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貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：研究報告書

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1．助成金額：600,000 円

2．研究テーマ

Ras・ヒト発癌過程における活性酸素産生遺伝子Nox1の役割の研究

3．成果の概要（100字程度）

Nox1によって産生されるROSの標的蛋白候補の一つ、小胞体蛋白ERp57の機能を解析した結果、ERp57は、Nox1と physical相互作用をし、細胞内で共局存在する事を見出した。このことからERp57は Nox1レドックスシグナリングを媒介することが考えられる。又、Nox1ファミリーの Nox4は、AKT-ASK1シグナルを介して、膵癌細胞の生存に寄与することが判明した。

4．研究業績

（1）学会における発表　　無　・　有（学会名・演題）

2005第64回癌学会
1. Inhibition of NADPH oxidase 4 activates apoptosis via the AKT/apoptosis signaling regulating kinase 1 pathway in pancreatic cancer PANC-1 cells
2. Downregulation of Rho by NADPH oxidase 1 is required for EGF-and Ras- induced actin depolynerization

2005 第78回日本生化学会
1. The superoxide generating oxidase Nox4 confers anti-apoptosis activity to pancreatic cancer cells via the AKT-ASK1 kinase signaling
2 Nox1-dependent redox regulation exerts an integral role in actin cytoskeleton organization of Ras-transformd cells by controling Rho signaling

（2）発表した論文　　無　・　有（雑誌名・題名）

(Anal Biochem. 2005 Jul 15;342(2):348-51.)
Development of a direct and sensitive detection method for DNA-binding proteins based on electrophoretic mobility shift assay and iodoacetamide derivative labeling.
(Oncogene 2006 in press)
Inhibition of NADPH oxidase 4 activates apoptosis via the AKT/apoptosis signaling regulating kinase 1 pathway in pancreatic cancer PANC- 1 cells

—日中医学協会助成事業—

Ras.ヒト発癌過程における活性酸素産生遺伝子 Nox1 の役割の研究

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Summary

Cellular target proteins for superoxide-generating oxidase Nox1, that may play a mediating role in Ras oncogene transformation and in some types of human cancers, have been analyzed. An endoplasmic reticulum protein Erp57 was identified to be oxidized by Nox1-generated ROS and interact with Nox1 proteins. This implicates Erp57 as a downstream effector for Nox1 redox signaling involved in tumor progression. In another experiment, we found that Nox4-produced ROS activate AKT-ASK1 kinase cascade and thereby lead to cell survival signaling in pancreatic cancer cells, indicating that Nox4 may in part confer pancreatic cancer cells resistance against apoptosis.

Key words

NADPH oxidase, Reactive Oxygen Species, Erp57, apoptosis, cancer

Background

Generation of intracellular reactive oxygen species (ROS) has been implicated in immortalization, invasion, and metastasis during the multistep cancer development, but its exact molecular mechanism has not been fully elucidated. Superoxide generated by the NADPH oxidase (Nox) family has recently been recognized as important signaling molecules in cellular processes (Lambeth, 2004). They share common structural similarities with six transmembrane domains and the cytoplasmic domain that comprises NADPH-and FAD-binding sites. Among them, Nox1 is unique in that it is involved in mitogenic regulation (Suh et al., 1999). We therefore initiated study on the functional role of Nox1 in carcinogenesis by utilizing Ras oncogene transformation as a model system. We found that Ras oncogene upregulates the expression of Nox1 via the MAP kinase kinase (MAPKK)-MAP kinase (MAPK) dependent pathway, and that

small interference RNA (SiRNA) targeting Nox1 blocks the Ras transformation phenotypes including anchorage-independent growth, morphological changes, and tumorigenecity (Mitsushita et al.,2004).The finding suggests that ROS generation by Ras-induced Nox1 is functionally required for Ras transformation.

Furthermore, the increased expression of Nox4, another member of the Nox family, was detectable in human pancreatic cancer cell line and inhibition of the Nox4 activity by Nox4 RNAi caused apoptosis of colon cancer cells, suggesting that Nox4 generated ROS are indispensable for cell survival of pancreatic cancer cells. Thus, our study provides a new concept that Nox-generated ROS, unlike a conventional view of genotoxity of ROS, are important signaling molecules in cell transformation process.

Material and Method

1. Detection of physical association of Erp57 with Nox1

Human (h) Nox1 cDNA and c-myc-tagged Erp57 cDNA were subcloned into pcDNA3.0 and pcDNA3.1, respectively. Erp57 was cotransfected into COS1 cells with Nox1 and its regulators p41 and p51.Cell lysates were subjected to coimmunoprecipitation study by using anti-c-myc-antibodies and anti-hNox1 antibodies.

2. Immunocytochemistry

Human colon cancer CACO-2 cells tranfected with hNox1 expression plasmids, fixed and permeabilized. Indirect immunostaining was performed with anti-hNox1 antibodies and anti-Erp57 antibodies, followed by staining with FITC-or Rhodamine-conjugated antibodies. The stained cells were observed under a Zeiss confocal microscope.

3. Analysis of AKT and ASK1 activities.

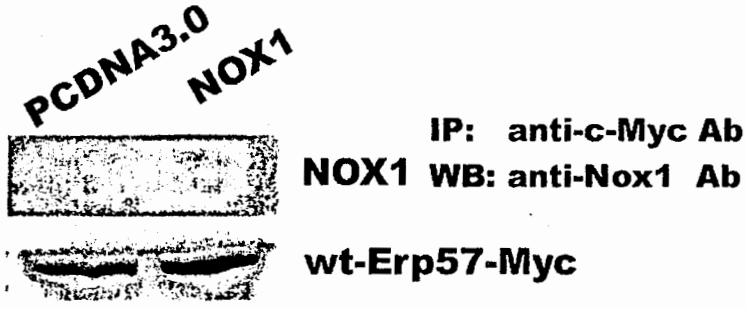
Pancreatic cancer PANC-1 cells were incubated with a NADPH oxidase inhibitor or transfected with psilencer vector carrying scrambled RNAi or Nox4 RNAi-1 and -2, and cell lysates were subjected to immunoblotting with anti-phospho-AKT antibodies and anti-phospho-ASK1 antibodies. In the control experiment, PANC-1 cells were transfected with pcDNA3.0 wt-AKT1 or AKT1Lys179Ala and cell lysates were subjected to immunoblotting with anti-phospho-AKT and anti-phospho-ASK1 antibodies.

Results

Specific aim 1: Identification of cellular target proteins for Nox1-generated ROS

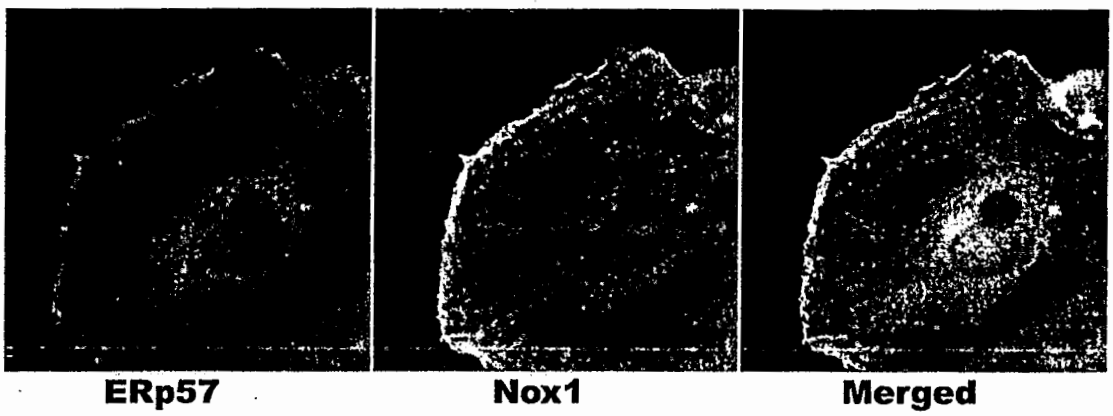
Because we previously identified an endoplasmic reticulum protein Erp57 as one of proteins oxidized by Nox1-generated ROS, we examined whether Erp57 interacts with Nox1.To this end,COS1 cells were transfected with Nox1 together with c-myc-tagged Erp57.Cell lysates were immunoprecipitated with anti-c-myc-antibodies and the immunoprecipitates were probed with anti-c-Myc antibodies. The data show that Erp57 and Nox1 were coimmunoprecipitated, suggesting the binding of Erp57 to Nox1 (figure.1).

Figure.1 Coimmunoprecipitation of Nox1 with wt-Erp57



To further assess the Nox1-Erp57 interaction, CACO2 cells were double-stained with mouse anti-Nox1-antibodies and rabbit anti-Erp57 antibodies. As shown in Figure.2,

Figure2 Colocolization of ERp57 with Nox1 on the Caco2 cell surface



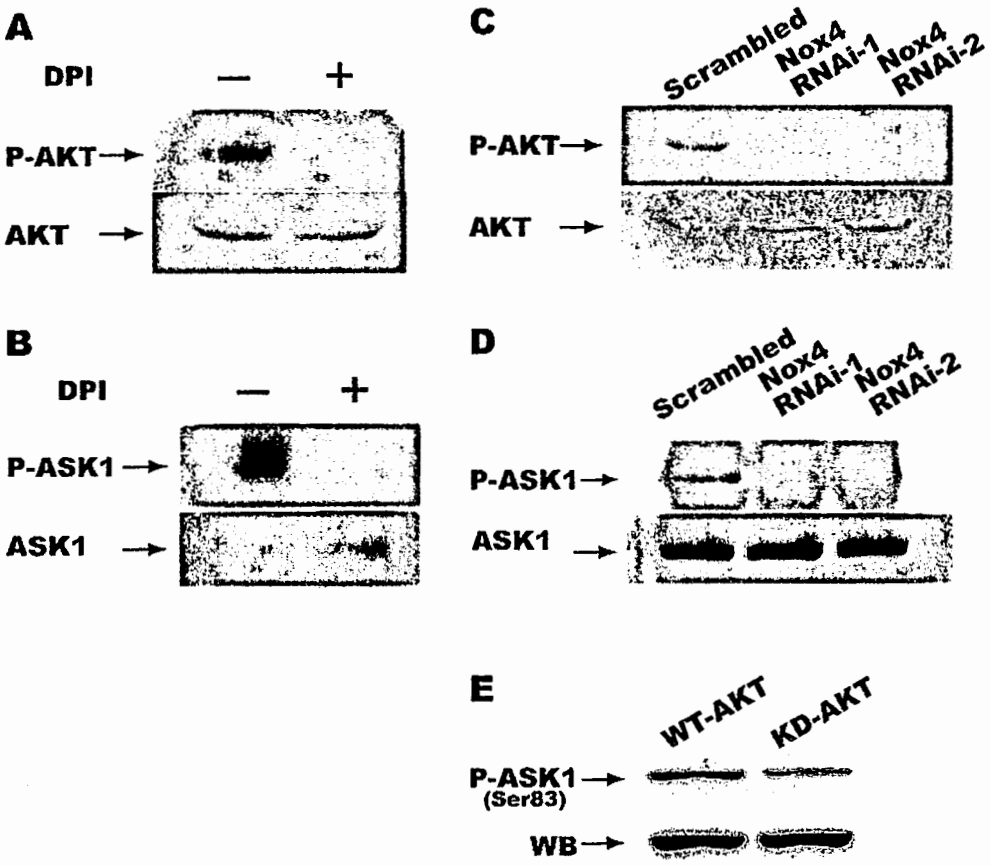
Erp57 was colocalized with Nox1 at the plasma membrane sites and the majority of Erp57, which is localized at ER, was not colocalized with Nox1.The data support the results obtained with above binding studies.

Specific aim 2: The dissection of Nox4-generated cell survival signaling pathway in Pancreatic cancer cells

Akt (protein Kinase B) is one of the crucial regulators of cell survival function in response to growth factor stimulation, and its genetic alteration is linked to progression of several human cancers. AKT is activated upon phosphorylation, which in turn inhibits apoptosis-inducing proteins by phosphorylating these proteins, resulting in promotion of cell survival. Conversely, conversion of the phosphorylated AKT to its dephosphorylated form is associated with induction of apoptosis. Previously we demonstrated that depletion of ROS by DPI, a Nox inhibitor treatment or introduction of small interference RNA (siRNA) for Nox4 induces apoptosis of PANC-1 cells. To investigate whether AKT is involved in cell death of PANC-1 cells caused by DPI treatment or siNox4 RNAs transfection, we examined the phosphorylation state of AKT under these experimental conditions. PANC-1 cells were incubated with DPI, cell lysates were prepared and the phosphorylation level of AKT was analyzed by immunoblotting with

anti-phospho-AKT (Thr308) antibodies. While AKT was found to be phosphorylated in untreated cells, addition of DPI markedly decreased its phosphorylation level (Figure 3).

Figure.3 DPI and siNox4RNAs inhibit AKT and ASK1 phosphorylation



DPI treatment did not affect the expression of AKT proteins. The results suggest that DPI attenuated AKT signaling during induction of apoptosis. Because recent findings suggest that ASK1, a key player in stress-responsive apoptosis, is one of the protein substrates for AKT, this raises the possibility that DPI-induced suppression of phosphorylation of AKT inhibits AKT-dependent phosphorylation of ASK1 on Ser-83. To test this possibility, lysates prepared from DPI-loaded PANC-1 cells were subjected to immunoblotting with antibodies to phospho-ASK1 antibodies, which specifically recognize ASK1 phosphorylated on Ser-83. PANC-1 cells exhibited a significant level of phosphorylation of ASK1, but this phosphorylation was clearly blocked by inhibition of cells with DPI (Figure 3), suggesting the involvement of DPI-inhibitable Nox-like enzyme in downregulation of an AKT-ASK1 signaling cascade. In fact, silencing of the endogenous Nox4 activity by Nox4 RNAi-1 and -2 reduced phosphorylation of AKT and hence phosphorylation of ASK1 on Ser-83, as shown in Figure 3. If ASK1 is a substrate for AKT, disruption of the AKT kinase activity should interfere with phosphorylation of ASK1. The overexpression of the kinase defective AKT mutant, AKT1Lys179Ala, yielded a significant reduction in ASK1 phosphorylation, as expected (Figure 3)

Discussion

Our studies indicate that Nox1 binds to an endoplasmic reticulum (ER) protein Erp57 and subsequently oxidize it. Erp57 is considered to play a role as oxidoreductase, disulfide isomerase, and molecular chaperone. Especially, Erp57 has been proposed to participate in correct protein folding at ER site through isomerase activity. However, not all of the Erp57 is confined to the area of ER and some of them are detectable at plasma membrane. In fact, our immunocytochemical study also revealed the membrane localization of Erp57 and importantly its association with Nox1. Thus, Erp57 could be a direct acceptor for Nox1-generated ROS that transmits redox-signaling to downstream molecules. It remains to be determined whether thiols of cysteine residues contained in Erp57 thioredoxin domains are oxidized by Nox1-generated ROS. Also, it remains to be elucidated whether the biochemical link of Nox1-Erp57 is required for Ras oncogene transformation and some of transformation phenotypes of human cancer cells.

Vaquero et al. (2004) initiated the study on the protective function of Nox4 against cell killing in pancreatic cells, but the signaling mechanism by which Nox4-generated ROS control cell survival was not fully elucidated in their study. Our study reveals that abrogation of Nox4-generated ROS induced inhibition of phosphorylation of AKT and subsequent suppression of AKT-mediated phosphorylation of ASK1 on Ser-83, and that the AKT phosphorylation-defective ASK1 Ser83Ala mutant alone can substitute for DPI and siNox3RNAi in apoptosis induction. Because phosphorylation of AKT on Thr-103 activates its kinase activity and the activated AKT decreases the ASK1 kinase activity through phosphorylation of ASK1 on Ser-83. It is most likely that inhibition of AKT phosphorylation by DPI and siNox4RNAs reduced its kinase activity, which in turn blocked AKT-catalyzed phosphorylation of ASK1, resulting in ASK1-mediated cell death. We therefore propose that Nox4-dependent cell survival signaling is mediated by the AKT-ASK1 kinase cascade and that antagonizing this signaling pathway by DPI or siNox4RNAs results in activation of apoptosis.

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