Nitric oxide protects endothelial cells from tumor necrosis factor-α-mediated cytotoxicity: possible involvement of cyclic GMP

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Abstract In cultured endothelial cells, incubation with TNF-α (50 ng/ml) for 48 h markedly reduced viability of endothelial cells. A 6 h preincubation with Sper/NO (0.03–1 μM) protected endothelial cells in a concentration-dependent manner and increased viability by 63% of control. The NO scavenger PTIO (30 μM) completely abolished cytoprotection by Sper/NO. A cytoprotective effect comparable to Sper/NO was observed when preincubating the cells with 8-bromo cyclic GMP (1–10 μM). Moreover, no protection by Sper/NO occurred in the presence of ODQ (0.1 μM), a selective inhibitor of soluble guanylyl cyclase. Our results demonstrate that NO produces a long-term endothelial protection against cellular injury by TNF-α, presumably via a cyclic GMP-dependent pathway.

Key words: Nitric oxide; Endothelial cell; Cyclic GMP; Cytoprotection; Tumor necrosis factor-α

1. Introduction

Nitric oxide (NO) is known as a cytotoxic effector molecule produced by the immune surveillance system [1]. Also, under conditions of ischemia, NO has been implicated as a mediator of tissue injury, e.g. during neuronal destruction in vascular stroke [2]. However, recent studies suggest that NO at lower non-toxic levels may also function as a cytoprotective and antioxidant agent in different models of oxidative stress. Thus, Wink and co-workers [3] reported NO to prevent cellular damage induced by hydrogen peroxide in lung fibroblasts. Similar results were published by Motterlini et al. [4] who found that pretreatment with NO donors rendered the vascular endothelium more resistant against the toxic effects of oxygen-free radicals, i.e. exogenously applied hydrogen peroxide. Possible NO-dependent cytoprotective mechanisms include direct neutralization of superoxide radicals [5] or activation of an antioxidant defense system such as heme oxygenase or ferritin [4,6,7].

Whereas the studies mentioned above demonstrate protective effects of NO against exogenous sources of oxygen radicals, little is known about the ability of NO to antagonize cytotoxicity induced by endogenous formation of oxygen radicals, e.g. in response to receptor-dependent agonists. Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine and mediator of septic shock with a direct toxic effect on the endothelium resulting in endothelial lesions which may favor thrombus formation, atherogenesis and vasodilation via induction of NO synthesis in the underlying smooth muscle cells [8,9]. This direct cytotoxicity of TNF-α has been attributed, at least in part, to the formation of reactive oxygen species [10–12]. Based on these observations and using the NO releasing agent spermine NONOate (Sper/NO), the present study addresses whether NO is capable of reducing cytotoxicity by TNF-α in endothelial cells.

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Abbreviations: NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one; PTIO, 2-Phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide; Sper/NO, spermine NONOate; TNF-α, tumor necrosis factor-α

2. Materials and methods

2.1. Materials

Bovine pulmonary artery endothelial cells (ATCC CCL 209) were obtained from the American Type Culture Collection, Rockville, MD, USA. Fetal bovine serum, Dulbecco's modified Eagle medium and penicillin-streptomycin were obtained from Gibco, Eggenstein, Germany. TNF-α was a gift of Knoll AG, Ludwigshafen, Germany. Sper/NO, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) and 2-Phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) were from Alexis Deutschland, Grünberg, Germany. Crystal violet and all other chemicals were bought from Sigma, Deisenhofen, Germany.

2.2. Cell culture

Endothelial cells were maintained and subcultured in Dulbecco's modified Eagle medium supplemented with 15% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin [11]. The cells were grown in a humidified incubator at 37°C and 5% CO_2.

2.3. Incubation procedure

Endothelial cells were seeded at 2×10^4 cells/well in 96 well microtiter plates in 100 μl of media containing 15% fetal bovine serum. After a 48 h incubation cells reached confluence and Sper/NO or 8-bromo cyclic GMP was added. PTIO and ODQ were added 10 min prior to Sper/NO. After an additional 6 h incubation TNF-α was added to the cells. Incubation was continued for 72 h, followed by a cytotoxicity assay.

2.4. TNF-α cytotoxicity assay

Cell viability was measured by staining with crystal violet as previously described [5,11,13]. After washing with phosphate buffered saline, cells were fixed with methanol for 5 min and then stained for 10 min with a 0.1% crystal violet solution. Following three washes with tap water, the dye was eluted with 0.1 mol/l trisodium citrate in 50% ethanol for 1 h. Optical density at 630 nm was measured using a microtiter plate reader.

3. Results

In endothelial cells, treatment with TNF-α (50 ng/ml) markedly reduced the number of viable cells (Fig. 1). A 6 h preincubation with Sper/NO (0.03–1 μM) protected endothelial cells from TNF-α-mediated cytotoxicity in a concentration-dependent manner and increased viability by up to 63% of control (Fig. 1). The specific NO scavenger PTIO (30 μM) completely abolished cytoprotection by Sper/NO (Fig. 2). A cytoprotective effect comparable to Sper/NO was observed

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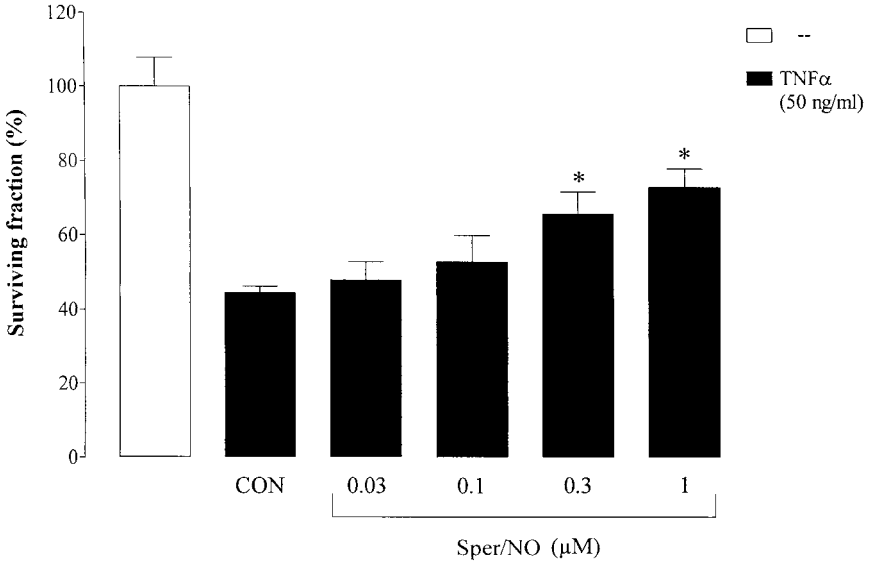

Fig. 1. Effect of Sper/NO on TNF-α-mediated cytotoxicity in endothelial cells. Incubations were carried out as described in Section 2. *: $P < 0.05$, Sper/NO vs. control (CON), two-tailed t-test. All data shown are mean ± SEM of $n = 6$ observations.

when preincubating the cells with 8-bromo cyclic GMP (1–10 μM, Fig. 3). Moreover, no protection by Sper/NO occurred in the presence of ODQ (0.1 μM), a selective inhibitor of the NO-sensitive soluble guanylyl cyclase (Fig. 2). Sper/NO, PTIO, ODQ, and 8-bromo cyclic GMP alone had no significant effect on cell viability under these conditions (not shown).

4. Discussion

The present study demonstrates protection from TNF-α-mediated toxicity in endothelial cells by the NO donor Sper/NO. The observed protection can be attributed to the free NO radical since Sper/NO-induced cytoprotection was completely abolished in the presence of the NO radical scavenger PTIO [14]. Moreover and as an additional control, NO-free spermine tetrahydrochloride did not reduce TNF-α-dependent endothelial toxicity (not shown). Antioxidant and cytoprotective effects of NO have been reported before, particularly with respect to exogenous sources of oxygen radicals such as hydrogen peroxide [4,5]. This is one of the first studies to show that NO reduces endothelial injury by TNF-α and may thus interfere with initiating events of inflammation and atherosclerosis.

We and others have previously shown that generation of oxygen radicals plays a crucial role in endothelial cell killing by TNF-α [10–12]. It is therefore conceivable that the known antioxidant effect of NO contributes to the increased resistance of endothelial cells against TNF-α-mediated toxicity. However, a direct quenching of superoxide radicals as under-

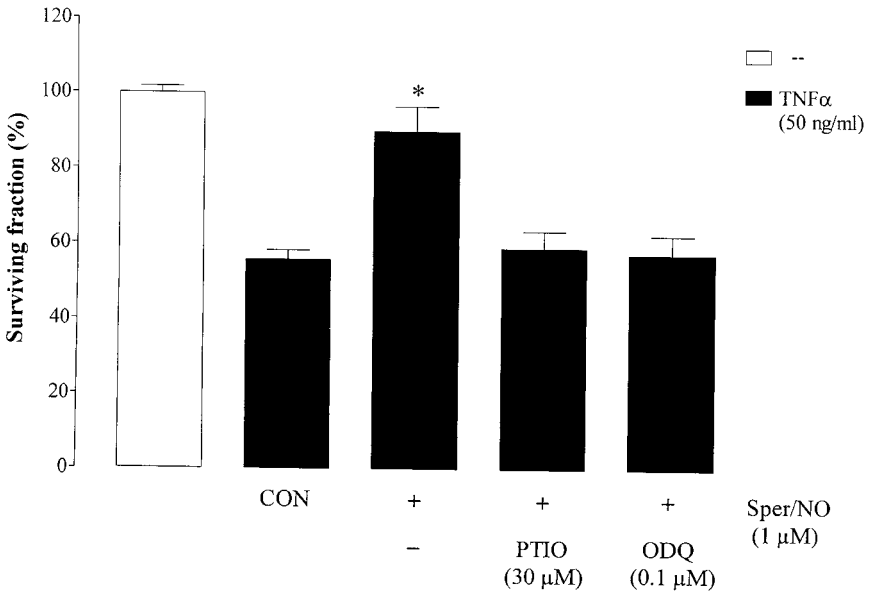

Fig. 2. Effect of PTIO and ODQ on Sper/NO-induced cytoprotection from TNF-α-mediated toxicity in endothelial cells. Incubations were carried out as described in Section 2. *: $P < 0.05$, treatment vs. control (CON), two-tailed t-test. All data shown are mean ± SEM of $n = 6$ observations.

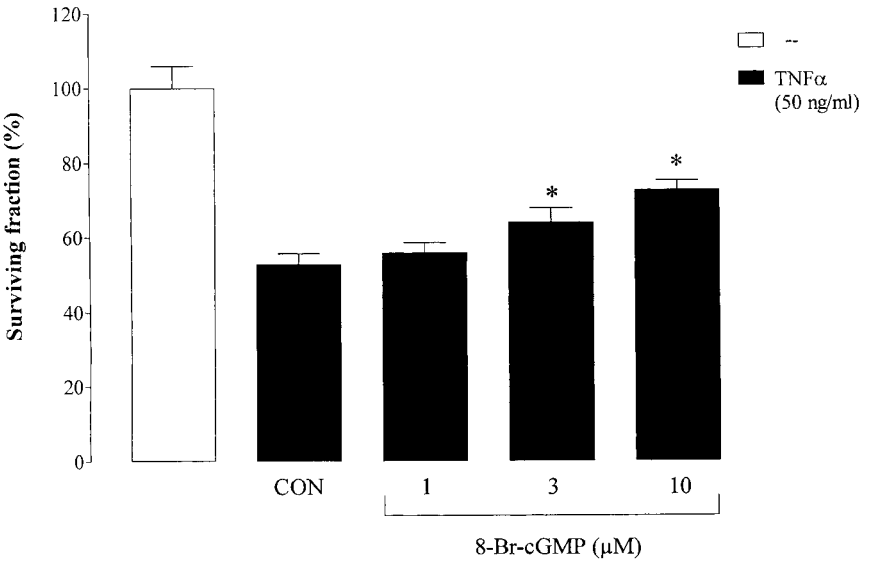

Fig. 3. Effect of 8-bromo cyclic GMP (8-Br-cGMP) on TNF-α-mediated cytotoxicity in endothelial cells. Incubations were carried out as described in Section 2. *: $P < 0.05$, 8-Br-cGMP vs. control (CON), two-tailed t-test. All data shown are mean ± SEM of $n = 6$ observations.

lying mechanism seems unlikely, since the endothelial protection was fully expressed only after 6 h of pretreatment with Sper/NO prior to the addition of TNF-α. Our data rather suggest NO to trigger long-term signal transduction processes which may lead to the up-regulation of protective proteins such as heme oxygenase, ferritin, or HSP70 [6,7,15]. This assumption is strongly supported by our finding that ODQ, a selective inhibitor of soluble guanylyl cyclase [16], abolished the endothelial protection by Sper/NO suggesting the second messenger cyclic GMP as responsible mediator. A signaling function for cyclic GMP can also be derived from our observation that the membrane penetrating analog 8-bromo cyclic GMP mimicked the cytoprotective action of Sper/NO. Thus, cyclic GMP-dependent pathways could represent alternative routes by which NO in addition to direct nitrosylation of protein thiols [15,17] modulates cellular adaptation to stress caused by TNF-α and other cytotoxic agents.

Together, our results show that NO reduces endothelial injury by TNF-α, possibly via a cyclic GMP-dependent mechanism. These observations underline the importance of NO in maintaining vascular wall integrity and point to the antiinflammatory/antiatherogenic potential of NO.

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