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Nitric oxide at a low concentration protects murine macrophage RAW264 cells against nitric oxide-induced death *via* cGMP signaling pathway

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1 We investigated the cytoprotective effect of low-dose nitric oxide (NO) on NO-induced cell death in mouse macrophage-like cell line RAW264.

2 Sodium nitroprusside (SNP), an NO donor, at a high concentration (4 mM) released cytochrome *c* from mitochondria and induced death in RAW264 cells. Acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspart-1-al (Ac-DEVD-CHO, 100 – 200 μM), a caspase-3 inhibitor, attenuated the SNP-induced cell death in a concentration-dependent manner.

3 Pretreatment with 100 μM SNP for 24 h, which had no effect on cell viability, attenuated the cell death and reduced cytochrome *c* release from mitochondria to the cytosol induced by 4 mM SNP.

4 LY83583 (1 – 3 μM) and 1*H*-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, 30 – 100 μM), soluble guanylate cyclase inhibitors, negated the protective effect of the 100 μM SNP pretreatment.

5 Pretreatment with 1 mM dibutylyl guanosine-3′,5′-cyclic monophosphate (DBcGMP), a cell-permeable guanosine-3′,5′-cyclic monophosphate (cGMP) analogue, for 24 h inhibited both cytochrome *c* release and cell death induced by SNP.

6 Protein kinase G inhibitor KT5823 (10 μM) significantly reduced the cytoprotective effects of low-dose SNP and DBcGMP.

7 These results indicate that low-dose NO protects RAW264 cells from NO-induced apoptosis through cGMP production and activation of protein kinase G.
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Abbreviations: Ac-DEVD-CHO, acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspart-1-al; cGMP, guanosine-3′,5′-cyclic monophosphate; DBcGMP, dibutylyl cGMP; ERK, extracellular signaling-regulated kinase; GSNO, *S*-nitrosoglutathione; MAP kinase, mitogen-activated protein kinase; MTT, 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide; NOC18, 1-hydroxy-2-oxo-3,3-bis-(2-aminoethyl)-1-triazene; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; TBS, Tris-buffered saline

Introduction

Nitric oxide (NO), a radical produced in mammalian cells from arginine in a reaction catalyzed by NO synthase (Moncada & Higgs, 1993), has been implicated in a variety of biological phenomena, such as vasodilation, platelet aggregation, synaptic transmission and cytotoxicity (Moilanen & Vapaatalo, 1995; Wang & Robinson, 1997; Pfeifer *et al*., 1998; Riddell & Owen, 1999). Most of these physiological events are exerted by an increase in the guanosine-3′,5′-cyclic monophosphate (cGMP) concentration *via* activation of soluble guanylate cyclase (sGC). Recent data indicate that endogenously generated or exogenously supplied NO induces characteristic apoptotic features, that is, chromatin condensation and DNA fragmentation. NO-induced apoptosis occurs in β cells of the pancreas (Ankarcrona *et al*., 1994; Kaneto *et al*., 1995), thymocytes (Fehsel *et al*., 1995; Sandau & Brune, 1996), hepatocytes (Kim *et al*., 1995), macrophages (von Knethen *et al*., 1999) and several other cells (Suenobu *et al*., 1999; Vincent & Maiese, 1999; Battinelli & Loscalzo, 2000; Taimor *et al*., 2000). NO inhibits mitochondrial respiration and disrupts energy metabolism and calcium homeostasis (Brown, 1999). In addition, NO modulates the mitochondrial permeability transition, a process that is linked to the release of apoptogenic factors such as cytochrome *c* (Brookes *et al*., 2000). The release of cytochrome *c* constitutes an important step in the activation of a specific subgroup, CPP32, of caspases, and thus promotes apoptosis (Nijihawan *et al*., 1997). On the other hand, an antiapoptotic role of NO in some cultured cells has also been demonstrated. NO at a low nontoxic concentration induces resistance to TNF-α-induced hepatotoxicity (Kim *et al*., 1997), inhibits Fas-induced apoptosis in B lymphocytes (Mannick *et al*., 1997), and modulates CD95-induced apoptosis in T lymphocytes (Sciorati *et al*., 1997).

Macrophages play a significant role in the host defense mechanism. When activated, they produce large amounts of NO that inhibit the growth of a wide variety of tumor cells and microorganisms (MacMicking *et al*., 1997). Since this NO

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production is also toxic for the macrophages themselves (Albina *et al*., 1993), they must have self-defense mechanisms against NO toxicity. Sensitivity of cells to NO toxicity seems to differ among cell types. For example, pancreatic beta cells and thymocytes are very susceptible to NO toxicity (Kroncke *et al*., 1993; Fehsel *et al*., 1995; Kaneto *et al*., 1995), but hepatocyte and mesangial cells are less susceptible to it (Kim *et al*., 1995; Muhl *et al*., 1996), suggesting the presence of protective mechanisms in the latter cell types. Their self-defense mechanisms may depend on antiapoptotic effects of NO. Actually, von Knethen *et al*. (1999) indicated that *S*-nitrosoglutathione (GSNO), an NO donor, at a low concentration inhibited apoptosis induced by high-dose GSNO in macrophages. The mechanism of the antiapoptotic effect of NO, however, is largely unknown. In this study, we investigated the protective effect of NO at a low concentration on NO-induced cell death in the mouse macrophage cell line RAW264.

Methods

Cell culture and treatment with drugs

Clonal murine macrophage-like RAW264 cells were cultured as described previously (Fujimori *et al*., 2001). Briefly, the cells were grown in Dulbecco's modified Eagle's medium (Sigma, St Louis, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL, Gaithersburg, U.S.A.) containing 100 μg ml^{-1} streptomycin, 100 IU ml^{-1} penicillin, and 1 μg ml^{-1} fungizone in a humidified atmosphere of 95% air – 5% CO_2 at 37°C. The cells were plated at a density of 2×10^4/well on 96-well tissue culture plates for viability experiments or 2×10^6 on 100-mm-diameter dishes for Western blotting experiments. LY83583 (Calbiochem, La Jolla, CA, U.S.A.), 1*H*-[1,2,4]oxadiazolo[4,3,-a]quinoxalin-1-one (ODQ, Calbiochem), PD98059 (Calbiochem), SB203580 (Wako Chemicals, Osaka, Japan), wortmannin (Sigma), acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspart-1-al (Ac-DEVD-CHO) (Peptide Institute, Osaka, Japan) or KT5823 (Calbiochem) was added 30 min before exposure to sodium nitroprusside (SNP) or dibutylyl guanosine-3′,5′-cyclic monophosphate (DBcGMP) (Sigma). All other reagents were of the highest grade of purity and were commercially available.

Cell viability

Cell viability was determined by a colorimetric 3,4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983) or LIVE/DEAD viability/cytotoxicity kit (Molecular Probe, Inc., Eugene, OR, U.S.A.) as described previously (Kanesaki *et al*., 1999; Koyama *et al*., 2000). Briefly, for MTT assay, cells were incubated with MTT (Nakarai Tesque, Kyoto, Japan) solution for 2 – 4 h. Then the medium was discarded, and the resulting dye was dissolved in dimethyl sulfoxide, and thereafter the absorbance was measured at 570 nm. MTT reduction was expressed as a percentage of that of the untreated control. When the LIVE/DEAD viability/cytotoxicity kit was used, cells were incubated for 30 min with calcein AM and ethidium homodimer. Then the numbers of both green and red fluorescent cells were counted under a fluorescence microscope.

Hoechst 33258 staining

Chromosomal condensation and DNA fragmentation were determined by using the chromatin dye Hoechst 33258. Cells were harvested and fixed with 1% glutaraldehyde for 30 min at 4°C. Then the cells were washed with phosphate-buffered saline (PBS) and stained with 100 μM Hoechst 33258/PBS as described previously (Koyama *et al*., 2000).

Western blot analysis

To prepare whole-cell lysate, we lysed cells with a buffer consisting of 1% nonidet p-40, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 150 mM sodium chloride, 5 mM sodium ethylenediaminetetraacetic acid, and 0.1 mM PMSF. Preparation of cytosolic S-100 fraction was performed as described by Hiura *et al*. (2000). In brief, RAW264 cells were lysed by three cycles of freeze – thawing in another lysis buffer (20 mM HEPES/KOH, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM sodium ethylenediaminetetraacetic acid, 1 mM DTT, 0.1 mM PMSF, 250 mM sucrose; pH 7.5). The cell lysates were then centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was collected, and the protein concentration was measured with a BCA protein assay kit (Pierce, Rochford, IL, U.S.A.). Aliquots of whole-cell lysate and the supernatant (30 μg protein) were separated by 15% SDS – PAGE and transferred onto Immobilon-P membranes (Millipore Corp., Waters Chromatography, Milford, MA, U.S.A.). The membranes were rinsed three times with Tris-buffered saline (TBS) and blocked for 30 min with 5% nonfat dry milk/TBS/0.1% Tween-20. The membranes were then incubated for 2 h at room temperature with a monoclonal antibody specific for cytochrome *c* (1 : 200 dilution, PharMingen, San Diego, CA, U.S.A.). Next, the membrane was rinsed five times with TBS/0.1% Tween-20, and incubated for 1 h with a horseradish peroxidase-conjugated anti-mouse IgG antibody (Cappel, Durham, NC, U.S.A.). Signals were detected with a chemiluminescence detection kit (NEN, Boston, MA, U.S.A.).

Statistical evaluation

The results were expressed as means ± s.e. for five – nine wells obtained from three – five independent experiments. One-way ANOVA and two-way ANOVA were used to test for differences between group means. When appropriate, *post hoc* multiple comparisons were performed to test for differences between experimental groups (Scheffe test). When the *P*-value was less than 0.05, the difference was considered to be significant.

Results

We estimated SNP-induced damage to RAW264 cells by using the MTT assay and LIVE/DEAD viability/cytotoxicity kit. Treatment of the cells with 4 mM SNP for 12 h caused loss of viability. A caspase-3 inhibitor, Ac-DEVD-CHO, at concentrations of 100 and 200 μM attenuated this decrease in the cell viability (Figure 1). Pretreatment of the cells with 100 μM SNP for 24 h before their exposure to 4 mM SNP significantly reduced the cell death (Figure 2). This protective effect of SNP pretreatment was concentration-dependent (1 – 100 μM), but

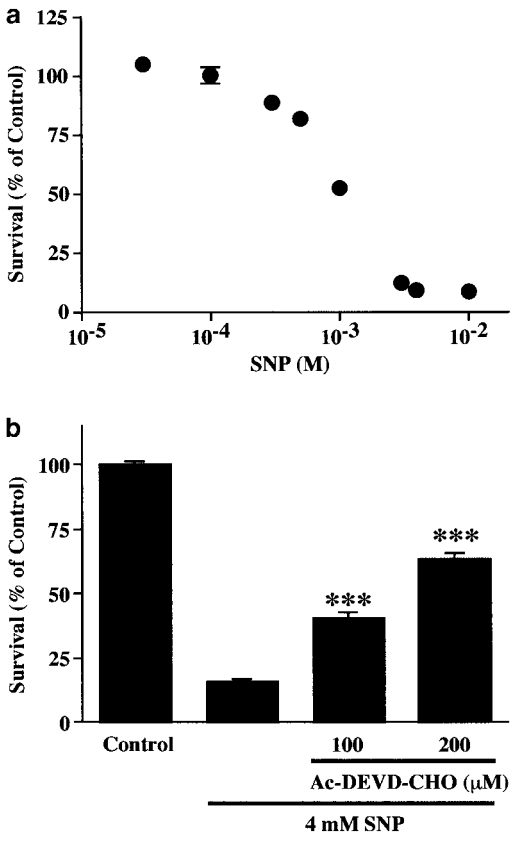

Figure 1 Effect of a caspase-3 inhibitor, Ac-DEVD-CHO, on cell death induced by 4 mM SNP in RAW264 cells. (a) Cells were treated with various concentration of SNP for 24 h. Cell viability was determined by the colorimetric MTT assay. Results show the mean ± s.e. for nine wells and were obtained from three independent experiments. (b) Cells were treated with 4 mM SNP in the absence or presence of Ac-DEVD-CHO for 12 h. Cell viability was determined by the colorimetric MTT assay. Results show the mean ± s.e. for nine wells and were obtained from three independent experiments. $***P<0.001$, significantly different from the values for cells without Ac-DEVD-CHO (Scheffe test).

no further protective effect was detected at concentrations higher than 300 μM (Figure 3). Furthermore, the pretreatment with low-dose SNP inhibited high-dose SNP-induced nuclear condensation (Figure 4).

Next, we examined the effects of sGC inhibitors on the protection against cell death by low-dose SNP. Both LY83583 (1 – 3 μM) and ODQ (30 – 100 μM) reduced the protective effect of low-dose SNP (Figure 5). DBcGMP (1 mM), a cell-permeable cGMP analogue, attenuated cell death induced by various NO donors, for example, SNP (4 mM), GSNO (3 mM) and 1-hydroxy-2-oxo-3,3-bis-(2-aminoethyl)-1-triazene (NOC18, 3 mM; Figure 6). It is well known that cytochrome *c* released from mitochondria into the cytosol can induce caspase-3 activation followed by apoptosis. To investigate effects of low-dose NO and cGMP on cytochrome *c* release induced by high-dose SNP, we measured the cytosolic and total levels of cytochrome *c*, using anti-cytochrome *c* antibody in the Western blotting procedure. The cytosolic level of cytochrome was increased by 4 mM SNP, and this increase was reduced by pretreatment of the cells with 100 μM SNP. The sGC inhibitor LY83583 (3 μM) markedly blocked the effect of 100 μM SNP pretreatment on the cytochrome *c* release (Figure 7a). DBcGMP (1 mM) diminished the cytochrome *c* release induced by NO donors (Figure 7b). Total cytochrome *c* levels were not affected by these NO donors, and cytochrome *c* levels in the cytosol after the treatment with SNP, GSNO, and NOC18, were 66.0 ± 6.6%, 67.8 ± 2.0% and 68.3 ± 8.3% of the total cytochrome *c*, respectively.

Finally, we examined the effect of a protein kinase G inhibitor, KT5823, on the protection against cell death by low-dose SNP or DBcGMP. KT5823 (10 μM) significantly reduced the protective effect of low-dose SNP (Figure 8), and also inhibited the effect of DBcGMP. In contrast, the mitogen-activated protein kinase (MAP kinase)/extracellular signaling-regulated kinase (ERK) kinase inhibitor PD98059 (50 μM), the p38 MAP kinase inhibitor SB203580 (10 μM), and the phosphatidylinositol 3 (PI 3)-kinase inhibitor wortmannin

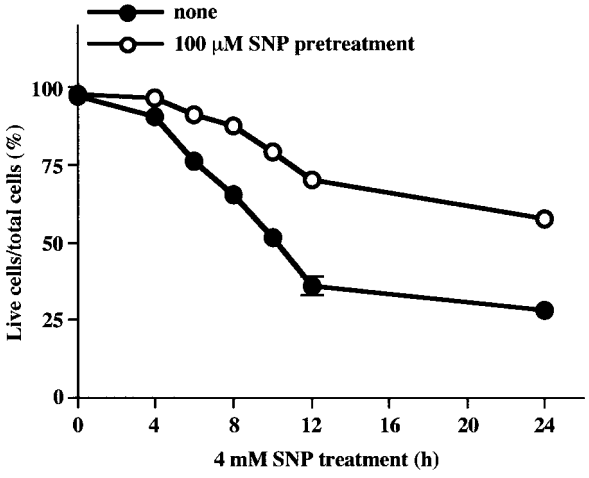

Figure 2 Effect of SNP at a low concentration on cell death induced by 4 mM SNP in RAW264 cells. Cells were treated with 100 μM SNP for 24 h. Then the cells were treated with 4 mM SNP for various times (0 – 24 h). Cell viability was determined with a LIVE/DEAD viability/cytotoxicity kit as described in Methods. Results show the mean ± s.e. for eight wells and were obtained from four independent experiments.

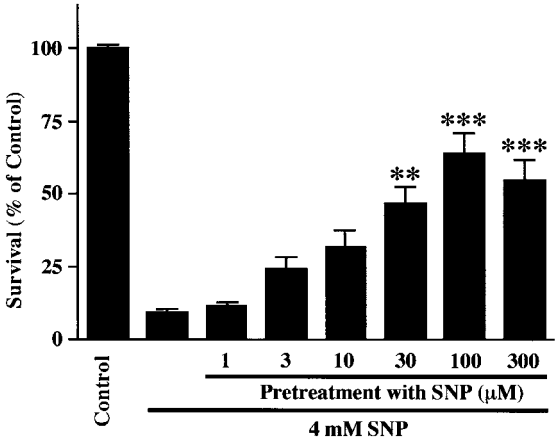

Figure 3 Concentration – survival relation for the effects of SNP pretreatment on SNP-induced cell death in RAW 264 cells. Cells were treated with the indicated concentrations of SNP for 24 h. Then the cells were treated with 4 mM SNP for 24 h. Cell viability was determined by using the colorimetric MTT assay. Results show the mean ± s.e. for nine wells and were obtained from three independent experiments. $**P<0.01$, $***P<0.001$, significantly different from values without SNP pretreatment (Scheffe test).

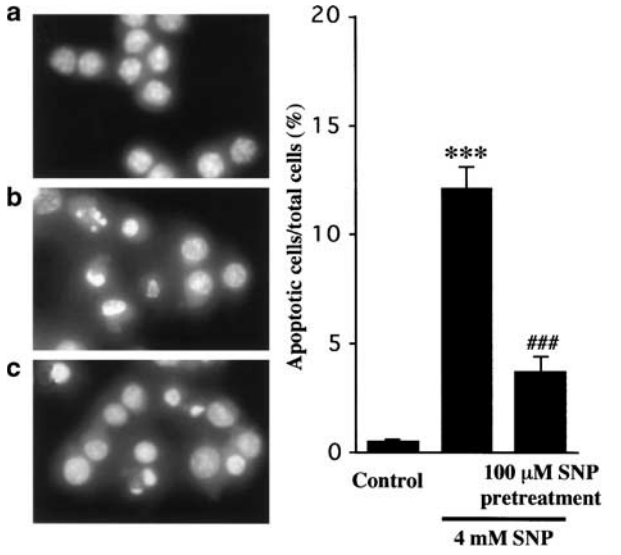

Figure 4 Effect of SNP pretreatment on apoptosis induced by 4 mM SNP in RAW264 cells. Cells were incubated with (c) or without 100 μM SNP (a, b) for 24 h. Then they were treated for 8 h with 4 mM SNP (b, c). The cells were fixed and stained with Hoechst 33258 as described in Methods (left panel). The results are representative of three independent experiments. Apoptotic cells exhibiting characteristic chromatin condensation were counted by fluorescence microscopy (right panel). Results show the mean $\pm$ s.e. of five different experiments. $^{***}P<0.001$, significantly different from the control, $^{\#\#\#}P<0.05$, significantly different from 4 mM SNP (Scheffe test).

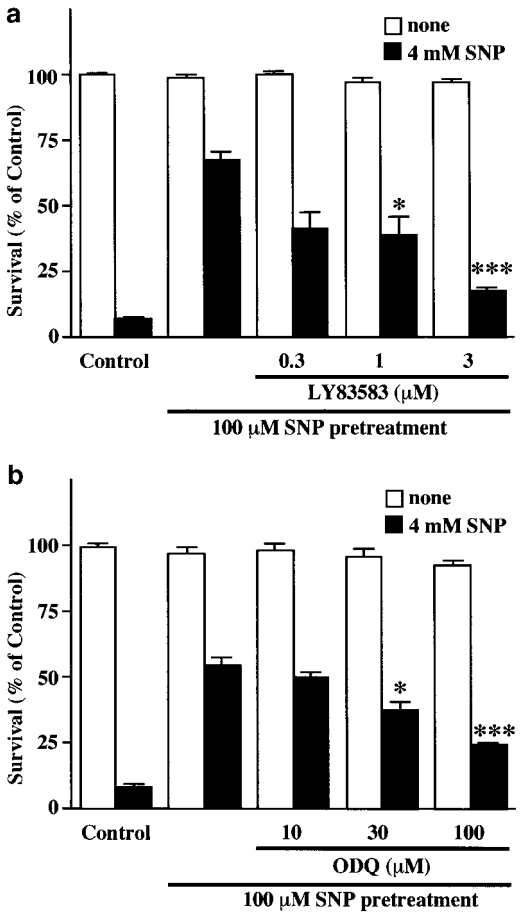

Figure 5 Effects of LY83583 and ODQ, sGC inhibitors, on the protection from cell death by SNP pretreatment of RAW264 cells. Cells were treated with 100 μM SNP for 24 h in the absence or presence of LY83583 (a) or ODQ (b). Then they were incubated with (solid columns) or without (open columns) 4 mM SNP for 24 h. Cell viability was determined by using the colorimetric MTT assay. Results show the mean $\pm$ s.e. for nine wells and were obtained from three independent experiments. $^{*}P<0.05$, $^{***}P<0.001$, significantly different from 100 μM SNP pretreatment + 4 mM SNP (Scheffe test).

(200 nM) did not affect the cytoprotective effect of low-dose SNP (Figure 9).

Discussion and conclusions

The major findings of the present study are that NO at a low concentration protects RAW264 cells against a toxic concentration of NO inducing apoptosis through cGMP production and activation of protein kinase G.

In rat mesangial cells and RAW264.7 cells, it was reported that a high concentration of NO induced both apoptosis and necrosis (Messmer & Brune, 1996; Sandau *et al*., 1997). In the present study, high-dose SNP induced cell death and cytochrome *c* release in RAW264 cells. The caspase-3 inhibitor Ac-DEVD-CHO, however, only partially inhibited the high-dose SNP-induced cell death, indicating that the cell death induced by SNP may include both apoptosis and necrosis. In endothelial cells and cardiomyocytes, NO-induced cell death was shown to be mediated through cGMP production (Suenobu *et al*., 1999; Taimor *et al*., 2000). We observed that LY83583 did not inhibit SNP-induced cell death and that DBcGMP did not induce cell death even at a concentration higher than 1 mM (data not shown). These results suggest that NO-induced cell death does not depend on cGMP production in RAW264 cells.

The pretreatment with SNP at a low concentration inhibited cytochrome *c* release, nuclear condensation, and fragmentation induced by high-dose SNP, suggesting that low-dose SNP inhibited the high-dose SNP-induced apoptosis in RAW264 cells. Pretreatment of the cells with potassium ferrocyanide or potassium ferricyanide, which are compounds structurally similar to SNP but devoid of NO, did not affect SNP-induced apoptosis (data not shown). This observation indicates that the cytoprotective effect of low-dose SNP is mediated through NO production. In some cells, NO prevents apoptosis *via* cGMP-dependent mechanisms (Beauvais *et al*., 1995; Genaro *et al*., 1995). In other cell lines, however, the antiapoptotic mechanism of NO is independent of cGMP (Mannick *et al*., 1994, 1997; Sata *et al*., 2000). In the present study, both LY83583 and ODQ inhibited the cytoprotective effect of low-dose SNP, and DBcGMP attenuated the cell death and cytochrome *c* release induced by NO donors. These results indicate that low-dose NO protects RAW264 cells against NO-induced death mostly through a cGMP-dependent mechanism.

The present study showed that high-dose NO-induced cytochrome *c* release was reduced by DBcGMP pretreatment and that a protein kinase G inhibitor significantly inhibited the effects of low-dose SNP or DBcGMP. These results suggest that NO at a low concentration protects RAW264 cells against NO-induced death partly due to inhibition of cytochrome *c* release by activation of protein kinase G. The molecular mechanism by which protein kinase G inhibits cytochrome *c*

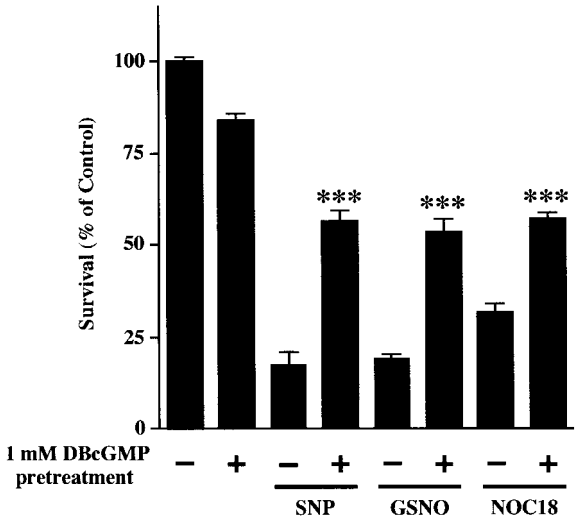

Figure 6 Effect of pretreatment with DBcGMP on death of RAW264 cells induced by NO donors. Cells were treated with 1 mM DBcGMP for 24 h. Then they were treated with 4 mM SNP, 3 mM GSNO, or 3 mM NOC18 for 24 h. Cell viability was determined by using the colorimetric MTT assay. Results show the mean ± s.e. for nine wells and were obtained from three independent experiments. ***$P<0.001$, significantly different from the values for cells without DBcGMP pretreatment (Scheffe test).

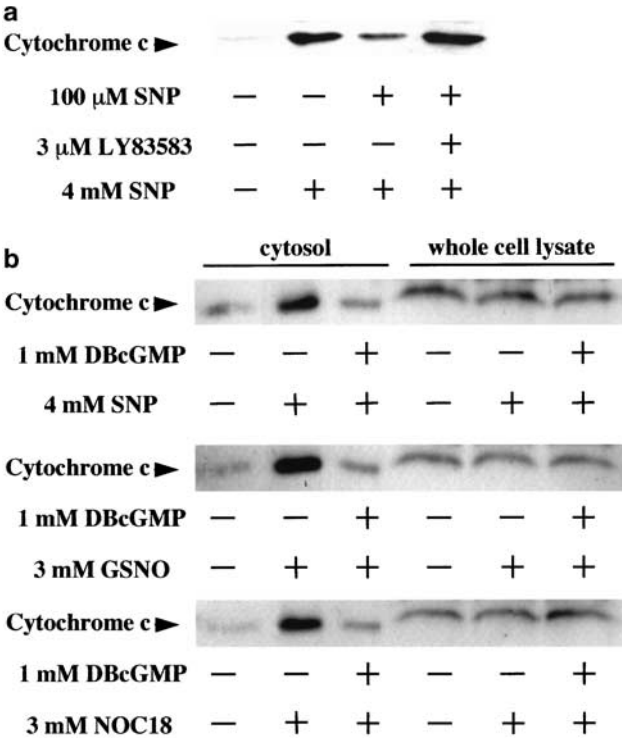

Figure 7 Western blot analysis of cytochrome *c* release. (a) RAW264 cells were treated with 100 μM SNP in the absence or presence of 3 μM LY83583 for 24 h. Then they were treated with 4 mM SNP for 4 h. After the cells had been lysed in a hypotonic buffer, the S-100 fraction was collected and subjected to Western blotting as described in Methods. (b) RAW264 cells were treated with 1 mM DBcGMP for 24 h. Then they were treated with 4 mM SNP, 3 mM GSNO or 3 mM NOC18 for 4 h. Cytosolic S-100 fraction and whole-cell lysate were subjected to Western blot analysis. Results are representative of three independent experiments.

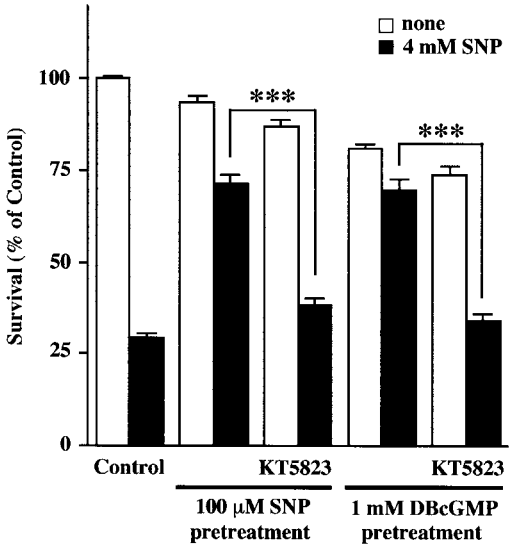

Figure 8 Effect of KT5823, a protein kinase G inhibitor, on the protection from death of RAW264 cells by SNP or DBcGMP pretreatment. Cells were treated with 100 μM SNP or 1 mM DBcGMP for 24 h in the absence or presence of KT5823 (10 μM). Then they were incubated with (solid columns) or without (open columns) 4 mM SNP for 24 h. Cell viability was determined by using the colorimetric MTT assay. Results show the mean ± s.e. for nine wells and were obtained from three independent experiments. ***$P<0.01$, significantly different from the values for cells without KT5823 (Scheffe test).

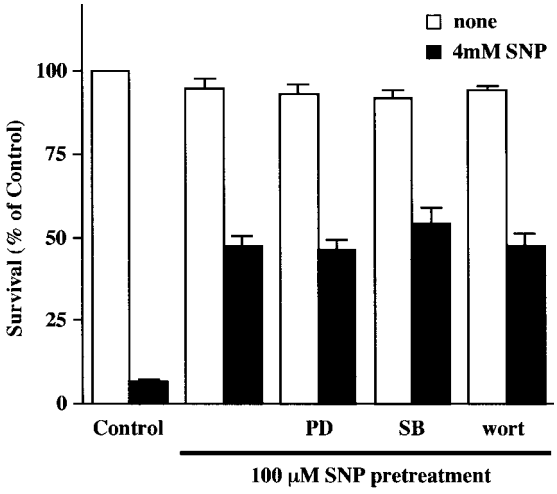

Figure 9 Effect of PD98059, SB203580 or wortmannin on the protection from death of RAW264 cells by SNP pretreatment. Cells were treated for 24 h with 100 μM SNP in the absence or presence of 50 μM PD98059 (PD), 10 μM SB203580 (SB), or 200 nM wortmannin (wort). Then they were incubated for 24 h with (solid columns) or without (open columns) 4 mM SNP. Results show the mean ± s.e. for nine wells and obtained from three independent experiments.

release is now under investigation. One possibility is that protein kinase G may phosphorylate some apoptosis-related protein that modulates cytochrome *c* release. In this context, it was demonstrated that protein kinase G directly inhibited the opening of the mitochondrial permeability transition pore; this opening results in apoptotic events (Takuma *et al*., 2001). Bcl-2 family proteins appear to be able to regulate mitochondrial integrity (Harris & Thompson, 2000). It is well known that the

PI 3-kinase/Akt pathway induces phosphorylation at Ser-112 and Ser-136 of Bad, a Bcl-2 family protein. In the absence of this phosphorylation, Bad is thought to induce cytochrome *c* release, possibly *via* the formation of heterodimers with another Bcl-2 family member, Bcl-xL (Zha *et al*., 1996; Datta *et al*., 1997; Soane *et al*., 2001). Recently, it was reported that Akt was activated by NO through cGMP signaling in cerebellar granule neurons (Ciani *et al*., 2002). In the present study, however, wortmannin did not inhibit the cytoprotective effect of low-dose SNP, suggesting that the PI 3-kinase/Akt pathway may not be involved in the effect of SNP. MAP kinases ERK and p38 MAP kinase are known to modulate apoptosis induced by NO (Kim *et al*., 2002). It was reported that these kinases were activated by NO through the NO/cGMP/protein kinase G pathway (Browning *et al*., 2000; Komalavilas *et al*., 1999). However, the cytoprotective effect of NO would appear to be independent of MAP kinase activation, since PD98059 or SB203580, a specific inhibitor of ERK phosphorylation or p38 MAP kinase, respectively, did not affect the cytoprotection by SNP in the present study. Another possibility is that activation of protein kinase G may induce expression of some as yet unknown gene whose product inhibits cytochrome *c* release. It was reported that over-expression of Bcl-xL or cyclooxygenase-2 (COX-2) prevented NO-induced cell death in macrophages (von Knethen & Brune, 1997; Okada *et al*., 1998). It was indicated that the expression of Bcl-xL and COX-2 was regulated by transcriptional factor NF-kappa B, which can be activated by protein kinase G (von Knethen *et al*., 1999; Kalra *et al*., 2000; Bui *et al*., 2001). Consistent with these reports, NF-kappa B inhibitors – *N*-acetylcysteine and pyrrolidine dithiocarbamate – each partially but not completely inhibited the cytoprotective effects of SNP or DBcGMP in our study (3 mM *N*-acetylcysteine caused 53.5 or 60.7% inhibition of the effect of low-dose SNP or DBcGMP, respectively; and 3 μM pyrrolidine dithiocarbamate, 36.2 or 44.3% inhibition, respectively). Cytoprotection through the NO/cGMP/protein kinase G pathway against NO toxicity may include the induction of these antiapoptotic proteins.

In conclusion, our present results demonstrate the existence of a potential self-defense mechanism against NO toxicity in macrophages through cGMP production and activation of protein kinase G.

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References

ALBINA, J.E.S., MAETO, R.B. & REICHNER, J.S. (1993). Nitric oxide-mediated apoptosis in murine peritoneal macrophages. *J. Immunol.,* **150,** 5080 – 5085.

ANKARCRONA, M., DYPBUKT, J.M., BRUNE, B. & NICOTERA, P. (1994). Interleukin-1 beta-induced nitric oxide production activates apoptosis in pancreatic RINm5F cells. *Exp. Cell Res.,* **213,** 172 – 177.

BATTINELLI, E. & LOSCALZO, J. (2000). Nitric oxide induces apoptosis in megakaryocytic cell lines. *Blood,* **95,** 3451 – 3459.

BEAUVAIS, F., MICHEL, L. & DUBERTRET, L. (1995). The nitric oxide donors, azide and hydroxylamine, inhibit the programmed cell death of cytokine-deprived human eosinophils. *FEBS Lett.,* **361,** 229 – 232.

BROOKES, P.S., SALINAS, E.P., DARLEY-USMAR, K., EISERICH, J.P., FREEMAN, B.A., DARLEY-USMAR, V.M. & ANDERSON, P.G. (2000). Concentration-dependent effects of nitric oxide on mitochondrial permeability transition and cytochrome *c* release. *J. Biol. Chem.,* **275,** 20474 – 20479.

BROWN, G.C. (1999). Nitric oxide and mitochondrial respiration. *Biochim. Biophys. Acta,* **1411,** 351 – 369.

BROWNING, D.D., MCSHANE, M.P., MARTY, C. & YE, R.D. (2000). Nitric oxide activation of p38 mitogen-activated protein kinase in 293T fibroblasts requires cGMP-dependent protein kinase. *J. Biol. Chem.,* **275,** 2811 – 2816.

BUI, N.T., LIVOLSI, A., PEYRON, J.F. & PREHN, J.H. (2001). Activation of nuclear factor kappaB and Bcl-x survival gene expression by nerve growth factor requires tyrosine phosphorylation of IkappaBalpha. *J. Cell Biol.,* **152,** 753 – 764.

CIANI, E., VIRGILI, M. & CONTESTABILE, A. (2002). Akt pathway mediates a cGMP-dependent survival role of nitric oxide in cerebellar granule neurones. *J. Neurochem.,* **81,** 218 – 228.

DATTA, S.R., DUDEK, H., TAO, X., MASTERS, S., FU, H., GOTOH, Y. & GREENBERG, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell,* **91,** 231 – 241.

FEHSEL, K., KRONCKE, K.D., MEYER, K.L., HUBER, H., WAHN, V. & KOLB-BACHOFEN, V. (1995). Nitric oxide induces apoptosis in mouse thymocytes. *J. Immunol.,* **155,** 2858 – 2865.

FUJIMORI, Y., MAEDA, S., SAEKI, M., MORISAKI, I. & KAMISAKI, Y. (2001). Inhibition by nifedipine of adherence- and activated macrophage-induced death of human gingival fibroblasts. *Eur. J. Pharmacol.,* **415,** 95 – 103.

GENARO, A.M., HORTELANO, S., ALVAREZ, A., MARTINEZ, C. & BOSCA, L. (1995). Splenic B lymphocyte programmed cell death is prevented by nitric oxide release through mechanisms involving sustained Bcl-2 levels. *J. Clin. Invest.,* **95,** 1884 – 1890.

HARRIS, M.H. & THOMPSON, C.B. (2000). The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. *Cell Death Differentiation,* **7,** 1182 – 1191.

HIURA, T.S., LI, N., KAPLAN, R., HORWITZ, M., SEAGRAVE, J.C. & NEL, A.E. (2000). The role of a mitochondrial pathway in the induction of apoptosis by chemicals extracted from diesel exhaust particles. *J. Immunol.,* **165,** 2703 – 2711.

KALRA, D., BAUMGARTEN, G., DIBBS, Z., SETA, Y.,SIVASUBRAMANIAN, N. & MANN, D.L. (2000). Nitric oxide provokes tumor necrosis factor-alpha expression in adult feline myocardium through a cGMP-dependent pathway. *Circulation,* **102,** 1302 – 1307.

KANESAKI, T., SAEKI, M., OOI, Y., SUEMATSU, M., MATSUMOTO, K., SAKUDA, M., SAITO, K. & MAEDA, S. (1999). Morphine prevents peroxynitrite-induced death of human neuroblastoma SH-SY5Y cells through a direct scavenging action. *Eur. J. Pharmacol.,* **372,** 319 – 324.

KANETO, H., FUJII, J., SEO, H.G., SUZUKI, K., MATSUOKA, T., NAKAMURA, M., TATSUMI, H., YAMASAKI, Y., KAMADA, T. & TANIGUCHI, N. (1995). Apoptotic cell death triggered by nitric oxide in pancreatic beta-cells. *Diabetes,* **44,** 733 – 738.

KIM, S.J., JU, J.W., OH, C.D., YOON, Y.M., SONG, W.K., KIM, J.H., YOO, Y.J., BANG, O.S., KANG, S.S. & CHUN, J.S. (2002). ERK-1/2 and p38 kinase oppositely regulate nitric oxide-induced apoptosis. *J. Biol. Chem.,* **277,** 1332 – 1339.

KIM, Y.M., BERGONIA, H. & LANCASTER JR., J.R. (1995). Nitrogen oxide-induced autoprotection in isolated rat hepatocytes. *FEBS Lett.,* **374,** 228 – 232.

KIM, Y.M., DE VERA, M.E., WATKINS, S.C. & BILLAR, T.R. (1997). Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-alpha-induced apoptosis by inducing heat shock protein 70 expression. *J. Biol. Chem.,* **272,** 1402 – 1411.

KOMALAVILAS, P., SHAH, P.K., JO, H. & LINCOLN, T.M. (1999). Activation of mitogen-activated protein kinase pathways by cyclic GMP and cyclic GMP-dependent protein kinase in contractile vascular smooth muscle cells. *J. Biol. Chem.,* **274,** 34301 – 34309.

KOYAMA, Y., KIMURA, Y., YOSHIOKA, Y., WAKAMATSU, D., KOZAKI, R., HASHIMOTO, H., MATSUDA, T. & BABA, A. (2000).

Serum-deprivation induces cell death of rat cultured microglia accompanied with expression of Bax protein. *Japan J. Pharmacol.,* **83,** 351 – 354.

KRONCKE, K.D., BRENNER, H.H., RODRIGUEZ, M.L., ETZKORN, K., NOACK, E.A., KOLB, H. & KOLB-BACHOFEN, V. (1993). Pancreatic islet cells are highly susceptible towards the cytotoxic effects of chemically generated nitric oxide. *Biochim. Biophys. Acta,* **1182,** 221 – 229.

MACMICKING, J., XIE, Q.W. & NATHAN, C. (1997). Nitric oxide and macrophage function. *Annu. Rev. Immunol.,* **15,** 323 – 350.

MANNICK, J.B., ASANO, K., IZUMI, K., KIEFF, E. & STAMLER, J.S. (1994). Nitric oxide produced by human B lymphocytes inhibits apoptosis and Epstein – Barr virus reactivation. *Cell,* **79,** 1137 – 1146.

MANNICK, J.B., MIAO, X.Q. & STAMLER, J.S. (1997). Nitric oxide inhibits Fas-induced apoptosis. *J. Biol. Chem.,* **272,** 24125 – 24128.

MESSMER, U.K. & BRUNE, B. (1996). Nitric oxide (NO) in apoptotic versus necrotic RAW 264.7 macrophage cell. *Arch. Biochem. Biophys.,* **327,** 1 – 10.

MOILANEN, E. & VAPAATALO, H. (1995). Nitric oxide in inflammation and immune response. *Ann. Med.,* **27,** 359 – 367.

MONCADA, S. & HIGGS, A. (1993). The L-arginine – nitric oxide pathway. *N. Engl. J. Med.,* **329,** 2002 – 2012.

MOSMANN, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods,* **65,** 55 – 63.

MUHL, H., NITSCH, D., SANDAU, K., BRUNE, B., VARGA, Z. & PFEILSCHIFTER, J. (1996). Apoptosis is triggered by the cyclic AMP signalling pathway in renal mesangial cells. *FEBS Lett.,* **382,** 271 – 275.

NIJIHAWAN, D., BUDIHARDJO, I., SRINIVASULA, S.M., AHMAD, M., ALNEMRI, E.S. & WANG, X. (1997). Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell,* **91,** 479 – 489.

OKADA, S., ZHANG, H., HATANO, M. & TOKUHISA, T. (1998). A physiologic role of Bcl-xL induced in activated macrophages. *J. Immunol.,* **160,** 2590 – 2596.

PFEIFER, A., KLATT, P., MASSBERG, S., NY, L., SAUSBIER, M., HIRNEISS, C., WANG, G.X., KORTH, M., ASZODI, A., ANDERSSON, K.E., KROMBACH, F., MAYERHOFER, A., RUTH, P., FASSLER, R. & HOFMANN, F. (1998). Defective smooth muscle regulation in cGMP kinase I-deficient mice. *EMBO J.,* **17,** 3045 – 3051.

RIDDELL, D.R. & OWEN, J.S. (1999). Nitric oxide and platelet aggregation. *Vitam. Horm.,* **57,** 25 – 48.

SANDAU, K. & BRUNE, B. (1996). The dual role of *S*-nitrosoglutathione (GSNO) during thymocyte apoptosis. *Cell Signal,* **8,** 173 – 177.

SANDAU, K., PFEILSCHIFTER, J. & BRUNE, B. (1997). The balance between nitric oxide and superoxide determines apoptotic and necrotic death of rat mesangial cells. *J. Immunol.,* **158,** 4938 – 4946.

SATA, M., KAKOKI, M., NAGATA, D., NISHIMATSU, H., SUZUKI, E., AOYAGI, T., SUGIURA, S., KOJIMA, H., NAGANO, T., KANGAWA, K., MATSUO, H., OMATA, M., NAGAI, R. & HIRATA, Y. (2000). Adrenomedullin and nitric oxide inhibit human endothelial cell apoptosis *via* a cyclic GMP-independent mechanism. *Hypertension,* **36,** 83 – 88.

SCIORATI, C., ROVERE, P., FERRARINI, M., HELTAI, S., MANFREDI, A.A. & CLEMENTI, E. (1997). Autocrine nitric oxide modulates CD95-induced apoptosis in gamma delta T lymphocytes. *J. Biol. Chem.,* **272,** 23211 – 23215.

SOANE, L., CHO, H.J., NICULESCU, F., RUS, H. & SHIN, M.L. (2001). C5b-9 terminal complement complex protects oligodendrocytes from death by regulating Bad through phosphatidylinositol 3-kinase/Akt pathway. *J. Immunol.,* **167,** 2305 – 2311.

SUENOBU, N., SHICHIRI, M., IWASHINA, M., MARUMO, F. & HIRATA, Y. (1999). Natriuretic peptides and nitric oxide induce endothelial apoptosis *via* a cGMP-dependent mechanism. *Arterioscler. Thromb. Vasc. Biol.,* **19,** 140 – 146.

TAIMOR, G., HOFSTAETTER, B. & PIPER, H.M. (2000). Apoptosis induction by nitric oxide in adult cardiomyocytes *via* cGMP-signaling and its impairment after simulated ischemia. *Cardiovasc. Res.,* **45,** 588 – 594.

TAKUMA, K., PHUAGPHONG, P., LEE, E., MORI, K., BABA, A. & MATSUDA, T. (2001). Anti-apoptotic effect of cGMP in cultured astrocytes: inhibition by cGMP-dependent protein kinase of mitochondrial permeable transition pore. *J. Biol. Chem.,* **276,** 48093 – 48099.

VINCENT, A.M. & MAIESE, K. (1999). Nitric oxide induction of neuronal endonuclease activity in programmed cell death. *Exp. Cell Res.,* **246,** 290 – 300.

VON KNETHEN, A. & BRUNE, B. (1997). Cyclooxygenase-2: an essential regulator of NO-mediated apoptosis. *FASEB J.,* **11,** 887 – 895.

VON KNETHEN, A., CALLSEN, D. & BRUNE, B. (1999). NF-kappaB and AP-1 activation by nitric oxide attenuated apoptotic cell death in RAW 264.7 macrophages. *Mol. Biol. Cell,* **10,** 361 – 372.

WANG, X. & ROBINSON, P.J. (1997). Cyclic GMP-dependent protein kinase and cellular signaling in the nervous system. *J. Neurochem.,* **68,** 443 – 456.

ZHA, J., HARADA, H., YANG, E., JOCKEL, J. & KORSMEYER, S.J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell,* **87,** 619 – 628.

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