CHEMILUMINESCENCE STUDY ON THE REGULATION OF NADPH OXIDASE ACTIVITY BY THIOREDOXIN REDUCTASE IN VASCULAR ENDOTHELIAL CELLS

XUN SHEN and ZHEN-BO LIU
Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

INTRODUCTION

The endothelium, which consists of a single layer of endothelial cells, lines the inner surface of all blood vessels and the heart, forming an important interface with circulating blood. The vascular endothelial cells have well-established roles in cardiovascular homoeostasis and the initiation of the inflammatory process such as artherosclerosis. It is now accepted that the expression of some inflammatory genes, regulated by reactive oxygen species (ROS), is considered as the molecular mechanism of the early atherosclerosis. A major source of ROS in vascular cells is the NAD(P)H oxidase,[1] which consists of the membrane subunits gp91phox and p22phox and the cytosolic subunits p67phox, p47phox, and the small GTPase rac1. Mechanisms that control activity of this multisubunit enzyme complex are incompletely understood.

Thioredoxin reductase (TrxR) is an antioxidant enzyme that participates in thiol-dependent cellular reductive processes.[2] Since the enzyme regenerates reduced thioredoxin that serves as reducing equivalent and may also scavenge ROS, TrxR has been considered as an enzyme to reduce the ROS level in cells including vascular endothelial cells. However, we studied the effect of TrxR on the NADPH oxidase activity (using a chemiluminesence method) of either overexpressing TrxR1 or knockdown the endogenous TrxR1 in the an endothelial cell line, and found that TrxR actually promotes ROS generation by upregulating the activity of NADPH oxidase.

MATERIALS AND METHODS

Cell culture and transfection. The endothelial-like cell line EA.hy926 cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 g/mL streptomycin at 37°C in a 5% CO_2-incubator. The cells overexpressing TrxR1 were established by stably transfecting the TrxR-expressing vector pIRESneo2-TrxR1, which contains the neomycin resistance gene and the wild-type human TrxR1 gene, and selected with G418 for 4 w. The cells expressing only neomycin resistance gene were used as control. In the experiments with the TrxR1-cells, the cells and their controls were incubated in the medium supplemented with 40 nM sodium selenite for 48 h before usage. The Trx1-knockdown cells were obtained by transfection of 150 nM TrxR1 siRNA (5'-AACACGTGCTTGTGGACATCA-3') for 72 h. The cells transfected with a scramble RNA were used as control. The cells having their endogenous p22phox

knocked down were obtained by transfecting siRNA for p22phox (5'-AACATGACCGCCGTGGTGAAG-3').

Horseradish peroxidase (HRP)-catalyzed chemiluminescence detection of the intracellular ROS.[3] The HRP-luminol solution was prepared by adding an adequate quantity of horseradish peroxidase (HRP) in luminol (0.1 mg/mL). The cuvette containing 2 mL of cell suspension (10^6 cells/mL) was placed in a home-made single-photon counter, and then, the photon emission from the cell suspension was recorded before and after addition of the HRP-luminol solution. The stationary photon emission reached after addition of HRP-luminol solution represents the ROS level in cell suspension.

RESULTS AND DISCUSSION

Expression and activity of TrxR1 in TrxR1-overexpressing cells. To know if TrxR1 plays any role in regulation of NADPH oxidase in vascular endothelium, a stable TrxR1-overexpressing EAhy926 cell line was established and is referred as EAhy-TrxR1 cells. The activity of TrxR 1 in the stably transfected cells was determined as a DTNB reduction rate after subtracting the contribution of other selenoproteins in the presence of the TrxR1 inhibitor gold thioglucose. As Fig.1A and 1B show, the activity of TrxR1 in EAhy-TrxR1 cells (the TrxR1-overexpressing cells) was 34% higher than that in control (the cells expressing only neomycin resistance gene, and being referred as EAhy-neo cells). The Western blot analysis also showed a 33% higher expression of TrxR1 in the TrxR1-overexpressing cells (Fig.1C and 1B).

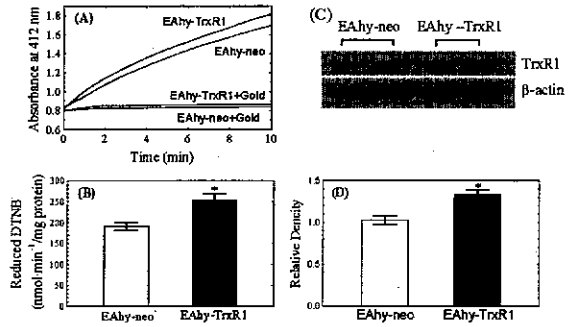

Fig. 1. Activity and expression of TrxR1 in TrxR1-overexpressing cells. (A) The activity of TrxR1 was determined by reduction rate of DTNB, the substrate of TrxR. (B) The relative TrxR activity in corresponding cell extracts after correction for the DTNB reduction in the presence of gold thioglucose. * indicates $P<0.05$ vs. neo cells. (C, D) A typical Western blot of the TrxR1 protein levels and relative expression of TrxR1 in the two cell lines

Chemiluminescence detection of ROS in the TrxR1-overexpressing cells. The enhanced chemiluminescence from the cells transfected with pIRESneo2-TrxR1 and

the controls (i.e. the cells transfected with pIRESneo2) was recorded (see Fig.2). It is clear that the enhanced chemiluminescence is very sensitive to the ROS in a cell suspension, and overexpression of TrxR1 enhanced intracellular generation of ROS in endothelial cells. The TrxR1-enhanced ECL almost disappeared when DPI, the inhibitor of NADPH oxidase, was added into the cell suspension, indicating that the TrxR1-enhaced ROS generation might be connected to activation of NADPH oxidase.

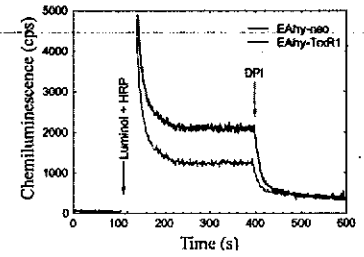

Fig. 2. HRP-catalyzed chemiluminescence from the cells overexpressing TrxR1 and their controls (expressing only neomycin resistance gene). HRP-luminol solution was added in cell suspension 100 s after the chemiluminescence was recorded. At 400 s, DPI, the NADPH oxidase inhibitor, was added into the mixture.

Regulation of the NADPH oxidase subunit p22phox expression by TrxR1. Increased ROS generation in TrxR1-overexpressing cells only suggests a possibility that thioredoxin reductase might upregulate the activity of NADPH oxidase. To know if and how thioredoxin reductase regulates NADPH oxidase activity, the protein level of the membrane subunit of NADPH oxidase, p22phox, in either TrxR1-overexpressing or TrxR1-knockdown cells was determined. Western blot analysis showed that thioredoxin redactase 1 upregulates the expression of the NADPH oxidase subunit (see Fig. 3). It was found that over-expression of TrxR1 led to upregulation, while knockdown of the endogenous TrxR1 down-regulated the

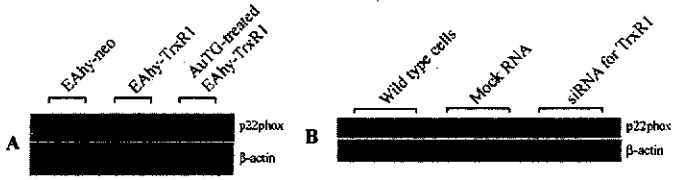

Fig. 3. Protein level of p22phox in TrxR1-overexpressing cells (A) and the cells having their endogenous TrxR1 knocked down (B). The cells transfected only with neomycin-resistance gene (EAhy-neo cells) and the cells transfected with a scramble RNA (Mock RNA) were used as control respectively.

expression of p22phox in the endothelial cells. It was interestingly noticed that the upregulation of p22phox in TrxR1-overexpressing cells can be abolished by incubation of the cells with inhibitor of thioredoxin reductase, gold thioglucose (AuTG). It suggests that the expression of p22phox is dependence of TrxR1 activity.

ROS generation in the p22phox-knockdown cells. To verify that the increased ROS generation in TrxR1-overexpressing cells is due to increased expression of p22phox, siRNA for p22phox was transfected into EAhy.926 cells to knock down endogenous p22phox, and to see if ROS generation is reduced. The results are shown in Fig. 4. It can be seen that knockdown of p22phox reduced intracellular ROS generation detected by chemiluminescence. Based on the observation that overexpression of TrxR1 upregulated p22phox expression and intracellular ROS generation as well as knockdown p22phox expression reduced ROS generation in cells, it is reasonable to suggest that thioredoxin reductase 1 upregulates cellular ROS generation by upregulation of, at least, expression of the NADPH oxidase subunit p22phox.

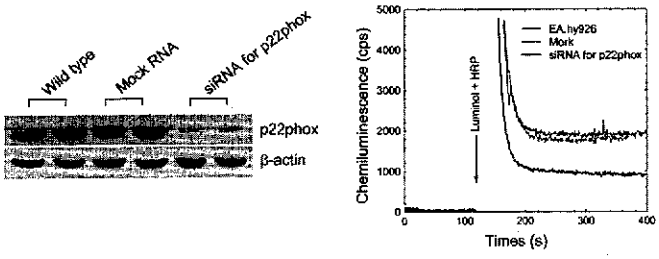

Fig. 4. Chemiluminescence detection of the ROS generated in the $p22^{phox}$-knockdown cells, the wild type cells and the cells transfected with mock RNA.

CONCLUSIONS

Thioredoxin reductase upregulates activity of NADPH oxidase by upregulating, at least, expression of its subunit p22phox. Enhanced chemiluminescence can be used as a sensitive method to detect intracellular ROS generation.

REFERENCES

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