). On the other hand, the total RSK1 at ~120 kDa remained unchanged by the treatment with Grx and GSH.

3.3. Cys223 is an essential site for inactivation of RSK1

We previously showed that Ca^{2+}/CaM-dependent protein kinase I is fully and reversibly inactivated by oxidative *S*-glutathionylation at Cys179 [19]. This Cys179 in the subdomain VIII, often mentioned as a key protein kinase catalytic domain indicator [20], is highly conserved among a number of kinases including RSK1 (Cys223: based on the mouse RSK1 sequence). We generated a mutant FLAG-RSK1 in which Ser was substituted for Cys223 (C223S) and characterized both activity and sensitivity to inhibition by GSH and diamide. FLAG-RSK1 wild-type (WT) or C223S mutant was overexpressed in cells and purified by FLAG–agarose gel from cell lysates, and gave a major band at ~90 kDa on Western blot with anti-RSK1 antibody (Fig. 3A). The enzyme activities of WT and C223S mutants were similar in an in vitro assay. With increasing

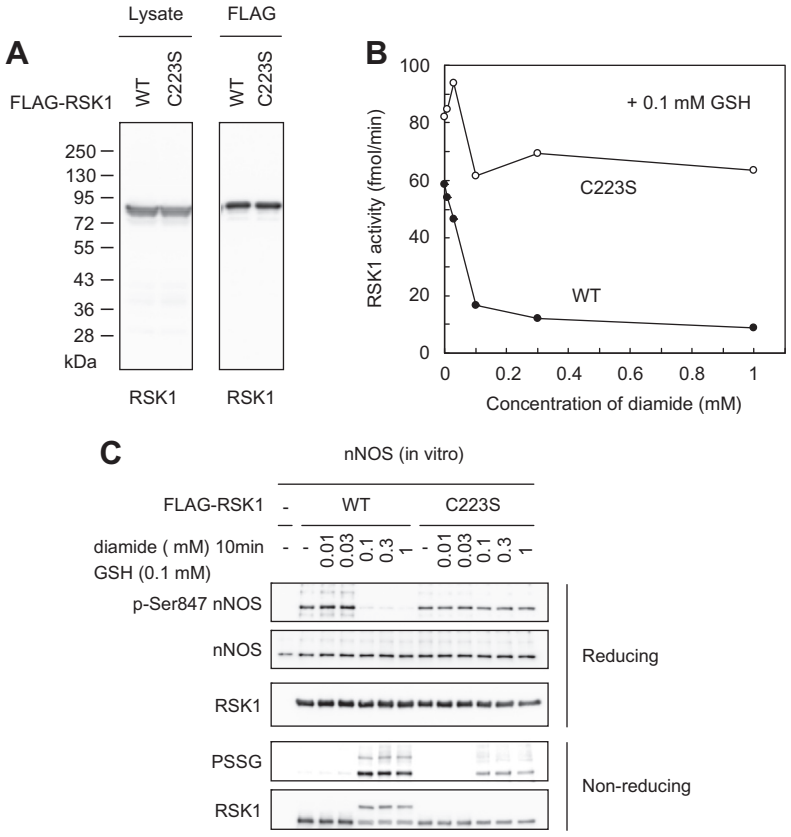

Fig. 3. Generation of a redox-resistant RSK1 mutant. (A) Equal amounts of RSK1 WT and the C223S mutant from transfected HEK293 cells were separated by 10% SDS–PAGE and stained with anti-RSK1 (RSK1) antibodies. (B) RSK1 WT or C223S mutant were incubated (10 min at 25 °C) with the indicated amounts of diamide (0.01–1 mM) in the presence of 100 μM GSH then assayed for activity as described for Fig. 1. The means ± S.E. of three independent experiments are shown. (C) RSK1 WT or C223S mutant were incubated (10 min at 25 °C) with the indicated amounts of diamide (0.01–1 mM) in the presence of 100 μM GSH. Each treated RSK1s were incubated (10 min 30 °C) with recombinant nNOS (0.6 μg) in a kinase reaction buffer. After terminating the reaction, each sample was subjected to Western blotting with anti-phospho-847Ser nNOS (NP847), anti-nNOS (nNOS), anti-GSH (PSSG) and RSK1 (RSK1) antibodies under reducing or non-reducing conditions. The data are representative of at least three independent experiments.

amounts of diamide (0.01–1 mM) in the presence of GSH (100 μM) no remarkable decrease in activity was noted with the C223S mutant, while the WT was significantly inactivated (Fig. 3B).

Our previous data showed that RSK1 inhibits neuronal NO synthase (nNOS) enzyme activity by adding phosphate groups to Ser847 on nNOS [7]. We further examined the effect of RSK1 inactivation by diamide and GSH on nNOS phosphorylation. Purified activated RSK1 was incubated with GSH (100 μM) and diamide (0.01–1 mM) for 10 min, and the phosphorylation of nNOS at Ser847 by the glutathionylated RSK1 was monitored using phospho-Ser847 nNOS antibody. In the absence of treatment, the nNOS phosphorylation at Ser847 was evident in both RSK1 WT and C223S mutant. Treatment of RSK1 WT with diamide (>100 μM)/GSH (100 μM) led to decrease in the nNOS phosphorylation (Fig. 3C). In contrast, C223S mutant proved resistant to diamide/GSH treatment. The treatment of diamide and GSH led to increase in *S*-glutathionylation of RSK1. Amount of *S*-glutathionylation of RSK1 WT was more than that of C223S mutant. The upper band at ~120 kDa of RSK1 was observed by the treatment of diamide/GSH in RSK1 WT but not in C223S mutant.

3.4. Cys223 is the site of S-glutathionylation on RSK1 in cells

Having discerned that *S*-glutathionylation of Cys223 inactivates RSK1, we tested whether RSK1 substrates (ATP, S6 peptide) can protect against inactivation. The inhibition of RSK1 by diamide and GSH was competitive with neither ATP nor S6 peptide. Table 1 summarized the properties of the RSK1 WT treated either with or without GSH and diamide.

Table 1
Kinetic properties of diamide/GSH-untreated (control activity) and -treated RSK1 phosphorylation reaction was carried out at 30 °C for 10 min in a solution containing 50 μM S6 peptide, and 10 μM [γ-^{32}P] ATP, as standard condition (standard). The values represent the means ± S.E. of three experiments.

	Control activity (fmol/min)	Diamide/GSH
Standard	27.21 ± 1.93	11.19 ± 0.15
Excess S6 peptide (500 μM)	52.71 ± 2.78	22.15 ± 0.84
Excess ATP (100 μM)	217.02 ± 3.23	75.70 ± 4.35

We further examined the working of this Cys223-sensitive redox signaling in situ. HEK293 cells were transfected with nNOS alone or with RSK1 WT or RSK1 C223S, and treated with combination of EGF and diamide. Then, cell lysates were immunoblotted with phospho-Ser847 nNOS antibody. When nNOS-expressing cells were treated with EGF, phosphorylation of Ser847 was observed as described previously (Fig. 4) [7]. Treatment of the cells with diamide almost completely blocked EGF-induced phosphorylation of Ser847. When nNOS/RSK1 WT or nNOS/RSK1 C223S -expressing cells were treated with EGF, phosphorylation of Ser847 was observed in both cells. Treatment of the cells expressing nNOS/RSK1 WT with diamide led to decrease in the EGF-induced nNOS phosphorylation (Fig. 4). In contrast, cells expressing nNOS/RSK1 C223S proved resistant to diamide. Note that EGF-, diamide-, and EGF/diamide-induced phosphorylation of ERK1/2, the

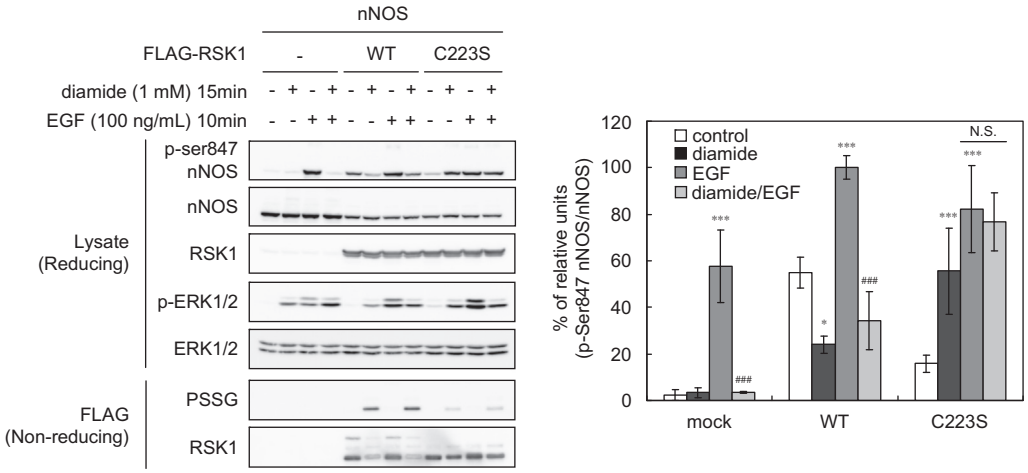

Fig. 4. Effects of diamide on RSK1-induced phosphorylation of nNOS at Ser847 in cells. HEK293 cells expressing nNOS with FLAG-tagged RSK1 (WT), or FLAG-tagged RSK1 C223S (C223S) were treated with buffer alone, 1 mM diamide for 15 min and 100 ng/ml EGF for 10 min. Cell lysates were immunoblotted with anti-phospho-847Ser nNOS (NP847), anti-nNOS (nNOS), anti-RSK1 (RSK1), anti-phospho-ERK1/2 (p-ERK1/2) or anti-ERK1/2 (ERK1/2). Expressed RSK1 was also affinity-purified from transfected cells using anti-FLAG-agarose chromatography technique. Purified RSK1 (FLAG) was immunoblotted with anti-GSH (PSSG) and anti-RSK1 (RSK1) under non-reducing conditions. The histogram shows amounts of phospho-nNOS relative to those of nNOS. Results are the mean ± S.E.M. of three independent experiments. $^{***}P < 0.001$ and $^{*}P < 0.05$ as compared with control of each transfected cells and $^{\#\#\#}P < 0.001$ as compared with EGF treatment of each transfected cells.

upstream activator of RSK1, were similar in all the cells. Diamide-induced *S*-glutathionylation of WT was more evident than C223S mutant.

4. Discussion

We showed here at the first time that RSK1 is sensitive to inhibition by direct *S*-glutathionylation of its Cys223 residue during oxidative stress. Activation of RSK1 is regulated by its upstream kinases, ERK1/2 and PDK1, by sequential phosphorylations. These sequential phosphorylations of the enzyme are initiated by ERK1/2 at Thr573 of CTKD leading to the autophosphorylation of RSK1 at Ser380. This phosphorylation allows the dockage of PDK1 and enables PDK1-dependent phosphorylation in the NTKD of RSK1 at Ser221, resulting in its maximal activation [1,2]. Since Cys575 in the CTKD of RSK1 is highly conserved among a number of kinases, we also generated mutant of RSK1 (C575S), in which Cys575 residue of CTKD was replaced by Ser and determined both activity and sensitivity to inhibition by GSH and diamide. The enzyme activities of WT and C575S mutant were similar in an in vitro assay. Treatment of the WT and C575S mutant with GSH (100 μM) and diamide (100 μM) for 10 min resulted in a nearly complete inhibition of enzyme activity using nNOS as a substrate (data not shown). The oxidative stress prior to EGF stimulation results in inhibition of the RSK1 activation in cells (Fig. 4), and the EGF-induced activation of RSK1 activity was also inhibited by oxidative stress in vitro (Figs. 1–3). Thus, inhibition of RSK1 by its *S*-glutathionylation at Cys223 appears to be dominant over activation of the kinase by its phosphorylation at Ser221. An additional ~120 kDa band of RSK1 WT was observed by the treatment of GSH/diamide but not in the case of C223S mutant, determined with non-reducing SDS–PAGE (Figs. 2–4). In addition, this ~120 kDa band of RSK1 was detected when glutathioylated RSK1 WT was treated with Grx and GSH (Fig. 2), indicating that Cys223 also contributes to another oxidative modification of the enzyme besides its *S*-glutathionylation. However, our data clearly indicate that the oxidative stress-induced inactivation of RSK1 is occurred via its *S*-glutathionylation at Cys223 in cells. Because diamide has many potential actions that could function to influence the ERK pathway, including inhibiting thiol containing protein phosphatases [21], additional studies are needed to elucidate how diamide is activating ERK. We also examined the working of this Cys223-sensitive redox signaling using H_2O_2 or NO as an oxidative stress. Although the sensitivity of RSK1 to regulation by NO was not observed, H_2O_2 treatment led to a decrease in the enzyme activity of RSK1 WT but not of the C223S mutant (data not shown). Thus, it remains to be determined whether other oxidative modifications of RSK1 at Cys223 were involved in an oxidative-induced inactivation of the enzyme besides its *S*-glutathionylation. Active ERK1/2 has many intracellular targets located in nucleus, cytoplasm and cell surface. All of these ERK substrates seem to play an important role in controlling cellular processes that occur on mitogenic stimulation. One of the ERK1/2 substrates, RSK1 is a central mediator of ERK1/2 during cell survival [22]. The phosphorylation of ERK1/2 was increased following treatment with either EGF or diamide in cells (Fig. 4). Treatment of the cells expressing nNOS with EGF resulted in an increase in the phosphorylation of nNOS at Ser847, but diamide had no effect on the nNOS phosphorylation. Thus, diamide is through to inhibit RSK1 activity via its *S*-glutathionylation at Cys223 in cells. Indeed, treatment of the cells expressing nNOS/RSK1 C223S with diamide resulted in a similar increase in the phosphorylation of nNOS at Ser847 as with EGF (Fig. 4).

Under what cellular conditions may RSK1 be glutathionylated and inactivated? RSKs are broadly expressed in the brain that regulate important cellular functions, including cell survival [23]. Inhibition of NOS has been shown to be neuroprotective, implicating NOS in the production of radicals which can produce peroxynitrite and H_2O_2 [24]. Following EGF treatment, nNOS was phosphorylated by RSK1 in rat hippocampal neurons and cerebellar granule cells, which suggests a novel role for RSK1 in the regulation of NO function in the brain [7]. We began to investigate whether diamide treatment is regulating NO production in WT and in mutant RSK1 expressing cells. The brain is highly susceptible to oxidative stress due to its requirement for excessive use of glucose for energy, as well as its inability to undergo cellular regeneration. Indeed, various indices of ROS damage have been reported within the specific brain region that undergoes selective neurodegeneration [25]. Huntington's disease is an autosomal dominant disease that causes degeneration of medium spiny GABAergic neurons, expressing nNOS in the striatum. The expression levels of and

the activities of RSK1/RSK2 are increased in the striatum of Huntington's disease animal models [26]. We showed here that EGF-induced nNOS phosphorylation at Ser847 was inhibited during oxidative stress through the glutathionylation of RSK1 at Cys223 in cells (Fig. 4). We speculate that the RSK1 Cys223 glutathionylation enhances the accumulation of toxic levels of NO in the brain. It needs to be determined in future study whether oxidative stress regulates NO production via RSK1 inactivation.

Acknowledgments

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