Sodium trisulfide, a sulfane sulfur donor, stimulates bovine aortic endothelial cell proliferation in culture

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ABSTRACT — Reactive sulfur species (RSS) include biological persulfide molecules that protect cells against oxidative stress and heavy metal toxicity. Vascular endothelial cells regulate blood coagulation and fibrinolytic activity, and prevent vascular disorders such as atherosclerosis. We hypothesized that RSS protect vascular endothelial cells not only from nonspecific cell damage but also from specific functional damage through regulation of specific cell functions. In the present study, cultured bovine aortic endothelial cells were treated with sodium trisulfide, a sulfane sulfur donor, and both [3H]thymidine incorporation and effects on cell cycle were analyzed. These results suggest that RSS stimulate vascular endothelial cell proliferation. RSS may reduce the functional cytotoxicity of antiproliferative agents.

Key words: Reactive sulfur species, Endothelial cell, Proliferation

INTRODUCTION

Reactive sulfur species (RSS) are persulfide-containing biological molecules that include hydrogen sulfide, cysteine persulfide, glutathione persulfide, and other protein persulfides. RSS production is catalyzed by enzymes such as cystathionine γ-lyase (Ida et al., 2014), cystathionine β-synthase (Ida et al., 2014), 3-mercaptopyruvate sulfurtransferase (Módis et al., 2013), and cysteinyl-tRNA synthetase (Akaike et al., 2017). We report a copper complex, copper diethyldithiocarbamate, that induces transcription of cystathionine γ-lyase in vascular endothelial cells (Fujie et al., 2020), suggesting that RSS production is regulated in response to exposure to chemical compounds, and may protect cells from toxic compound exposure. RSS are known to reduce cadmium cytotoxicity in vascular endothelial cells (Shinkai et al., 2017).

Two types of cytotoxicity occur: nonspecific damage to biomolecules including proteins and DNA caused by cellular malignancies, and specific toxicities mediated by signaling pathways (Kanno, 2016). We hypothesized that RSS protect vascular endothelial cells from nonspecific cell damage, and specific pathway-mediated damage. Vascular endothelial cells cover the luminal surface of blood vessels in a monolayer and regulate the blood coagulation-fibrinolytic system. When the monolayer is severely or repeatedly damaged, vascular lesions, such as atherosclerosis, are initiated by the damage. Because proliferation is crucial to repair of damaged monolayers, organic/inorganic compounds that inhibit endothelial cell proliferation can act as risk factor for vascular lesions.

If RSS exert a stimulatory effect on endothelial cell proliferation, RSS may counter the proliferation inhibiting effects of toxic compounds. The purpose of the present study was to clarify RSS function in vascular endothelial cell proliferation.

MATERIALS AND METHODS

[3H]Thymidine incorporation assay

Bovine aortic endothelial cells (Cell Applications, San Diego, CA, USA) were cultured until confluent in a humid atmosphere containing 5% CO2 at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. These cells were seeded into 6-well plates at 1 × 10^4 cells/cm², and incu-
bated at 37°C for 24 hr in DMEM supplemented with 10% fetal bovine serum. While still sparse, cell cultures were treated with sodium trisulfide (20, 50, or 100 µM) (Dojindo, Kumamoto, Japan) in 12.5 mM HEPES buffer (pH 7.0) for 24 hr and labeled with 500 kBq/mL [methyl-\(^{3}\text{H}\)]thymidine (2.74 TBq/mmol, Moravek Biochemicals, Brea, CA, USA) for the last 3 hr of treatment. Incorporation of [\(^{3}\text{H}\)]thymidine into the 5% trichloroacetic acid-insoluble fraction of the cell homogenates was determined as described previously (Nakamura et al., 2020).

**Cell cycle analyses**

Vascular endothelial cells at 1 × 10⁴ cells/cm² in 100-mm dishes were treated with sodium trisulfide (5, 10, 20, 50, or 100 µM) for 24 hr. Cell suspensions were prepared with 0.25% trypsin-0.02% EDTA, fixed with 70% ethanol, and treated with 10 mg/mL RNase A (Sigma-Aldrich, St. Louis, MO, USA) in 50 mM Tris-HCl buffer containing 50% glycerol (pH 7.4) at 37°C for 20 min. After adding propidium iodide (50 µg/mL), cells were immediately subjected to flow cytometry (FACSCalibur Flow Cytometer, Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Data were analyzed using FlowJo software (FlowJo, Becton, Dickinson and Co.).

**Statistical analyses**

Data were analyzed for statistical significance using analysis of variance and Bonferroni-type multiple \(t\)-tests for multiple comparisons with Statcel3 (OMS, Tokyo, Japan) when possible. Statistical significance was set at \(p < 0.05\).

**RESULTS AND DISCUSSION**

Figure 1 shows the effect of sodium trisulfide on [\(^{3}\text{H}\)]thymidine incorporation into the acid-insoluble fraction of vascular endothelial cell lysates. [\(^{3}\text{H}\)]Thymidine incorporation was significantly increased by 24-hr incubation with sodium trisulfide at 50 and 100 µM in a concentration-dependent manner, suggesting that sodium trisulfide promotes endothelial cell DNA synthesis. These results suggest that RSS produced by sodium trisulfide stimulate vascular endothelial cell proliferation. Although RSS have multiple detoxifying functions, including protection against heavy metal cytotoxicity (Shinkai et al., 2017), oxidative stress (Ida et al., 2014), and 1-methyl-4-phenylpyridinium (Marutani et al., 2014), few reports indicate that RSS regulates specific cell functions such as proliferation.

To confirm the stimulatory effects of sodium trisulfide on vascular endothelial cell proliferation, the effect of treatment with sodium trisulfide on vascular endothelial cell distribution across cell cycle phases was analyzed by flow cytometry (Fig. 2, upper panels). Quantitative evaluation (Fig. 2, lower panels) revealed that the proportions of cells in G0/G1 and S phases were unchanged, while that of cells in G2/M phase was significantly increased in a concentration-dependent manner by sodium trisulfide, suggesting that RSS promote cell division just after stimulating DNA synthesis in vascular endothelial cells. The present data support the hypothesis that RSS stimulate vascular endothelial cell proliferation.

Regulation of vascular endothelial cell proliferation by RSS appears to be relevant from two viewpoints. Relevant to physiology, vascular endothelial cell proliferation is regulated by growth factors such as fibroblast growth factor-2 (FGF-2) (Sato and Rifkin, 1988) and transforming growth factor-\(\beta\) \(1\) (TGF-\(\beta\) \(1\)) (Fräter-Schröder et al., 1986). FGF-2 and TGF-\(\beta\) \(1\) exert stimulatory and inhibitory effects on proliferation, respectively. Intracellular RSS levels may contribute to regulation of endothelial cell proliferation by these growth factors. Relevant to toxicology, we have previously reported that lead, a toxic heavy metal, inhibits vascular endothelial cell proliferation without nonspecific cell damage (Kaji et al., 1995). Lead may decrease cellular RSS levels. RSS may in turn ameliorate negative effects of lead. Further studies on the regulation and misregulation of RSS and RSS-produc-
ing enzyme expression should be performed to understand the roles of RSS in physiological and toxicological responses of vascular endothelial cells to changes in their microenvironment.

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Conflict of interest—The authors declare that there is no conflict of interest.

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