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Modulation of AP-1 activity by nitric oxide (NO) in vitro: NO-mediated modulation of AP-1

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Abstract To understand the role of nitric oxide (NO) in controlling the specific DNA-binding activities of transcriptional factors, we investigated the in vitro effect of the NO-donor sodium nitroprusside (SNP) on the AP-1 activity of cultured mouse cerebellar granule cells. A gel-mobility assay showed that SNP inhibited AP-1 activity in the presence, but not the absence of dithiothreitol (DTT). This DTT-dependent inhibition of AP-1 activity by SNP corresponded with the activation of the chemical reactivity of SNP with DTT, which can be monitored by the production of nitrite (NO_2^-). In contrast, diamide, a typical sulfhydryl oxidizing agent, inhibited AP-1 activity in the absence of DTT and its inhibitory effect was reversed competitively by DTT. Studies using structurally or functionally related analogues of SNP demonstrated that S-nitrosylation of the AP-1 moiety mediated by some NO-carriers but not by free NO, which can be produced by the chemical reaction of SNP with DTT, was responsible for the inhibition of AP-1 activity, suggesting NO-mediated regulation of the AP-1 transcriptional factor.

Key words: Nitric oxide (NO); AP-1; Redox regulation; S-nitrosylation; Sodium nitroprusside (SNP); Transcriptional factor

1. Introduction

Nitric oxide (NO), a short-lived, free-radical gas produced by NO synthase (NOS) in a variety of cells, has been implicated as a diffusible intercellular mediator in diverse biological phenomena such as vasodilation, macrophage cytotoxicity, neurotoxicity and synaptic transmission [1–4]. NO can modulate the enzymatic activities of guanylate cyclase [5] and aconitase [6], the latter exhibiting IRF (iron responsive factor)-RNA binding activity [7], through interaction with heme and iron-sulfur centers. Furthermore, the S-nitrosylation of proteins such as serum albumin [8], glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [9, 10] and tissue-type plasminogen activator [11] seems to modulate their functions. However, it is not exactly known which intracellular target molecules interacting with NO are included in the cellular milieu or how NO mediates this variety of cellular responses.

The specific DNA-binding activities of transcriptional factors such as NF-κB and AP-1 are modulated by oxidative stress to the cells [12]. Furthermore, the DNA-binding activities of these transcriptional factors can be modulated by the redox (reduction-oxidation) state of their components in vitro [13,14]. AP-1 transcriptional factors are members of the Fos/Jun families and have high binding affinity to the 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) [15]. Single conserved cysteine residues in the DNA-binding domain of c-Fos and c-Jun, major components of AP-1, are responsible for the redox regulation of AP-1 activity [13]. Thus transcriptional factors can be modulated by cellular redox mechanisms. However, it is still unknown whether NO produced intracellularly is involved in these processes.

Sodium nitroprusside (SNP: $Na_2[Fe(CN)_5NO]$), a potent vasodilator, is a typical NO-donor [16], and the process of NO release from SNP after its reaction with thiols of reducing agents has been described in detail by Bates et al. [17]. Furthermore, SNP is reported to modulate the enzymatic activity of GAPDH in vitro in the presence of dithiothreitol (DTT), probably through S-nitrosylation at the active-site cysteine residues of GAPDH [9,10]. In this study we added SNP directly to a DNA-binding reaction mixture containing a nuclear extract of cultured mouse cerebellar granule cells and measured the resulting level of AP-1 activity by a gel-mobility assay.

2. Materials and methods

2.1. Preparation of nuclear mini-extracts

The conditions for culturing mouse cerebellar granule cells and preparing nuclear mini-extracts were as described [18]. Nuclear mini-extracts were extracted from cultured granule cells with extraction buffer consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) 1 h after stimulating the cells with 100 μM *N*-methyl-D-aspartate (NMDA). The nuclear fraction was precipitated and then suspended in nuclear buffer consisting of 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM PMSF. Buffers depleted of dithiothreitol (DTT) were used for all steps of preparation of nuclear mini-extracts. Nuclear extracts of NIH3T3 cells were prepared by the same procedure as used for the granule cells.

2.2. Gel-mobility assay

The DNA-binding reaction was performed for 15 min at 25°C in a standard reaction mixture (20 μl) containing 20 mM HEPES (pH 7.9), 1 mM DTT, 0.3 mM EDTA, 0.2 mM EGTA, 80 mM NaCl, 2 μg poly[dI-dC], 10% glycerol, 0.2 mM PMSF, 0.2–0.4 ng of ^{32}P-labeled oligonucleotide probe, and nuclear mini-extract (5 μg of protein). Where indicated as DTT-free, we omitted DTT from the standard reaction mixture. Before starting the DNA-binding reaction at 25°C, SNP and other agents were added to the mixture at the concentrations indicated in the Figures. The gel-mobility profiles were quantified using an imaging scanner (Fuji Bas 2000, Japan). Oligonucleotides (TRE: 5′-GATTCGTGACTCAGCACAGG-3′, MYC: 5′-AAGCAGACCACGTGGTCAGGGAT-3′) [15,19], synthesized using a DNA synthesizer (MilliGen/Biosearch), were used as DNA probes for the gel-mobility assay [18]. Sodium nitroprusside (SNP) and azodicarboxylic acid bis(dimethylamide) (diamide) were purchased from Sigma. Superoxide dismutase was from Wako Co. (Japan). 3-Morpholino-sydnonimine (SIN-1) was a gift from R. Henning (Leiter der Pharma-Forshung, Frankfurt, Germany).

2.3. Assay of NO_2^- levels

NO_2^- production in the DNA-binding reaction containing SNP was monitored by a colorimetric procedure based on the Griess reaction [20].

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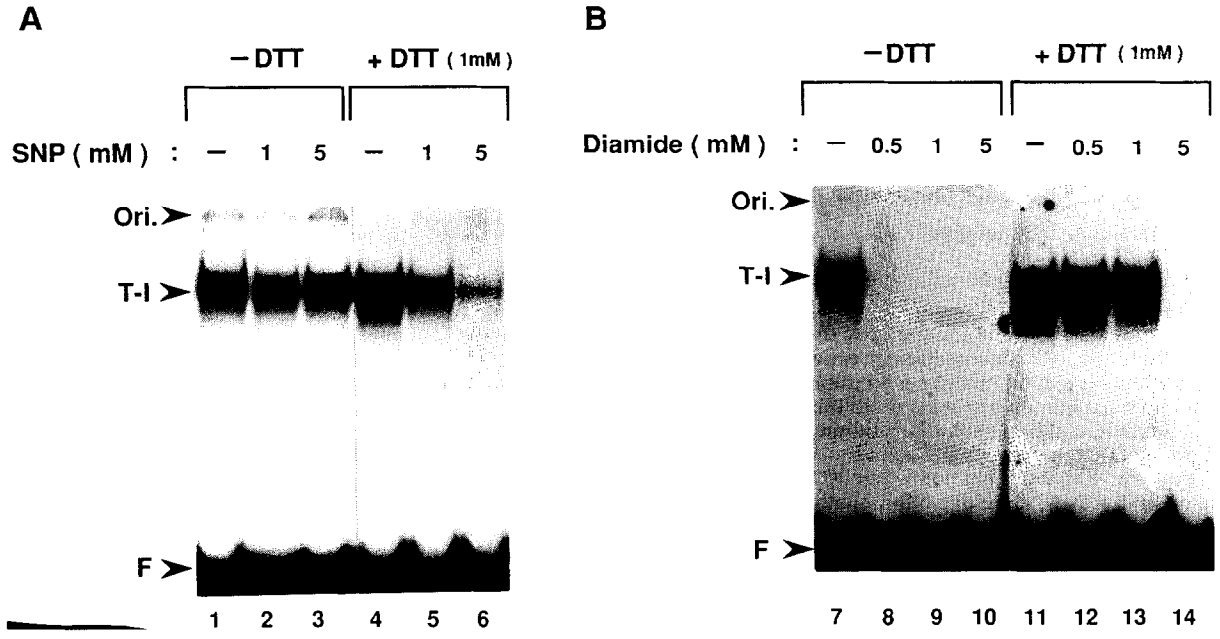

Fig. 1. Inhibitory effects of SNP and diamide on AP-1 activity in the presence and absence of DTT. (A) Sodium nitroprusside (SNP) or (B) diamide was added at the indicated concentrations to the reaction mixture without DTT (lanes 1–3 and lanes 7–10), or with 1 mM DTT (lanes 4–6 and lanes 11–14). Nuclear extracts prepared using DTT-free extraction buffers were assayed by DNA-binding reactions in the presence or absence of DTT. T–I, DNA-protein complexes formed on the TRE by AP-1 complexes; F, free labeled TRE probe.

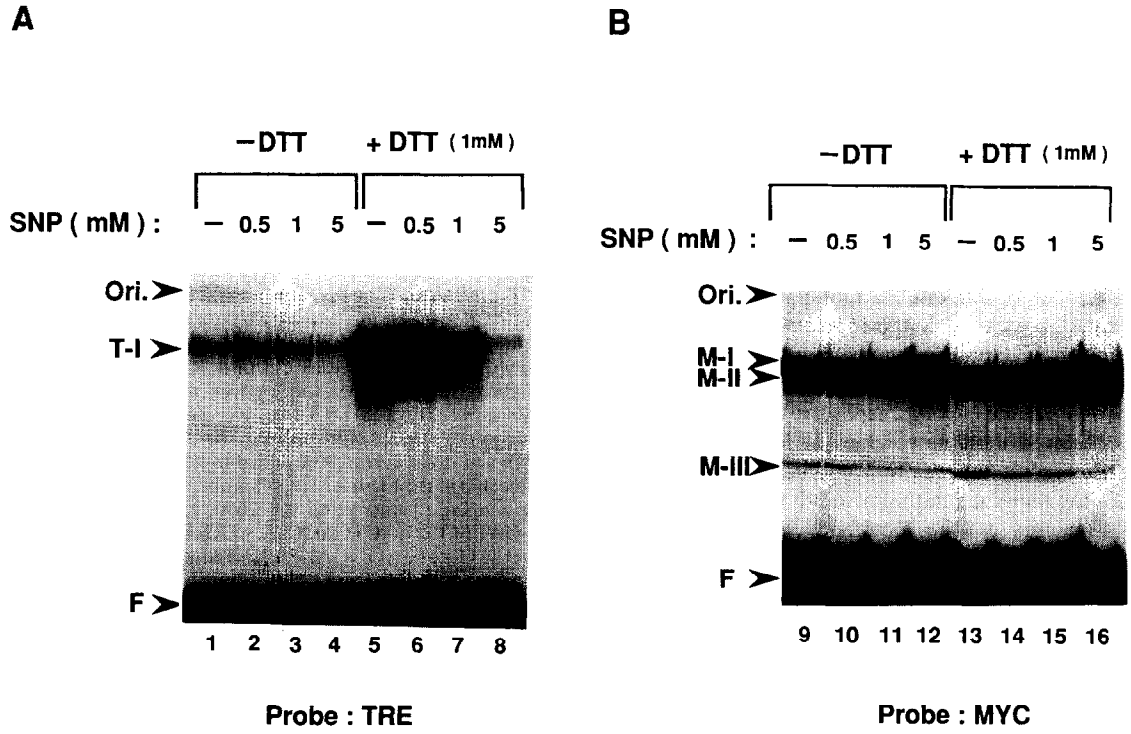

Fig. 2. Effects of SNP on AP-1 activity of NIH3T3 cells and c-Myc-binding activity of cerebellar granule cells. (A) TRE-binding activities, and (B) c-Myc-binding activities were measured with increasing concentrations of SNP in reaction mixture containing nuclear mini-extracts prepared from NIH3T3 cells (A) and cerebellar granule cells (B). The DNA-binding reaction was carried out in the absence (lanes 1–4 and 9–12) or presence (lanes 5–8 and 13–16) of 1 mM DTT. M-I, -II and -III, DNA-protein complexes specifically formed on MYC.

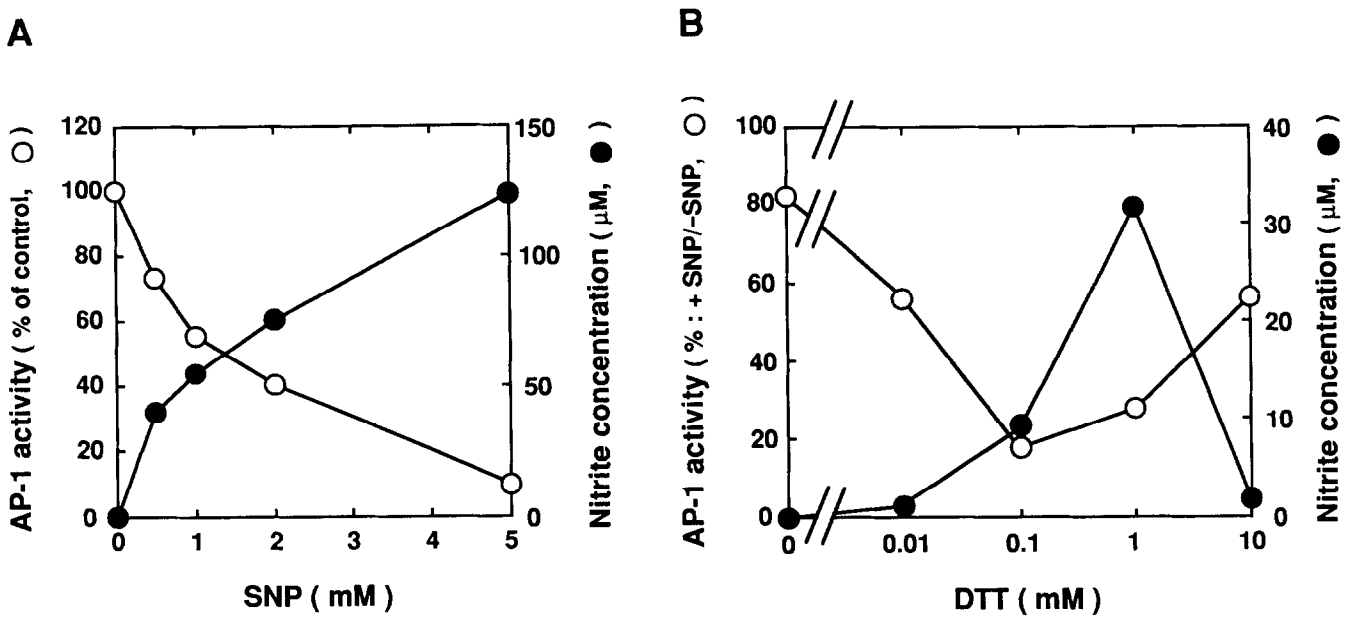

Fig. 3. Correlation between inhibition of AP-1 activity and NO_2^- production from SNP. (A) The concentration of SNP was varied in the presence of 1 mM DTT. Open circles indicate AP-1 activities determined by gel-mobility assay and imaging scanning. The AP-1 activity at each concentration of SNP is indicated as a percentage of the control, which was measured in the absence of SNP. Filled circles indicate the concentrations of nitrite (NO_2^-) produced in 20 mM HEPES containing SNP at the indicated concentrations (pH 7.9), measured as the absorbance at 541 nm after the Griess reaction [18]. (B) The concentration of DTT was varied in the presence of 1 mM SNP. At each concentration of DTT, the AP-1 activity was measured in the presence and absence of SNP. The AP-1 activity in the presence of SNP as a percentage of that in the absence of SNP is indicated by open circles (%, +SNP/−SNP). Filled circles indicate the concentration of NO_2^- produced in 20 mM HEPES (pH 7.9) containing DTT at the indicated concentrations. The levels of AP-1 activity and the NO_2^- production were measured separately.

3. Results and discussion

We have detected enhanced AP-1 activities in nuclear mini-extracts prepared from cultured cerebellar granule cells stimulated with 100 μM NMDA [18]. In a standard DNA-binding reaction containing 1 mM DTT, addition of SNP decreased the AP-1 activity dose-dependently (Fig. 1A, lanes 4–6). But when DTT was omitted from the standard reaction, no inhibition of AP-1 activity was observed, even with 5 mM SNP (Fig. 1A, lanes 1–3). These results indicated that the inhibition of AP-1 activity by SNP (the SNP-effect) depended on the presence of DTT. Addition of β-mercaptoethanol as a reducing agent resulted in only slight inhibition of AP-1 activity by SNP (data not shown).

In contrast, the typical sulfhydryl oxidizing agent diamide strongly inhibited AP-1 activity in the absence of DTT (Fig. 1B, lanes 7–10), probably due to oxidation of cysteine residues in AP-1 moieties [13]. The inhibition of AP-1 by diamide was competitively counteracted by added DTT at a concentration above or equal to that of diamide (Fig. 1B, lanes 11–13), but was complete at a DTT concentration of less than that of diamide (Fig. 1B, lane 14). Thus, the inhibitory effect of diamide on AP-1 activity was due to its oxidation of AP-1 moieties. Therefore, the effect of SNP, which depended on the presence of DTT, was not due to an oxidative effect, unlike the inhibition of AP-1 by diamide.

SNP also inhibited the AP-1 activity in a preparation from NIH3T3 cells in the presence, but not in the absence of DTT (Fig. 2A), suggesting that the SNP-effect on AP-1 activity is common to a variety of cells. The higher AP-1 activity detected with DTT than without DTT (compare lane 5 with lane 1) was due to the reducing effect of DTT on AP-1 activity as described by Abate et al. [13]. The c-Myc-binding activity was not inhibited, but rather stimulated by SNP, regardless of the presence of DTT (Fig. 2B), suggesting that SNP specifically affects the DNA-binding activity of AP-1.

Since SNP can react with thiol groups of reducing agents and release NO [17], we examined the relationship between the inhibition of AP-1 activity by SNP and the reactivity of SNP with DTT. The latter reaction was monitored by the production of nitrite (NO_2^-), an aerobic reaction product of NO. In the absence of SNP or DTT, the NO_2^- production was undetectable (Fig. 3A,B). Addition of increasing amounts of SNP in the presence of 1 mM DTT (Fig. 3A) resulted in gradual decrease in AP-1 activity, but increase in production of NO_2^-. When the concentration of DTT was varied in the presence of 1 mM SNP (Fig. 3B), AP-1 activity was effectively inhibited between 0.1 and 1 mM DTT. A higher concentration of DTT (10 mM) resulted in less inhibition of AP-1 activity by SNP. In the presence of 1 mM SNP, the production of NO_2^- was increased with 0.1 mM DTT, reached a maximum with 1 mM DTT, and was less with a higher concentration of DTT (10 mM). Bates et al. also reported lower reactivity of 1 mM SNP at a higher concentration of DTT (10 mM) [17]. Thus, the inhibition of AP-1 by SNP almost coincided with the production of NO_2^- from SNP, indicating that the SNP-effect depended upon the chemical reaction of SNP with DTT.

To determine whether free NO released from SNP is responsible for the inhibition of AP-1 activity, we investigated the effect of SIN-1, another typical NO-donor [16], on the AP-1

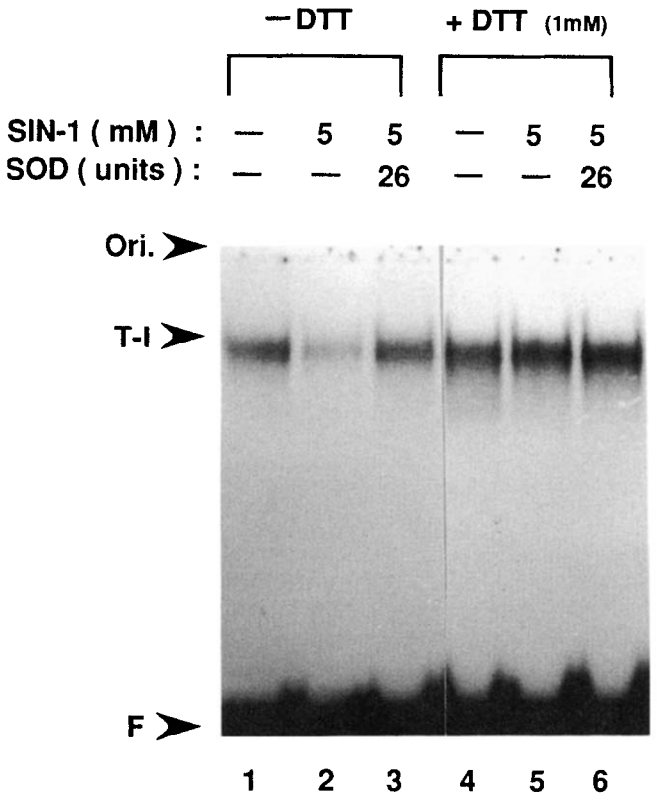

Fig. 4. Effects of SIN-1 on AP-1 activity in the presence and absence of DTT. SIN-1 was added at a final concentration of 5 mM (lanes 2, 3, 5 and 6) to reaction mixture in the absence (lanes 1–3) or presence (lanes 4–6) of 1 mM DTT. SOD (26 units) was added before SIN-1 (lanes 2 and 5). T-I and F are as described in the legend to Fig. 1. The intensities of the T-I bands relative to that of the control (lane 1) were 1, 0.6, 1.1, 1.5, 1.8 and 1.9 (lanes 1–6), respectively.

activity. Fig. 4 shows that SIN-1 inhibited the AP-1 activity only in the absence of DTT (lane 2). Since the addition of superoxide dismutase (SOD), which prevents the effects of the superoxide ion (O_2^-) produced during SIN-1 decomposition [16], restored the AP-1 activity (Fig. 4, lane 3), the inhibition of AP-1 activity by SIN-1 was due to the oxidizing effect of O_2^-. As SIN-1 did not inhibit AP-1 activity in the presence of DTT (Fig. 4, lanes 4–6), irrespective of the active production of NO as measured by the NO_2^- level (data not shown), it is unlikely that free NO mediates the SNP-effect.

Potassium ferrocyanide, which is structurally similar to SNP but devoid of NO, did not affect AP-1 activity (data not shown). This fact do not support the notion that the SNP-effect is due to the ferrocyanide ion. The addition of other agents which might explain the SNP-effect, such as $NaNO_2$, $NaNO_3$ and KCN, did not influence the AP-1 activity with or without DTT (data not shown), excluding the contributions of NO_2^-, NO_3^- and CN^-, which are also formed during or after the reaction of SNP with DTT.

A nitrosonium form of SNP, $[Fe(CN)_5NO^+]^{2-}$, which is stable in neutralized solutions [17], also did not seem to cause the SNP-effect because in the absence of DTT, SNP did not inhibit the AP-1 activity (Fig. 1A). The reaction of SNP with thiols of DTT yields the intermediate radical form, $[Fe(CN)_5NO^{\bullet}]^{3-}$ [17]. This radical form of SNP is then processed, releasing NO after CN^- [17]. Since free NO was not responsible for the SNP-effect, as described above, we prefer the notion that the chemical reaction of SNP with thiols of DTT produces not only an intermediate of SNP but also that of *S*-nitrosodithiothreitol [10], either of which could be required as an intermediate carrier of NO from SNP to the AP-1 moiety.

Modulation of the enzymatic activity by SNP with DTT has been fully investigated with GAPDH [7,8]. SNP causes a decrease of GAPDH activity with modification by nicotinamide adenine dinucleotide (NAD) in vitro, depending upon the presence of DTT [10]. Under these conditions, GAPDH is thought to be S-nitrosylated by SNP at its active-site cysteine residues, leading to the covalent binding of NAD through an NO-dependent thiol intermediate. Thus it is likely that the inhibition of AP-1 activity by SNP is caused by S-nitrosylation of AP-1 moieties. We do not yet know whether AP-1 moieties are covalently modified with NAD.

Although the precise molecular mechanism of the SNP-effect is still unknown, we speculate that a mechanism modulating the AP-1 activity might work in vivo coupled with the redox mechanisms of cells. Stimulation of cerebellar granule cells with NMDA leads not only to AP-1 induction [18] but also to the activation of NO production [21]. Thus, NO produced intracellularly could cause fine modulation of AP-1 activity in vivo through unknown intermediate carriers, the functions of which might be mimicked by SNP and DTT in vitro. Since c-*fos* induction can be prolonged prior to excitotoxic neuronal cell death [22], such NO-mediated modulation of AP-1 activity in neuronal cells might act as a neuroprotective effector.

Thus, we predict that NO-mediated regulation of the AP-1 transcriptional factor in vivo might contribute to the control of gene expression in a variety of cellular responses.

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References

[1] Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1991) Pharmacol. Rev. 43, 109–142.
[2] Nathan, C.F. and Hibbs Jr., J.B. (1991) Curr. Opin. Immunol. 3, 65–70.
[3] Lipton, S.A., Choi, Y.-B., Pan, Z.-H., Lei, S.Z., Vincent Chen, H.-S., Sucher, N.J., Loscalzo, J., Singel, D.J. and Stamler, J.S. (1993) Nature 364, 626–631.
[4] Bredt, D.S. and Snyder, S.H. (1992) Neuron 8, 3–11.
[5] Murad, F., Mittal, C.K., Arnold, W.P., Katsuki, S. and Kimura, H. (1978) Adv. Cyclic Nucleotide Res. 9, 145–158.
[6] Drapier, J.-C., Hirling, H., Wietzerbin, J., Kaldy, P. and Kuhn, L.C. (1993) EMBO J. 12, 3643–3649.
[7] Weiss, G., Goosen, B., Doppler, W., Fuchs, D., Pantopoulos, K., Werner-Felmyer, G., Wachter, H. and Hentze, M.W. (1993) EMBO J. 12, 3651–3657.
[8] Stamler, J.S., Jaraki, O., Osborne, J., Simon, D.I., Keaney, J., Vita, J., Singel, D., Valeri, C.R. and Loscalzo, J. (1992) Proc. Natl. Acad. Sci. USA 89, 7674–7677.
[9] Vedia, L.M., McDonald, B., Reep, B., Brune, B., Silvio, M.D., Billiar, T.R. and Lapetina, E.G. (1992) J. Biol. Chem. 267, 24929–24932.
[10] McDonald, L.J. and Moss, J. (1993) Proc. Natl. Acad. Sci. USA 90, 6238–6241.
[11] Stamler, J.S., Simon, D.I., Jaraki, O., Osborne, J.A., Francis, S., Mullins, M., Singel, D. and Loscalzo, J. (1992) Proc. Natl. Acad. Sci. USA 89, 8087–8091.
[12] Meyer, M., Schreck, R. and Baeuerle, P.A. (1993) EMBO J. 12, 2005–2015.

[13] Abate, C., Patel, L., Rauscher III, F.J. and Curran, T. (1990) Science 249, 1157–1161.
[14] Toledano, M. and Leonard, W.J. (1991) Proc. Natl. Acad. Sci. USA 88, 4328–4332.
[15] Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987) Cell 49, 729–739.
[16] Southam, E. and Garthwaite, J. (1991) Neurosci. Lett. 130, 107–111.
[17] Bates, J.N., Baker, M.T., Guerra Jr., R. and Harrison, D.G. (1991) Biochem. Pharmacol. 42, s157–s165.
[18] Sakurai, H., Kurusu, R., Sano, K., Tsuchiya, T. and Tsuda, M. (1992) J. Neurochem. 59, 2067–2075.
[19] Halazonetis, T.D. and Kandil, A.N. (1991) Proc. Natl. Acad. Sci. USA 88, 6162–6166.
[20] Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Anal. Biochem. 126, 131–138.
[21] Kiedrowski, L., Costa, E. and Wrobleski, J.T. (1992) J. Neurochem. 58, 335–341.
[22] Smeyne, R.J., Vendrell, M., Hayward, M., Baker, S.J., Miao, G.G., Schilling, K., Robertson, L.M., Curran, T. and Morgan, J.I. (1993) Nature 363, 166–169.