Oxygen free radicals enhance the nitric oxide-induced covalent NAD⁺-linkage to neuronal glyceraldehyde-3-phosphate dehydrogenase

Philippe MARIN,*‡ Marion MAUS,* Joël BOCKAERT,† Jacques GLOWINSKI* and Joël PRÊMONT*

*Chaire de Neuropharmacologie, INSERM U114, Collège de France, 11 Place Marceau Berthelot, 75231 Paris Cedex 05, France, and †CNRS UPR 9023, CCIPE, Rue de la Cardonille, 34094 Montpellier Cedex 5, France

Nitric oxide (NO) induces a covalent modification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from various tissues. This phenomenon, which has previously been interpreted as an auto-ADP-ribosylation, is in fact a covalent binding of NAD⁺ to the enzyme. In the present study, we show that 3-morpholino-sydnonimine (SIN-1) is much more efficient than sodium nitroprusside (SNP) in stimulating the covalent labelling of GAPDH from cultured striatal neurones in the presence of [adenylate-32P]NAD⁺ (877 ± 110 and 266 ± 33 % increase in NAD⁺-labelling induced by maximally effective concentrations of SIN-1 and SNP respectively). The difference in the efficacy of both NO-generating compounds could be due to the additional release of superoxide by SIN-1, since superoxide dismutase and the nitrine 5,5'-dimethyl pyrroline-1-oxide markedly inhibited the SIN-1-induced covalent binding of NAD⁺ to GAPDH. Catalase and selective scavengers of hydroxyl radicals, mannitol and dimethyl sulfoxide, did not alter the SIN-1-induced covalent modification of GAPDH, ruling out the involvement of hydroxyl radicals in this phenomenon. Supporting further a role of oxygen free radicals in the NAD⁺ linkage to GAPDH, pyrogallol, a superoxide generator, which alone was ineffective, potentiated the SNP-evoked response. The NAD⁺ linkage to neuronal GAPDH measured in the presence of NO and superoxide probably involves sulphhydryl groups, since the radiolabelling of the protein was reversed by exposure to HgCl₂ and prevented by pretreatment with the alkylating agent N-ethylmaleimide. Moreover, the NO-induced inhibition of GAPDH activity was enhanced by pyrogallol, which was ineffective alone. In conclusion, the present study indicates that superoxide anions potentiate NO-induced covalent NAD⁺-linkage to GAPDH and enzyme inactivation.

INTRODUCTION

In addition to its roles in the vascular and immune systems, nitric oxide (NO) has been shown to be an important neuronal messenger in both the peripheral and central nervous systems, acting as a non-typical neurotransmitter or a trans-cellular second messenger [1–4]. In the brain, NO seems to play a crucial role in long-term plasticity events, such as long-term potentiation in the CA1 area of the hippocampus [5–8] and long-term depression in the cerebellum [9]. Moreover, studies both in vitro and in vivo have suggested the involvement of NO in neurotoxicity events and, in particular, those following the activation of glutamatergic N-methyl-D-aspartate (NMDA) receptors [10–14]. However, the role of NO in glutamate-induced neuronal death is still debated [15,16]. In fact, recent findings indicate that oxygen free radicals, in particular superoxide anions (O₂⁻), which are also produced in neurones in response to NMDA-receptor stimulation, could also contribute to this phenomenon [17,18]. Alternatively, in some but not all neurones, the neurotoxicity triggered by NO and O₂⁻ may result from their interaction leading to the formation of peroxynitrite [11,19,20].

In neurones, NO is synthesized by a constitutive and Ca²⁺-calmodulin-activated NO-synthase [3], an enzyme which also releases oxygen-derived free radicals when the concentrations of its substrate L-arginine or of the cofactor (6R)-tetrahydro-L-3-bioterin are suboptimal [21–23]. In a previous study, we have demonstrated that glutamate, by acting at both NMDA and ionotropic non-NMDA receptors, stimulates the production of NO in cultured striatal neurones, leading to the accumulation of intracellular cyclic GMP (cGMP) [24]. Besides the stimulation of cytosolic guanylate cyclase, NO has been shown to block the NMDA-receptor channel in these neurones through a negative-feedback process [25]. Moreover, glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12), a cytosolic protein composed of four identical 37 kDa subunits which is involved in the glycolytic cascade, has been identified as an additional target of NO in several cell types [26–29] as well as in brain homogenates [30]. Indeed, NO stimulates the S-nitrosylation of GAPDH, and this effect is associated with an NAD⁺–dependent covalent modification of this enzyme [28]. This modification, previously believed to be an auto-ADP-ribosylation of GAPDH [26–28,30], appears in fact to be a covalent NAD⁺ linkage to the enzyme [29]. In the present study, we show that the covalent [32P]NAD⁺ labelling of GAPDH from cultured striatal neurones is stimulated by the NO-generating compounds sodium nitroprusside (SNP) and 3-morpholinosydnonimine (SIN-1), the latter compound being the more efficient. Since SIN-1 generates both NO and O₂⁻ [31], particular attention is focused on the role of oxygen free radicals in the NAD⁺-dependent covalent modification of neuronal GAPDH as well as the associated changes in GAPDH activity.

EXPERIMENTAL

Materials

Swiss mice and rats were obtained from Iffa Credo (Lyon, France), culture media from Gibco and fetal calf serum (FCS).

Abbreviations: cGMP, cyclic GMP; DMPO, 5,5'-dimethyl pyrroline-1-oxide; DMSO, dimethyl sulfoxide; DT1, diethiothreitol; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEF, isoelectric focusing; NMDA, N-methyl-D-aspartate; NO, nitric oxide; PGA, 3-phosphoglycerate; PGK, 3-phosphoglycerate kinase; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; SOD, superoxide dismutase; TCA, trichloroacetic acid; 2D, two-dimensional; pl, isoelectric point; ANOVA, analysis of variance.

†To whom correspondence should be addressed.
from Dutcher (Brumath, France). Reagents for polyacrylamide gels were purchased from Serva, ampholines from Pharmacia LKB Biotechnology Inc. and [adenylate-32P]NAD+ (800 Ci/mmol) from NEN-Dupont. Horseradish peroxidase-linked antirabbit antibodies, enhanced chemiluminescence (ECL) detection kit, nitrocellulose sheets (Hybond C), X-ray films (Hyperfilms MP) and kits for cGMP determination were obtained from Amersham. 5,5'-Dimethyl pyrrole-1-oxide (DMPO) was obtained from Aldrich and 3-phosphoglycerate kinase (PGK) from Boehringer, Mannheim. SIN-1 and its derivative SIN-1C were provided by Mrs Winicki (Hoechst Laboratories, Paris). All other chemicals and reagents used in the present study were purchased from Sigma.

Primary cultures of striatal neurones

Primary neuronal cultures were prepared as previously described [32]. Briefly, striata were removed from 14- to 15-day-old mouse embryos and cells were seeded on 100 mm culture dishes (25 × 104 cells/dish containing 10 ml of culture medium) or on 12-well culture dishes (104 cells/well containing 1 ml of medium), previously coated successively with poly-L-ornithine (15 μg/ml, 40 kDa) and culture medium containing 10% FCS. The culture medium included a 1:1 mixture of Dulbecco's modified Eagle's medium and F12 nutrient, supplemented with glucose (33 mM), glutamine (2 mM), NaHCO3 (3 mM) and a mixture of salt and hormones containing insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM) and NaSeO3 (30 nM). Two days after seeding, 2% FCS was included in the culture medium. Under these conditions, after 12-13 days in vitro, the cultures were shown to be highly enriched in neurones which form mature and functional synapses [32], and contained only 7% of astrocytes [33].

Cytosol preparation

Neurones were washed twice and scraped into PBS, and then centrifuged for 10 min at 100 g. The cell pellets were resuspended and homogenized in ice-cold lysing buffer containing 50 mM Tris-HCl (pH 7.5), 3 mM EDTA, 5 μg/ml soybean trypsin inhibitor and 100 μM phenylmethanesulphonyl fluoride in a volume giving a final protein concentration of 1–2 mg/ml and centrifuged for 6 min at 45 000 g. Supernatants (cytosols) were stored at −80°C before use. For the preparation of cytosols from whole rat and mouse brain, rat or mouse brains were homogenized in 7 and 1 ml of lysing buffer respectively.

[32P]NAD+ labelling of GAPDH

Neuronal cytosolic proteins (about 10 μg) were incubated for 60 min at 30°C in a final volume of 60 μl of a medium containing 120 mM phosphate buffer (pH 7.5), 1 mM EDTA, 10 mM thymidine, 2 mM MgCl2, 1 mM ATP, 0.1 mM GTP and [α-32P]NAD+ (0.5 μM, –2 μCi), 1 mM dithiothreitol (DTT) (unless otherwise indicated) and drugs at the indicated concentrations. The reaction was stopped by adding 20 μl of SDS (2%) plus BSA (0.1 mg/ml) followed by overnight precipitation of proteins with ice-cold trichloroacetic acid (TCA, 10%).

One- and two-dimensional gel analysis

For one-dimensional gel analysis, the TCA precipitate, washed with water-saturated ether, was resolubilized in 50 mM Tris-HCl (pH 6.8), 5% SDS and 50 mM DTT for 5 min at 90°C and mixed with Laemmli sample buffer [34]. Samples were then loaded onto 10% polyacrylamide gels for SDS/PAGE. Gels were stained with Coomassie Blue, dried and exposed to X-ray films for 24-48 h. Bands of interest were excised and counted for radioactivity. Two-dimensional (2D) gel analysis consisted of isoelectrofocusing (IEF) followed by SDS/PAGE. IEF gels, containing 2% ampholines (pH 3.5-10), were prepared according to the procedure described by O’Farrell [35]. The TCA precipitates were solubilized in 25 ml of urea (9.95 M), Nonidet P40 (4%), DTT (100 mM) and ampholines pH 6–8 (2%). 2D gels were silver stained or stained with Coomassie Blue and then exposed to X-ray films for 48 h.

Immunoblots

Proteins were transferred onto nitrocellulose sheets. The sheets were washed twice with TBST (200 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 0.2% Tween-20) before blocking non-specific binding of antibodies with TBST supplemented with 5% non fat dry milk (TBSTL) for 12 h at 4°C. Sheets were then incubated for 1 h in TBSTL in the presence of rabbit antiserum raised against pig skeletal muscle GAPDH (1:100 000). This antiserum was shown to recognize a single protein with apparent molecular mass of 39 kDa in cytosols from mouse neurones as well as from mouse or rat brain. The antibodies bound to GAPDH were detected by chemiluminescence coupled to peroxidase activity (using the ECL kit). The amount of GAPDH in cytosolic preparations was estimated using a standard curve constructed with skeletal muscle GAPDH (0.1, 0.2, 0.5, 1 and 2 μg).

Intracellular cGMP measurements

Neurones, grown for 12-13 days in 12-well culture dishes were washed three times at 5 min intervals with 1 ml of Krebs bicarbonate buffer (in mM: NaCl 124; KCl 3.5; K-HPO4, 1.25; NaHCO3, 26.3; CaCl2, 1.2, MgCl2, 1.2; glucose, 10, previously equilibrated with 95% O2/5% CO2) and then incubated for 5 min in this medium in the presence of drugs and 1 mM of the non-selective inhibitor of phosphodiesterases, 3-isobutyl-1-methylxanthine. The incubation was stopped by replacing the medium with 0.5 ml of ethanol/formic acid (95%/5%, v/v) and samples were freeze-dried. cGMP was estimated by radioimmunoassay.

GAPDH activity determination

Neuronal cytosolic proteins (20 μg) were incubated for 1 h with NO and superoxide generating compounds in the medium used for [32P]NAD+ labelling experiments. GAPDH activity was estimated by measuring the conversion of NADH and 1,3-diphosphoglycerate, produced from 3-phosphoglycerate (PGA) by PGK, into NAD+ and 3-phosphoglyceraldehyde respectively. GAPDH activity was monitored in triethanolamine buffer (50 mM, pH 7.5) containing EDTA (1 mM), MgCl2 (10 mM), ATP (3 mM), NADH (0.1 mg/ml), PGA (2 mM) and PGK (5 μg/ml) by reading the absorbance at 334 nm.

RESULTS

NO-generating compounds stimulate the [32P]NAD+ labelling of a neuronal cytosolic 39 kDa protein

Two NO generating compounds, SNP and SIN-1, stimulated the covalent labelling of a single cytosolic protein from cultured striatal neurones in the presence of [adenylate-32P]NAD+, SIN-1 being much more efficient than SNP (maximal stimulatory effects: 877±110% and 266±33% of control 32P incorporation, mean ± S.E.M. of n = 6 independent experiments respectively).
Nitric oxide and oxygen free radicals-included NAD⁺ linkage

The [32P]NAD⁺-labelled protein is GAPDH

Previous studies have demonstrated that NO can induce an NAD⁺-dependent covalent modification of the glycolytic enzyme GAPDH in several peripheral tissues and in whole rat brain homogenates [26–30]. This modification has been identified as a covalent NAD⁺ linkage to the enzyme [29]. Under our experimental conditions, SNP and SIN-1 also stimulated the [32P]NAD⁺ labelling of porcine skeletal muscle GAPDH (results not shown). Furthermore, Western-blot analysis indicated that a specific rabbit antibody generated against purified porcine GAPDH recognized the labelled 39 kDa protein from mouse neurones, as well as from mouse or rat whole brain (Figures 3a and 3b). Finally, autoradiograms of 2D-PAGE showed the same migration pattern for the 32P-labelled muscle and neuronal GAPDH [four spots of isoelectric point (pl) between 8.5 and 9.5] (Figures 3c and 3d). Additionally, silver-stained 2D-PAGE of native muscle GAPDH (not incubated with NAD⁺) revealed the presence of the four spots corresponding to the labelled isoforms of the protein and of an additional more basic isoform (Figure 3e).

The stoichiometry of the NAD⁺-dependent covalent modification of GAPDH induced by NO-generating compounds was very low (0.7 and 2.5 mmol NAD⁺/mol of neuronal GAPDH incorporated in the presence of 200 μM SNP and SIN-1 respectively). In addition, the radiolabelling of GAPDH was proportional to the amount of neuronal cytosolic proteins as well as of purified muscle GAPDH (results not shown), indicating a first-order reaction.

Role of superoxide in the covalent NAD⁺-linkage to GAPDH

The higher efficacy of SIN-1 compared with that of SNP on the [32P]NAD⁺ labelling of GAPDH did not appear to be linked to a larger amount of NO released by this compound. Indeed, the SNP response was saturable, the maximal effect being reached at 100 μM (Figure 1); this saturation was probably not related to an inhibitory effect of the ferrocyanide moiety of SNP, since K₅[Fe(CN)₆]₃ (up to 200 μM) did not decrease the response evoked by the same concentration of SIN-1 (Figure 2, lane 6). In addition, the observed saturation of the SNP response was not due to the reversible loss of 32P-labelling of GAPDH, since the addition of an excess of unlabelled NAD⁺ (10 mM for 2 h) at the end of the incubation period did not decrease the amount of radioactive NAD⁺ bound to GAPDH (results not shown). Kinetic experiments indicated that the SNP-induced NAD⁺ linkage to GAPDH was maximal after 1 h (Figure 4), suggesting that the difference in the efficacy of both NO donors used in this study was not linked to a slower release of NO by SNP. Finally, in contrast to the effects evoked by both NO generating compounds on GAPDH, SNP (200 μM) induced a larger activation of guanylate cyclase in striatal neurones (a response reflecting the amount of NO available) than an identical concentration of SIN-1 (Table 1), suggesting that the amount of NO released is not a limiting factor in the SNP-induced modification of GAPDH.

A recent study has shown that besides NO, SIN-1, but not SNP, can also release superoxide anions [31]. This led us to suppose that O₂⁻ contributes to the SIN-1-induced NAD⁺-dependent covalent modification of GAPDH. In agreement with this hypothesis, superoxide dismutase (SOD, 500 IU/ml), which alone did not alter the spontaneous [32P]NAD⁺-labeling of GAPDH, strongly inhibited the SIN-1 effect (Figure 5, lanes 3 and 5). As a control, SOD, inactivated by heating at 100 °C for 15 min, was totally ineffective. Moreover, the nitrone DMPO (100 mM), a non-selective superoxide scavenger which is not capable of spin trapping NO [36], also markedly inhibited the

![Figure 1](image1.png)

**Figure 1** SNP and SIN-1 stimulate the [32P]NAD⁺ labelling of a 39 kDa cytosolic neuronal protein

Cytosolic fractions containing 10 μg of proteins prepared from cultured striatal neurones were incubated for 1 h in the presence of [32P]NAD⁺ (0.5 μM, 1 μCi) and increasing concentrations of SNP (○) and SIN-1 (●) as described in the Experimental section. Data (radioactivity incorporated into the 39 kDa protein) are expressed in d.p.m. and are the mean±S.E.M. of results obtained in three independent experiments.

![Figure 2](image2.png)

**Figure 2** Involvement of NO in the [32P]NAD⁺ labelling of the 39 kDa neuronal protein

Cytosolic fractions containing 10 μg of proteins prepared from striatal neurones were exposed to the following compounds in the presence of [32P]NAD⁺: 1. control; 2. SIN-1 (200 μM); 3. SIN-1C (200 μM); 4. SNP (200 μM); 5. K₅[Fe(CN)₆]₃ (200 μM); 6. SIN-1 + K₅[Fe(CN)₆]₃; 7. reduced haemoglobin (Hb, 10 μM); 8. SNP + Hb; 9. SIN-1 + Hb. The autoradiograph of an SDS/PAGE obtained in a typical experiment is represented. Two other experiments performed independently yielded similar results.

This protein showed an apparent molecular mass of 39 kDa by SDS/PAGE (Figure 2). The SNP- and SIN-1-induced effects appeared to involve NO, since K₅[Fe(CN)₆]₃, a compound structurally similar to SNP but incapable of generating NO, and SIN-1C, the breakdown product of SIN-1, used at identical concentrations (200 μM), were totally ineffective in increasing the radiolabelling of the 39 kDa protein (Figure 2, lanes 3 and 5). In addition, reduced haemoglobin (10 μM), an NO scavenger, totally suppressed the effects induced by 200 μM of either SNP or SIN-1 (Figure 2, lanes 7–9). The covalent labelling induced by both NO generators was not linked to a change in the NAD⁺/NADH ratio in the neuronal cytosol samples, since identical results have been obtained on dial lysed cytosolic preparations (results not shown).
Figure 3  The labelled 39 kDa neuronal protein is GAPDH

(a) and (b) 1 µg of purified muscle GAPDH (lane 1), 50 µg of cytosolic proteins from mouse striatal neurones (lane 2), whole adult mouse (lane 3) or rat (lane 4) brain were incubated in the presence of [32P]NAD" and 200 µM SIN-1. The proteins were resolved on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose sheets. (a) Autoradiograph of the nitrocellulose sheet. (b) The same membrane was incubated with anti-GAPDH antibodies (1/100000) which were revealed with chemiluminescence linked to peroxidase. (c) Autoradiograph of 2D-PAGE performed with 50 µg of neuronal cytosolic proteins exposed to SIN-1 (200 µM). (d) Inset of an autoradiographed 2D gel performed with 1 µg of purified GAPDH exposed to SIN-1 (200 µM). (e) Inset of a silver-stained 2D gel performed with 1 µg of purified GAPDH. The arrows indicate the different labelled (c and d) and unlabelled (e) isoforms of GAPDH. The data illustrated are representative of three experiments performed independently.

Figure 4  Time-course of the SNP- and SIN-1-induced covalent NAD" labelling of neuronal GAPDH

Neuronal cytosolic fractions (10 µg of proteins) were incubated for the indicated times in the presence of either 200 µM SNP (○) or 200 µM SIN-1 (●). (a) Data, expressed in d.p.m. incorporated into GAPDH, are the mean±S.E.M. of results obtained in three independent experiments.

SIN-1 response (Figure 5, lanes 7 and 8). Although SOD could inhibit NO release by SIN-1 (by affecting the O"-dependent oxidation of the SIN-1 product, SIN-1A) [31,37], the enzyme instead markedly enhanced cGMP accumulation in striatal neurones treated with 200 µM SIN-1 (Table 1). The stimulatory effect is presumably due to the ability of SOD to limit scavenging of NO by O"- [19,31,37]. Finally, in agreement with a role of oxygen free radicals in the NAD" linkage to GAPDH, the superoxide generator pyrogallol (100 µM) [38], which alone was unable to stimulate the radiolabelling of GAPDH, potentiated the SNP response (Table 2). As expected, the enhancing effect of pyrogallol on the NAD" linkage was totally suppressed by SOD (Table 2). However, since SOD also inhibited pyrogallol oxidation itself (results not shown), the precise role of superoxide in this process cannot be assessed directly. Nonetheless, the stimulation of NAD" linkage with the pyrogallol-SNP system coupled with the ability of SOD to inhibit SIN-1-induced linkage, are both consistent with a role of superoxide in the NO-dependent process. Interestingly, SOD and DMPO slightly decreased the labelling of GAPDH induced by SNP alone (Figure 5, lanes 9–11). This observation indicates that O"- also contributes, at least in part, to the SNP response and suggests the presence of endogenous oxygen free radicals in the neuronal cytosolic preparation. According to this hypothesis, it should be noted

Table 1  Effects of NO donors and SOD on intracellular cGMP accumulation in striatal neurones

Striatal neurones (10⁶ neurones/well) were incubated for 5 min with NO donors and SOD at the indicated concentrations in the presence of 3-isobuty1-1-methylxanthine (1 mM) and the cGMP content was estimated by radioimmunoassay. Results are the mean±S.E.M. of four independent determinations. * Significantly different (P < 0.01) from the basal cGMP level (ANOVA followed by Student-Newman-Keuls test). † Significantly different (P < 0.001) from the cGMP level measured in the presence of SIN-1 alone.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cGMP (pmol/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.42±0.04</td>
</tr>
<tr>
<td>SNP (200 µM)</td>
<td>6.51±0.40*</td>
</tr>
<tr>
<td>SIN-1 (200 µM)</td>
<td>4.65±0.95*</td>
</tr>
<tr>
<td>SOD (500 IU/ml)</td>
<td>1.46±0.06*</td>
</tr>
<tr>
<td>SIN-1 (200 µM)+SOD (500 IU/ml)</td>
<td>11.60±0.48†</td>
</tr>
</tbody>
</table>
that SOD alone increased the level of cGMP in striatal neurones (Table 1), indicating an endogenous production of $O_2^-$ (and NO) in neurones.

Superoxide anions can react with NO to form peroxynitrite, leading to the production of an oxidant species having a reactivity similar to hydroxyl radicals (‘OH’) (reaction 1) [39,40, see also 41]. OH$^-$ can also be produced directly from $O_2^-$ by the iron-catalysed Haber–Weiss and Fenton reactions (reactions 2–4) [39].

$$O_2^- + NO \rightarrow ONOO^- + H^+ \rightarrow ONOOH$$

$$\rightarrow 'OH' + NO_2^- \rightarrow NO_2^- + H^+$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$Fe^{3+} + H_2O_2 \rightarrow OH^- + OH^- + Fe^{2+}$$

Hydroxyl radicals did not apparently contribute to the covalent labelling of GAPDH, since the SIN-1 response was not further decreased by the degradation of $H_2O_2$ by catalase (100 IU/ml) both in the absence (results not shown) and the presence of SOD (Figure 5, lanes 5 and 6). Furthermore, dimethyl sulphoxide (DMSO) and mannitol, two selective OH$^-$ scavengers, did not modify the SIN-1 response [98 ± 5 and 94 ± 3 % (n = 3) of the SIN-1-induced $^{32P}]NAD^+$ labelling of GAPDH measured in the presence of 100 mM DMSO and mannitol respectively].

**Table 2** Potentiation of the SNP-stimulated $^{32P}NAD^+$ covalent labelling of neuronal GAPDH by the superoxide generator pyrogallol

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$^{32P}NAD^+$ GAPDH (d.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>SNP</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>SNP + SOD</td>
<td>42 ± 4</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>SNP+pyrogallol</td>
<td>245 ± 10*</td>
</tr>
<tr>
<td>SNP + pyrogallol + SOD</td>
<td>47 ± 5†</td>
</tr>
<tr>
<td>SIN-1</td>
<td>189 ± 25</td>
</tr>
</tbody>
</table>

**Figure 5** Involvement of oxygen free radicals in the covalent $NAD^+$ labelling of neuronal GAPDH

Neuronal cytosolic proteins (10 µg) were incubated for 1 h with $^{32P}NAD^+$ under the following conditions: 1, control; 2, SIN-1 (200 µM); 3, SOD (500 IU/ml); 4, SOD + catalase (100 IU/ml); 5, SIN-1 + SOD; 6, SIN-1 + SOD + catalase; 7, DMPO (100 mM); 8, SIN-1 + DMPO; 9, SNP (200 µM); 10, SNP + SOD; 11, SNP + DMPO. Data, expressed in d.p.m. incorporated into GAPDH, are the mean ± S.E.M. of results obtained in three independent experiments. The inset illustrates a representative experiment. * Significantly different (P < 0.001) from the radiolabelling of GAPDH induced by SIN-1 alone [analysis of variance (ANOVA) followed by Student–Newman–Keuls test].

**Table 2** Potentiation of the SNP-stimulated $^{32P}NAD^+$ covalent labelling of neuronal GAPDH by the superoxide generator pyrogallol

Neuronal cytosolic proteins (10 µg) were incubated for 1 h with $^{32P}NAD^+$ in the presence of drugs used at the following concentrations: SNP, 200 µM; SOD, 500 IU/ml; pyrogallol, 100 µM; SIN-1, 200 µM. Results, expressed in d.p.m. incorporated into GAPDH, are the mean ± S.E.M. of data obtained in three independent experiments. It should be noted that the combined addition of pyrogallol and SNP led to a partial degradation of proteins. Therefore, data have been calculated as a function of the remaining amount of GAPDH estimated by immunoblot. * Significantly different (P < 0.001) from the radiolabelling of GAPDH induced by SNP alone (ANOVA followed by Student–Newman–Keuls test). † Significantly different (P < 0.001) from the radiolabelling of GAPDH induced by SNP in the presence of pyrogallol.

**Superoxide anions potentiate the NO-induced inhibition of GAPDH activity**

Previous studies performed in other laboratories have indicated that NO inhibits the catalytic activity of GAPDH [27,29]. Confirming these results, SNP dose-dependently inhibited the dehydrogenase activity of neuronal GAPDH (Figure 6), and this effect was reversed by haemoglobin (≥ 10 µM, results not shown). Interestingly, as observed for the SNP-induced covalent $NAD^+$ linkage to the enzyme, pyrogallol (100 µM) markedly enhanced the inhibition of GAPDH activity triggered by SNP (Figure 6). Moreover, SIN-1 almost totally inhibited GAPDH activity (7 ± 1 % of the basal activity was measured after a 1 h treatment with 200 µM SIN-1, n = 3), and thus reproduced the inhibition evoked by the combined application of SNP (200 µM) and pyrogallol (100 µM). Similar results have been obtained with purified skeletal muscle GAPDH (not shown). It should be noted that pyrogallol alone did not alter the activity of GAPDH (Figure 6). Although the covalent $NAD^+$ linkage only represents a minor fraction of the protein, these results suggest that an $O_2^-$/NO generating system more efficiently inactivates the enzyme than does NO alone.

**Cysteine residues are involved in the NO- and superoxide-induced covalent $NAD^+$ linkage to GAPDH**

As previously shown for the NO-induced covalent modification of GAPDH from various tissues, a cysteine residue is involved in the effects of NO and oxygen free radicals on neuronal GAPDH [26–30], probably that present in the active site of the enzyme [42]. Indeed, a 1 h treatment with $HgCl_2$ (2 mM), which is known to cleave ADP-ribosylcysteine linkages as well as other covalent linkages to sulphhydryl groups [29], removed 96 ± 5 % and 95 ± 3 % (n = 3) of the radioactive $NAD^+$ covalently bound to GAPDH in the presence of 200 µM SNP and SIN-1 respectively, compared with the values measured after a 2 mM NaCl treatment (instead of $HgCl_2$). Further supporting the involvement of thiol groups, the pretreatment of neuronal cytosolic preparations with the alkylating agent N-ethylmaleimide (3 mM) inhibited by 97 ± 4 % and 98 ± 2 %, (n = 3) the $^{32P}NAD^+$-labelling of GAPDH induced by 200 µM SNP and SIN-1 respectively. Interestingly, after a 2 h pretreatment of GAPDH in the presence of $HgCl_2$ (up to 10 mM), the five isoforms of the enzyme were still observed on silver-stained 2D-gels (results not shown). In addition, this treatment did not change their respective pls.

Therefore, the four most acidic isoforms of GAPDH (Figures 3d and 3e) correspond to both $NAD^+$-linked and unlinked proteins and do not derive from the most basic isoforn (Figure 3e) via the binding of $NAD^+$. 

2O$_2^-$ + 2H$^+$ → H$_2$O$_2$ + O$_2$  

(2)

O$_2^-$ + Fe$^{3+}$ → O$_2$ + Fe$^{2+}$  

(3)

Fe$^{3+}$ + H$_2$O$_2$ → OH$^-$ + OH$^-$ + Fe$^{2+}$  

(4)
Neuronal cytosolic proteins (10 μg) were incubated for 1 h in the medium used for NAD⁺ labelling in the presence of 1 mM DTT and the indicated concentrations of SNP and pyrogallol: ○, control; ●, +100 μM pyrogallol. GAPDH activity was then estimated as indicated in the Experimental section. Data have been calculated as a function of the remaining amount of GAPDH estimated by immunoblot. Results are the mean ± S.E.M. of three determinations and are expressed as a percentage of basal GAPDH activity (0.97 ± 0.09 IU/mg neuronal cytosolic protein, n = 3, measured in the absence of SNP and pyrogallol). * Significantly different (P < 0.01) from GAPDH activity measured in the presence of the same concentration of SNP without pyrogallol (ANOVA followed by Student–Newman–Keuls test).

Table 3. Effects of DTT on the spontaneous and SNP- or SIN-1-stimulated covalent NAD⁺ labelling of neuronal GAPDH

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No DTT</th>
<th>DTT (1 mM)</th>
<th>DTT (10 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>31 ± 2</td>
<td>21 ± 2†</td>
<td>7 ± 2†</td>
</tr>
<tr>
<td>SNP (200 μM)</td>
<td>35 ± 4</td>
<td>58 ± 7*</td>
<td>24 ± 2*</td>
</tr>
<tr>
<td>SIN-1 (200 μM)</td>
<td>124 ± 15*</td>
<td>189 ± 25*</td>
<td>106 ± 14*</td>
</tr>
</tbody>
</table>

Role of reducing agents

Reducing agents such as DTT (1 mM, Table 3) or reduced glutathione (1mM, results not shown) were required for stimulation of the radiolabelling of GAPDH by SNP. On the contrary, SIN-1 was effective alone, but its response was enhanced by a low concentration of DTT (1 mM). DTT, as well as reduced glutathione, can bind NO to form nitrosothiols, which could be intermediate carriers of NO to GAPDH. This interpretation is in agreement with the involvement of the S-nitrosylation of a cysteine residue in the covalent NAD⁺-binding to the protein [28]. However, when higher concentrations of DTT ( > 2 mM) were added, the effects of both NO donors were strongly reduced. These effects of high concentrations of DTT on NO donor-induced GAPDH labelling could reflect a competition for NO between the sulphhydril groups of the reducing agent and those present in GAPDH to form S-nitrosothiols. Interestingly, the spontaneous NAD⁺ linkage to GAPDH (i.e. measured in the absence of NO donors) was markedly decreased in the presence of DTT (up to 10 mM, Table 3). This inhibition may indicate that GAPDH had been, at least in part, previously nitrosylated by endogenous NO.

DISCUSSION

Our study demonstrates for the first time that superoxide anions enhance the NO-induced covalent linkage of NAD⁺ to GAPDH from cultured striatal neurones. This conclusion is supported by the following observations. (1) SIN-1, which is known to release both radical species, was more efficient than SNP, which mainly produces NO, in increasing the [³²P]NAD⁺ labelling of GAPDH. (2) SOD and the non-selective superoxide scavenger DMPO markedly reduced the SIN-1 response. However, the SNP response was also slightly reduced in the presence of SOD and DMPO. This last result could be explained by endogenous production of a small amount of superoxide by cytosolic enzymes such as aldehyde oxidases and xanthine oxidase [40] or resulting from free arachidonic acid metabolism [18]. An alternative explanation of the inhibition by SOD of the SNP response may be the conversion by this enzyme of NO⁻ into NO⁺ [43], which should be ineffective as a mediator of NAD⁺ labelling of GAPDH. Such a mechanism could also account for the decrease of the SIN-1 response in the presence of SOD. (3) Nevertheless the superoxide generator pyrogallol, potentiated the SNP-induced covalent modification of GAPDH to a level similar to that observed in the presence of SIN-1 and this effect was suppressed by SOD.

It is noteworthy that NO is required for the superoxide effect. Indeed, the generation of superoxide alone by pyrogallol did not increase the binding of [³²P]NAD⁺ to GAPDH. Furthermore, the non-selective NO scavenger, reduced haemoglobin, totally suppressed the SIN-1 response, proposed to result from the combined action of NO and O₂⁻. Therefore, this study indicates that superoxide anions only enhance the NO-induced NAD⁺ linkage to GAPDH.

At least two alternative hypothesis can be proposed to explain this observation. (1) Superoxide anions facilitate, through an unknown mechanism, the NO-induced NAD⁺ linkage to GAPDH. (2) Peroxynitrite anions, resulting from the reaction of NO with O₂⁻, could be more efficient than NO in inducing GAPDH covalent modification. Indeed, SIN-1, which generates NO and O₂⁻ stoichiometrically and concurrently, thus leading to the formation of peroxynitrite [44], was more efficient than SNP. In agreement with the role of peroxynitrite in the SIN-1-induced NAD⁺-linkage to GAPDH, SOD decreased this SIN-1 effect. Moreover, this SOD inhibition was not due to a decrease in the amount of available NO, since, on the contrary, this enzyme enhanced the SIN-1-induced cGMP formation in neurones.

As previously shown for the NO-induced covalent modification of GAPDH from various tissues [26–30,42], a cysteine residue (probably that present in the active site of the enzyme) is involved in the effects of NO and oxygen free radicals on neuronal GAPDH. Peroxynitrite has been shown to release compounds that could have strong nitrosylating properties, such as NO₂⁻. Assuming that the S-nitrosylation of GAPDH is involved in the covalent NAD⁺ linkage [28], one can speculate that peroxynitrite was more efficient than NO in nitrosylating the cysteine residue which binds NAD⁺. It should be noted that SOD also partially decreased the SNP response, suggesting that peroxynitrite, resulting from the reaction of NO and endogenous superoxide, could also contribute to the SNP-induced covalent NAD⁺ linkage.
The effects of DTT on both the spontaneous and NO donor-induced NAD⁺-linkage to GAPDH are in agreement with the hypothesis of the S-nitrosylation of the protein. Indeed, DTT, at low concentrations, could serve as an intermediate carrier of NO to nitrosylate GAPDH, whereas, when used at higher concentrations, it could compete with the cysteine residues present in the protein to bind NO and form nitrothiols. The permissive effect of low concentrations of DTT or reduced glutathione on the SNP-induced NAD⁺ labelling of GAPDH could also be related to the requirement of reductants for the release of NO from SNP.

2D gel analysis indicated that among the five detectable isoforms of GAPDH, only the four most acidic isoforms could be covalently labelled in the presence of [³²P]NAD⁺. Interestingly, in contrast to the ADP-ribosylation of cysteine or arginine residues of α-subunits of G-proteins [45], the covalent linkage of NAD⁺ to these isoforms of GAPDH did not alter their pI. Indeed, a 2 h treatment of GAPDH with HgCl₂, which cleaves covalent linkages to sulphhydryl groups, did not change the pI of the five isoforms of the enzyme. The absence of a modification of pI is quite surprising and raises the question as to the nature of the chemical bond existing between GAPDH and the NAD⁺ residue.

Confirming the results obtained by several investigators in other preparations [26–30], the stoichiometry of the labelling of the neuronal GAPDH in vitro was very low (less than 1% of GAPDH was modified). A possible explanation of this result is that an important proportion of the protein has been previously linked covalently to endogenous NAD⁺. However, NO has been shown to inhibit markedly the catalytic activity of GAPDH [27,28]. In this study, we have further demonstrated that supernoxide anions, which alone are ineffective, strongly enhance the NO-induced inactivation of GAPDH. As previously suggested, the strong inhibition of GAPDH activity in the presence of NO and superoxide seems to result from the S-nitrosylation of the cysteine present in the active site of the enzyme rather than the covalent NAD⁺-linkage [28,42] and may be related to the formation of strong nitrosylating compounds in the presence of both radical species. Although the low stoichiometry of the NAD⁺-linkage to GAPDH in vitro raises the question of the physiological relevance of this phenomenon, this covalent modification appears to be modulated in intact cells. Indeed, preliminary results have indicated that the pretreatment of intact striatal neurones with either glutacl or NMDA, which stimulate the production of both NO and superoxide in neuronal cells [3,18], decreases the subsequent [³²P]NAD⁺ labelling of GAPDH measured in vitro, suggesting that GAPDH is an endogenous target of these excitatory amino acids in intact neurones. The respective contribution of NO and superoxide in this glutamate response is currently under investigation. Similarly, a recent study has shown that the induction of long-term potentiation in the CA1 area of the rat hippocampus, which probably involves NO formation following the activation of NMDA receptors [5,6], is associated with endogenous NAD⁺-dependent covalent modification of several proteins including GAPDH [46]. These data, together with the recent observation that the stimulation of endogenous NO production in pancreatic β-cells, induced by exposure to the cytokine interleukin-1β, is accompanied by an increase in the NAD⁺-linkage to GAPDH [47], suggest a physiological relevance of this phenomenon.

Besides its glycolytic activity, GAPDH has been implicated in several other cellular functions, such as microtubule bundling [48], phosphorylation processes [49] and DNA repair [50]. In addition, a recent study has revealed that GAPDH can bind tRNAs and is involved in their translocation from the nucleus to the cytosol [51]. Interestingly, the binding of GAPDH to nucleic acids was inhibited by NAD⁺, suggesting that it occurs at the NAD⁺-binding sites of the protein [50,51]. We can thus speculate that the covalent NAD⁺-linkage to GAPDH induced by NO and superoxide also alters the tRNA export and DNA repair processes. Finally, GAPDH has also been shown to interact with the recombinant cytoplasmic domain of Alzheimer’s β-amyloid precursor protein [52]. The covalent modification of GAPDH induced by NO and oxygen free radicals could also alter the proteolysis of this peptide and thus be involved in the development of this neurodegenerative disease.

The authors would like to thank Drs. H. Cheneviex and M. Lévy-Stauss for helpful discussions. This research was supported by grants from Institut National de la Santé et de la Recherche Médicale (INSERM), Direction des Recherches, Etudes et Techniques (DRET, contract No. 90/078) and Rhône Poulenc Rorer.

REFERENCES

4 Snyder, S. H. (1992) Science 257, 494–496


Received 8 August 1994/6 April 1995; accepted 7 April 1995