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Nitric Oxide Production Correlates with Cell Death of Fibroblasts Treated by *Bacillus pumilus* Ribonuclease

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Abstract NO is a crucial messenger in tumor cell signaling. High levels of nitric oxide synthase expression may be cytostatic or cytotoxic for tumor cells, whereas low levels can have the opposite effect and promote tumor growth. Bioimaging is a major technique to visualize the nitric oxide level in living cells and to compare it with physiological outcomes. In this report, we used two fluorescent probes, DAA and DAF-FM diacetate, in order to visualize NO levels in normal and *ras*-transformed fibroblasts treated by the bacterial ribonuclease binase (*Bacillus pumilus* RNase). To assess selective toxicity of binase towards cells expressing the *ras* oncogene, a fluorescent live/dead dye was applied. Here we compared the NO levels in normal and *ras*-transformed fibroblasts to elucidate the role of NO in the apoptotic signaling cascade induced by binase.

Keywords Nitric oxide · Binase · Toxicity · *Ras*

1 Introduction

Nitric oxide and nitric oxide synthases are ubiquitous in malignant tumors and are known to exert pro- and anti-tumor effects [1]. With its rapid synthesis, high permeation and a short half-life, NO is a highly effective molecule with respect to local and transient signaling. NO activity is surprisingly long-termed and more potent in comparison with reactive oxygen species (ROS) [2]. New approaches for live-cell NO visualization were recently developed owing to the important roles of this radical in chemical industry, environmental ecology, and, especially, in biology [3].

Biological signaling through NO is suggested to be mediated by cyclic guanosine monophosphate (cGMP) synthesized by NO-activated guanylyl cyclases. cGMP operates through three main groups of cellular targets: cGMP-dependent protein kinases (PKGs), cGMP-gated cation channels, and cyclic nucleotide phosphodiesterases capable to degrade cGMP [4]. cGMP-binding activates PKG, which phosphorylates serines and threonines in many cellular proteins, and changes activity, functions, subcellular localization, and modulation of regulatory pathways. PKG-dependent regulation of calcium homeostasis and calcium sensitivity of cellular protein modulation is particularly important [4]. Thus, PKG promotes calcium-activated potassium channels opening which leads to cell's hyperpolarization and relaxation and blocks the agonistic activity of phospholipase C, reducing release of stored calcium ions by inositol triphosphate in smooth muscle tissue [5]. Another effect of NO/cGMP/PKG signaling includes PKG-mediated inactivation of the *ras* homolog gene family, member A (RhoA), in many biological processes [4].

Based on these data, we investigated some key molecules important for NO implementation in apoptotic processes, particularly cGMP, Ca^{2+}, and *ras* proteins and their connection with binase-induced apoptosis. Recently, we reported the triple-negative breast cancer BT-20 cell line, carrying a PI3K/AKT-activating mutation of PIK3CA, to be sensitive to apoptotic action of binase [6]. Binase also was shown to induce apoptosis in leukemic Kasumi cells [7], in lung adenocarcinoma cells A549 [8], and the ovarian cancer cell lines SKOV3 and OVCAR5 [9]. The selective cytotoxicity of binase towards cancer cells was partially determined by *ras* oncogene expression [10]. Moreover, binase directly interacts

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with the KRAS protein. This interaction interferes with guanine nucleotide exchange factor SOS1 function and stabilizes inactive GDP-bound conformation of *ras*, inhibiting thereby MAPK/ERK signaling [11].

Our previous research demonstrated exogenous RNases involvement in the regulation of Ca^{2+}-activated potassium channels [12]. By implementing an enzyme-linked immunosorbent assay, we found that during catalytic cleavage of RNA by binase cyclic intermediate 2′,3′-cGMP is maintained in reaction mixture for about 1 h. Activation of phosphodiesterases did not lead to a complete elimination of 2′,3′-cGMP [13]. Soluble guanylate cyclase, a critical component of key signaling pathways, is a hemoprotein activated through the specific interaction with NO [14]. Thus, inactivation of *ras* oncogene, increased cytoplasmic Ca^{2+} level, and production of positional isomer of 3′,5′-cGMP are involved in binase-induced cell death. This suggested the possible participation of NO in these processes.

Therefore, the questions whether NO participates in cell death induced by binase and how cytotoxic activity of binase correlates to NO production level remain unclear. Here, using the fluorescent dyes DAA and DAF-FM diacetate, we showed for the first time NO biosynthesis to be involved in binase-exerted cytotoxicity. Understanding NO role in tumor cell death will have profound therapeutic implications in diagnostics and treatment of this disease.

2 Methods

Fluorescent probes DAA and DAF-FM diacetate for NO detection and the live/dead viability/cytotoxicity kit were purchased from Sigma-Aldrich, Molecular Probes, and Invitrogen, correspondingly. DAPI for nuclei visualization was purchased from Sigma-Aldrich. Dulbecco's modified Eagle medium (DMEM), L-glutamine, and penicillin-streptomycin were from Invitrogen. Fetal calf serum was purchased from HyClone. Mouse NIH3T3 fibroblasts were purchased from ATCC, *ras*-transformed NIH3T3 fibroblasts were obtained by transfection with retroviral stock from v-Ha-*ras* transfected psi-2-cells [10].

NIH3T3 cells were cultured in DMEM supplemented with 1% L-glutamine, penicillin-streptomycin (100 U × mL^{-1} and 100 μg × mL^{-1}, respectively) and 10% fetal calf serum. The cells were grown at 37 °C in a 5% CO_2 atmosphere in Nunc flasks. Subcultures (1:3) were made every 3 days by dislodging the cells from the flask surface using a cell scraper.

Imaging with DAA was performed according to [15], imaging with DAF-FM diacetate and live/dead reagents and DAPI were performed according to manufacturer's recommendations with Leica DM 6000B fluorescence microscope. Data were evaluated for each variant for at least 30 fields of view.

To obtain the arbitrary intensity values, the fluorescence analysis data were calculated using Leica FW4000 software. All data analysis was performed in a double-blind fashion.

All data are expressed as the means ± standard deviations (SD). Student's *t* test was used to compare mean values between two groups. A P value of < 0.05 was considered statistically significant.

3 Results and Discussion

Although the fundamental roles of NO in biology are evident, many questions remain unsolved mainly due to limitations of NO detection methods available. Modern fluorescent probes provide valuable tools for intracellular NO level detection. Employing two different probes for NO-level detection indicated that binase either induced NO production in normal and *ras*-transformed cells or elevated the NO level by decreasing the cleaving activity of PDEs. Minimal NO level was detected in non-treated cells which did not undergo apoptosis (Figs. 1 and 2). After 72 h of binase treatment, cell death level was two times less in normal NIH3T3 fibroblasts than in *ras*-transformed cells (Fig. 1). The viability of NIH3T3 fibroblasts treated with binase was 98%, while the viability of *ras*-NIH3T3 after 72 h of incubation with RNase did not exceed 64%. These data corresponded with previously obtained results regarding fibroblasts viability [10]. On the contrary, the level of NO in non-treated *ras*-NIH3T3 was about 300% higher as compared to normal NIH3T3-cells. NO level in normal binase-treated cells was the same as in non-treated *ras*-NIH3T3 (Fig. 2). High-NO level in treated *ras*-NIH3T3 correlates with a markedly increased rate of cell death (Fig. 2).

On the contrary, the level of NO in non-treated ras-NIH3T3 was about 300% higher as compared to normal NIH3T3-cells, showing at basal conditions already values comparable to normal cells after treatment with RNase. Depending on a variety of factors, e.g., cell type, cellular redox status, flux, or local dose, NO is known to promote or suppress cell death [16, 17]. NO affects a variety of physiological processes, including cGMP-dependent and cGMP-independent apoptosis pathways. For example, NO inhibits platelet apoptosis through cGMP-dependent mechanisms inducing inhibition of phosphatidylserine externalization. Decrease in mitochondrial membrane potential inhibition was also cGMP-dependent at low-NO concentration but became cGMP-independent at high-NO concentrations [18]. Antiapoptotic actions of NO are numerous, ranging from an immediate interference with proapoptotic-signaling cascades to long-lasting effects based on expression of cell-protective proteins modulating the ability of reactive NO species to block caspases by S-nitrosylation or S-nitrosation [19]. On the other hand, it was established that endothelium-derived NO is a proapoptotic factor for vascular smooth muscle cells [20]. Mediated by NO, cGMP-dependent

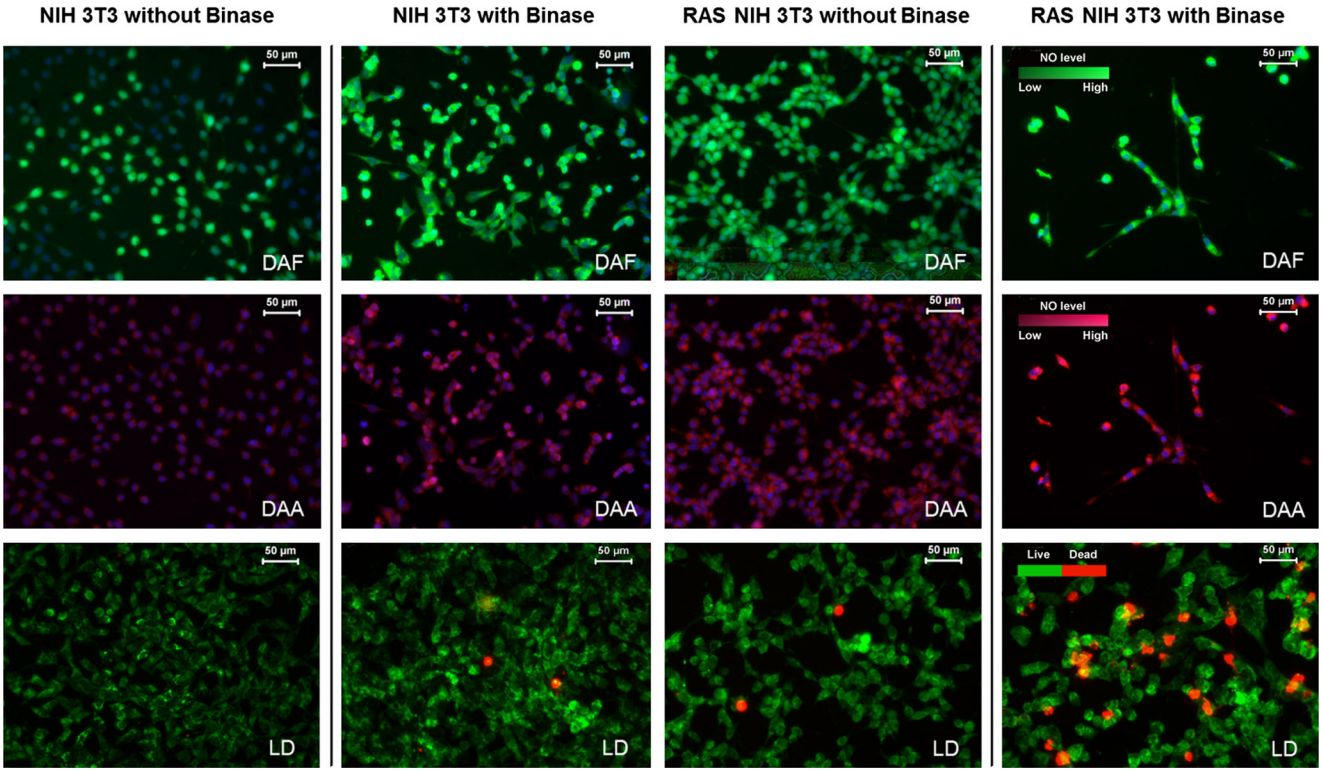

Fig. 1 Visualization of NO-production (DAF-FM diacetate, DAA) and viability (LIVE/DEAD Viability/Cytotoxicity Kit) of NIH 3T3 cells and *ras*-transformed NIH 3T3 cells after 72-h treatment with binase (500 µg/ml)

protein kinase PKG induces apoptosis in various cancer cell lines through phosphorylation of its substrate, death-associated protein kinase 2 (DAPK2), which is a Ca^{2+}/calmodulin-regulated serine/threonine kinase involved in modulating cellular calcium levels [21]. Proapoptotic pathways of NO are compatible with established signaling

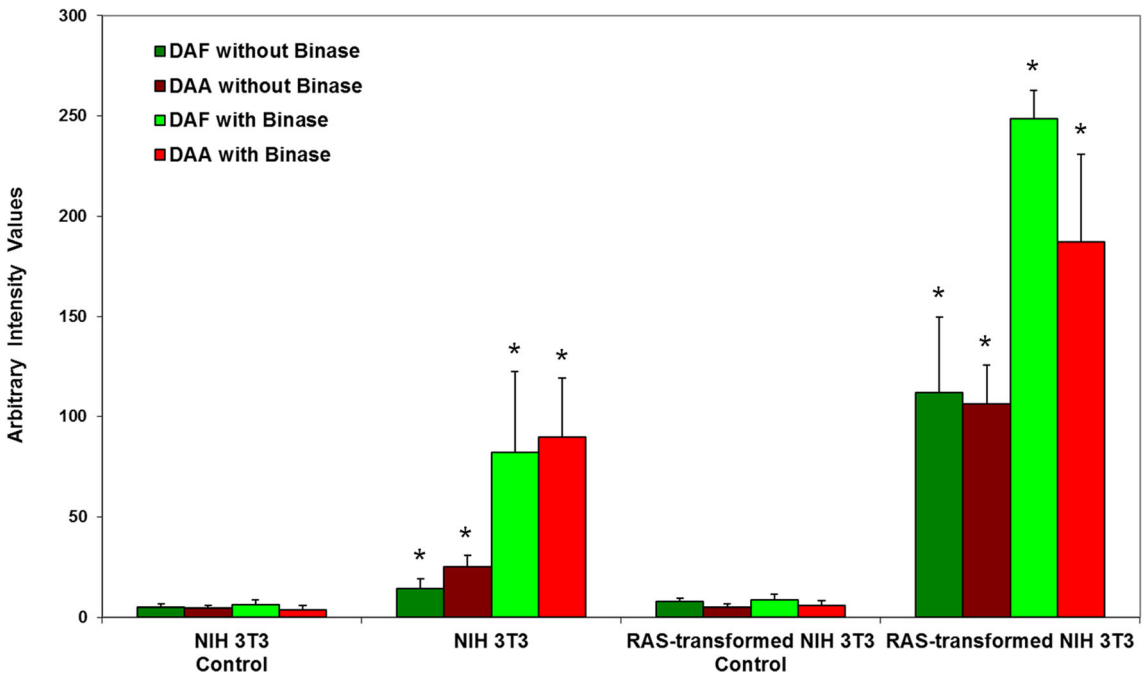

Fig. 2 NO measurement in NIH 3T3 fibroblasts and *ras*-transformed NIH 3T3 fibroblasts with and without binase (500 µg/ml) treatment for 72 h. Control represents the basal autofluorescence level (*$P < 0.05$)

pathways associated with mitochondria-dependent pathways of cell death, with some emphasis on the upregulating of the tumor suppressor p53 as a target during cell death execution [19]. Earlier, binase was found to induce increased expression of p53 mRNA [22]. We cannot distinguish the two death pathways under binase exposure, as binase induces apoptosis in a specific way that is characterized by cross-talk of genes involved in mitochondrial (intrinsic) and cell death receptors (extrinsic) apoptosis [7]. However, taking into account the participation of mitochondria-dependent pathways in binase-induced cell-death, increased NO biosynthesis in fibroblasts underdoing apoptosis was expected. Indeed, fluorescent microscopy results clearly indicated a positive correlation between NO production level and level of apoptosis with *ras*-transformed fibroblasts more susceptible to binase. Thus, we can conclude that cytotoxic ribonuclease binase influence intracellular NO level, affecting the cellular viability through regulation of apoptotic pathways.

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