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Modification of macrophage glyceraldehyde-3-phosphate dehydrogenase in response to nitric oxide

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Abstract

A potential cytotoxic, self-destructive role of endogenously generated and exogenously supplied nitric oxide (NO) was studied in two mouse monocytic macrophage cell lines (RAW 264.7 and J774.1). Our attention centered on NO-mediated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) modification and inhibition of the Krebs cycle enzyme, aconitase, related to macrophage cell death. NO formed by an active inducible nitric oxide synthase significantly decreased cell viability in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay. Similarly, cell viability was inversely and dose-dependently correlated to increasing concentrations of the NO-releasing compound, sodium nitroprusside. Biochemically, we noticed a correlation between endogenously derived or exogenously generated NO and inhibition of GAPDH as well as aconitase enzyme activity. The involvement of NO was further substantiated by the use of N^G-monomethyl-L-arginine. Associated with decreased GAPDH enzyme activity, ^{32}P-NAD$^+$-dependent modification of the enzyme in the cytosol of pretreated cells was hindered. This reflects intracellular protein modification as a result of NO signalling. Using sodium nitroprusside we achieved GAPDH translocation from the cytosol to the plasma membrane or the nucleus of treated cells. However, despite GAPDH modification, lactate production was not rate limiting during NO intoxication. Furthermore, blocking the iron-sulfur-containing enzyme, aconitase, is insufficient to produce macrophage cell death. Although RAW 264.7 and J774.1 cells show substantial variation in their sensitivity towards NO it can be concluded that NO-mediated macrophage cell death is not linked to energy depletion. For GAPDH, NO-mediated protein modification may be related to functions of the enzyme, other than its glycolytic role.

Keywords: Nitric oxide (NO); Glyceraldehyde-3-phosphate dehydrogenase; Aconitase; Cell death

1. Introduction

Nitric oxide (NO) is gaining recognition as an important biological mediator (Nathan, 1992; Moncada et al., 1991; for review). The expression of a variety of physiological and pathophysiological effects is achieved through both cyclic GMP (cGMP)-dependent as well as cGMP-independent signalling. Molecular mechanisms include NO's interaction with targets via ligand interaction at various redox centers. Heme proteins like soluble guanylyl cyclase, iron-sulfur proteins i.e. aconitase, complex I and II of the mitochondrial respiratory chain or ribonucleotide reductase (Nathan, 1992; for review) are examples of metal-containing target sites. Nitrosative chemistry at nucleophilic centers with prevalence of thiols over other biological nucleophiles supports *S*-nitrosothiol (RS-NO) formation. Nitration reactions of i.e. tyrosine residues, the interaction with oxygen (O_2) or superoxide (O_2^-) with the formation of various NO_x compounds or peroxynitrite ($ONOO^-$), respectively, enlarge potential patho-physiological signalling mechanisms (Stamler, 1994).

Nitric oxide synthases are dimeric, Ca^{2+}-calmodulin-dependent P450-like hemoproteins with several tightly bound redox cofactors, i.e. FAD, FMN and tetrahydrobiopterin required during L-arginine-dependent NO formation. NO synthase isoenzymes can broadly be categorized based on the biochemical feature of intracellular Ca^{2+} transients required for full activity as virtually Ca^{2+}-dependent or Ca^{2+}-independent forms. The latter are also known as cytokine and/or bacterial lipopolysaccharide inducible

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enzymes. Extending this characterization allows the approximation of a low (constitutive) versus a high output (inducible) system for NO. A further determinant is the duration of NO synthase activity under physiological conditions. Constitutive enzymes normally terminate activity after several minutes (Snyder and Bredt, 1991), whereas inducible enzymes are active much longer, up to several days. Physiological signalling seems involved in neurotransmission, peristalsis, and blood pressure control linked to endothelium-derived relaxation. Activation of soluble guanylyl cyclase, formation of cGMP and downstream phosphorylation events are key transduction steps (Ignarro, 1990). Pathophysiological concentrations of NO are generated by the high-output NO synthase system. Cytotoxic mechanisms are directed against tumor cells, invading bacteria or protozoans as a first line immunological host defense system. Moreover, enhanced NO formation is detected during endotoxic shock (Moncada et al., 1991), neuronal damage, autoimmune diseases, immune rejections of allografted organs or insulin-dependent diabetes mellitus type I, when upregulated NO synthesis becomes self-destructive (Nathan, 1992 for references). The toxicity of NO under these conditions is dependent on the chemistry it undergoes in a given biological milieu, mainly attributed to inhibition of iron-sulfur proteins, like aconitase.

One-electron oxidation of $NO^{\cdot}$ to NO^{+} (nitrosonium) generates an efficient nitrosating species which likely targets thiol anions (RS^{-}) in various proteins. This is exemplified for tissue plasminogen activator, cathepsin B (Stamler et al., 1992), the glutamate receptor and enzymes like protein kinase C (Gopalakrishna et al., 1993), γ-glutamylcysteinyl synthase or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mohr et al., 1994). GAPDH modification, addressed as an ADP-ribosylation-like reaction has received particular attention in view of evidence that NO stimulates an NAD^{+}-dependent modification of active site thiol in association with loss of enzyme activity (Dimmeler et al., 1992). Further studies, however, suggest the covalent binding of both ADP-ribose and nicotinamide in the presence of NO (McDonald and Moss, 1993). GAPDH modification due to NO action has been investigated mostly in several in vitro systems and RINm5F cells (Dimmeler et al., 1993). Since β cells (RINm5F cells) are known for their vulnerability to NO we intended to study biochemical alterations in response to NO in cells equipped with the machinery of massive NO production. Therefore, two mouse monocytic leukaemia cell lines, RAW 264.7 and J774.1 macrophages were employed.

Our study focused on GAPDH activity, NAD^{+}-dependent modification of the protein and intracellular GAPDH redistribution between the cytosolic and particulate compartment following cytokine-induced endogenous NO generation compared to the action of exogenously supplied NO. Potential cytotoxic mechanisms of NO in NO-producing macrophages related to cell viability, aconitase activity, and glycolysis are discussed.

2. Materials and methods

2.1. Materials

SIN-1 (3-morpholinosydnonimine) was provided by Cassella-Pharma, Frankfurt, Germany. ^{32}P-NAD^{+} (800 Ci/mmol) and ^{125}I-protein A (9.01 μCi/μg) were bought from Du Pont-New England Nuclear. Lipopolysaccharide (*Escherichia coli*, serotype 0127:B8), glyceraldehyde-3-phosphate, sodium nitroprusside, N^{G}-monomethyl-L-arginine and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were from Sigma, Deisenhofen, Germany. Lactate dehydrogenase (from rabbit muscle), recombinant murine interferon-γ (IFN-γ), isocitrate dehydrogenase, NAD^{+}, and $NADP^{+}$ were purchased from Boehringer Mannheim, Mannheim, Germany. RAW 264.7 cells were kindly provided by Prof. A. Wendel, University of Konstanz, Konstanz, Germany. J774.1 cells were generously given by Prof. R. Busse, University of Frankfurt, Frankfurt, Germany. All other chemicals were of the highest purity available and obtained from local commercial sources.

2.2. Cell culture

The mouse monocyte/macrophage cell lines, RAW 264.7 and J774.1, were maintained in RPMI 1640 (with 0.532 g/l *N*-acetyl-L-alanyl-L-glutamine) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (complete RPMI). All experiments were performed using complete RPMI. For enzyme modification, cells were cultured in 6-well culture plates (10^{6} cells/ml, 4 ml/well), allowed to adhere overnight, and stimulated with the inducers or inhibitors as indicated, after change of the medium. Stimulation of endogenous NO production was achieved with a combination of lipopolysaccharide (10 μg/ml) and IFN-γ (10–100 U/ml) applied for 24 h. The NO synthase inhibitor (N^{G}-monomethyl-L-arginine; 1 mM) was added simultaneously with lipopolysaccharide/IFN-γ.

2.3. Glyceraldehyde-3-phosphate dehydrogenase enzyme activity

GAPDH activity was monitored by following the reduction of NAD^{+} to NADH, recording the fluorescence signal above 430 nm with excitation at 313 and 366 nm, respectively. 8×10^{6} cells from individual experiments were scraped off the culture plate, centrifuged for 10 min at $800 \times g$ and resuspended in 500 μl homogenization buffer (100 mM Hepes, pH 7.4, 2 mM dithiotreitol, 1 mM phenylmethanesulfonylfluoride). This was followed by sonication (Branson sonifier; output control 3, duty cycle 100%, three times for 15 s), and centrifugation (10 min at $10\,000 \times g$ and 30 min at $100\,000 \times g$) in order to recover the cytosolic fraction (supernatant) or the membrane frac-

tion (pellet). For activity measurements 1–10 μg cytosolic protein was incubated in 50 mM Tris buffer, pH 7.6, 50 mM arsenate, 1 mg/ml glyceraldehyde-3-phosphate and 2.4 mM glutathione for 5 min at 37°C prior to the addition of 250 μM NAD^+, to start the enzymatic reaction. The linear part of the reaction was used for calculations.

2.4. Covalent NAD^+-dependent GAPDH modification

The covalent NAD^+-dependent protein modification was mainly carried out as outlined previously (Dimmeler and Brüne, 1992). Cytosolic protein (supernatant; 50 μg) was incubated in 100 mM Hepes buffer, pH 7.5, 2 mM dithiotreitol, 1 μM cold NAD^+ and 0.25 μCi ^{32}P-NAD^+ in a total volume of 100 μl for 30 min at 37°C. Proteins were precipitated with 500 μl 20% (w/v) trichloroacetic acid, washed twice with cold water-saturated ether, and were resolved in 10% sodium dodecylsulfate (SDS)-polyacrylamide gels (Laemmli, 1970). The 'differential radioactive labelling method' assumes that endogenously formed NO leads to a covalent modification of GAPDH using intracellular, unlabelled NAD^+, while a subsequent ^{32}P-NAD^+-dependent modification of GAPDH in the cytosolic fraction in the presence of the NO donor SIN-1 results in decreased incorporation of radioactivity from ^{32}P-NAD^+ into GAPDH.

2.5. Western blot

Proteins (100 μg/lane) were resolved on 10% sodium dodecylsulfate-polyacrylamide gels, and blotted onto nitrocellulose sheets using the semi-dry blot system from Pharmacia (0.8 mA/cm^2, 1.25 h, 25 mM Tris/192 mM glycine as buffer system). The sheets were washed twice with Tris-buffered saline (TBS) (140 mM NaCl, 50 mM Tris, pH 7.2) containing 0.1% Tween 20 before blocking unspecific binding with TBS/2% bovine serum albumin for 1 h at 20°C. The rabbit antiserum raised against human erythrocyte GAPDH (Dimmeler et al., 1993) was added (1:500 in TBS/0.2% bovine serum albumin) and incubated overnight at 4°C. Thereafter sheets were washed (five times, 15 min) in order to block unspecific binding. Detection was performed with ^{125}I-protein A (2 ng/ml protein A, 1 μCi in TBS/0.06% Tween 20/0.1% bovine serum albumin), incubated for 2 h. Afterwards the blots were washed three times and radioactivity was detected using the phosphor image system (Molecular Dynamics) (Johnston et al., 1990).

2.6. Aconitase activity

Aconitase catalyses the isomerization of citrate to isocitrate. Activity determination is coupled to the conversion of isocitrate to α-ketoglutarate using isocitrate dehydrogenase following the reduction of $NADP^+$ to NADPH. The accumulation of NADPH was monitored by recording the fluorescence signal above 457 nm after excitation at 340 nm. For each experiment 4×10^6 cells were scraped off the culture plate, centrifuged at $800 \times g$ for 5 min and resuspended in 400 μl homogenization buffer (100 mM Hepes, pH 7.4, 2 mM dithiotreitol, 1 mM phenylmethanesulfonyl fluoride, 50 μM leupeptin). The cells were sonicated (Branson sonifier; output control 3, duty cycle 100%, three times for 15 s) to disrupt cell, mitochondrial, and nuclear membranes. The homogenate was centrifuged for 10 min at $10000 \times g$ to remove cell debris. The supernatant was used for total aconitase activity (cytosolic and mitochondrial) and protein (Bradford assay (Bradford, 1976)) determination. Aconitase assays were performed in a total volume of 2 ml containing 50 mM Tris/HCl, pH 7.4, 2 mM $MnCl_2$, 250 μM $NADP^+$, 0.7 U/ml isocitrate dehydrogenase and 250 μg cytosolic protein at 25°C. After a preincubation period of 5 min the reaction was started by addition of 100 μM sodium citrate and following the change in fluorescence for 3 min.

2.7. Nitrite determination

Nitrite, a stable NO oxidation product, was determined using the Griess reaction (Ding et al., 1988). Cell-free culture supernatants were collected (200 μl), adjusted to 4°C, mixed with 20 μl sulphanilamide (dissolved in 1.2 M HCl) and 20 μl *N*-naphthylethylenediamine dihydrochloride. After 5 min at room temperature the absorbance was measured at 560 nm with a reference wavelength at 690 nm. Nitrite concentrations were calculated using a $NaNO_2$ standard.

2.8. MTT cytotoxicity assay

To test the viability of metabolically active cells, the enzymatic reduction of the yellow MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to a blue formazan product in intact cells was used (MTT cytotoxicity assay) (Mosmann, 1983). 2×10^5 cells/200 μl were grown in 96-well, flat bottomed microtiter plates and incubated for 24 h using the substances indicated. Afterwards, MTT (5 mg/ml, 20 μl/well) was added and incubation went on for 3 h at 37°C. After removal of the supernatant, the cells were lysed (100 μl 0.04 M HCl in isopropanol) and absorbance was determined at 560 nm.

2.9. Measurement of LDH release

Plasma membrane integrity was determined by measuring culture medium LDH (lactate dehydrogenase) activity. The activity was monitored by NADH oxidation following the decrease in absorbance at 334 nm. Reactions were carried out in a KP_i buffer, pH 7.5, containing 0.24 mM NADH, 0.62 mM pyruvate. The percentage of LDH released was defined as the ratio of LDH activity in the supernatant to the sum of the LDH activity released plus

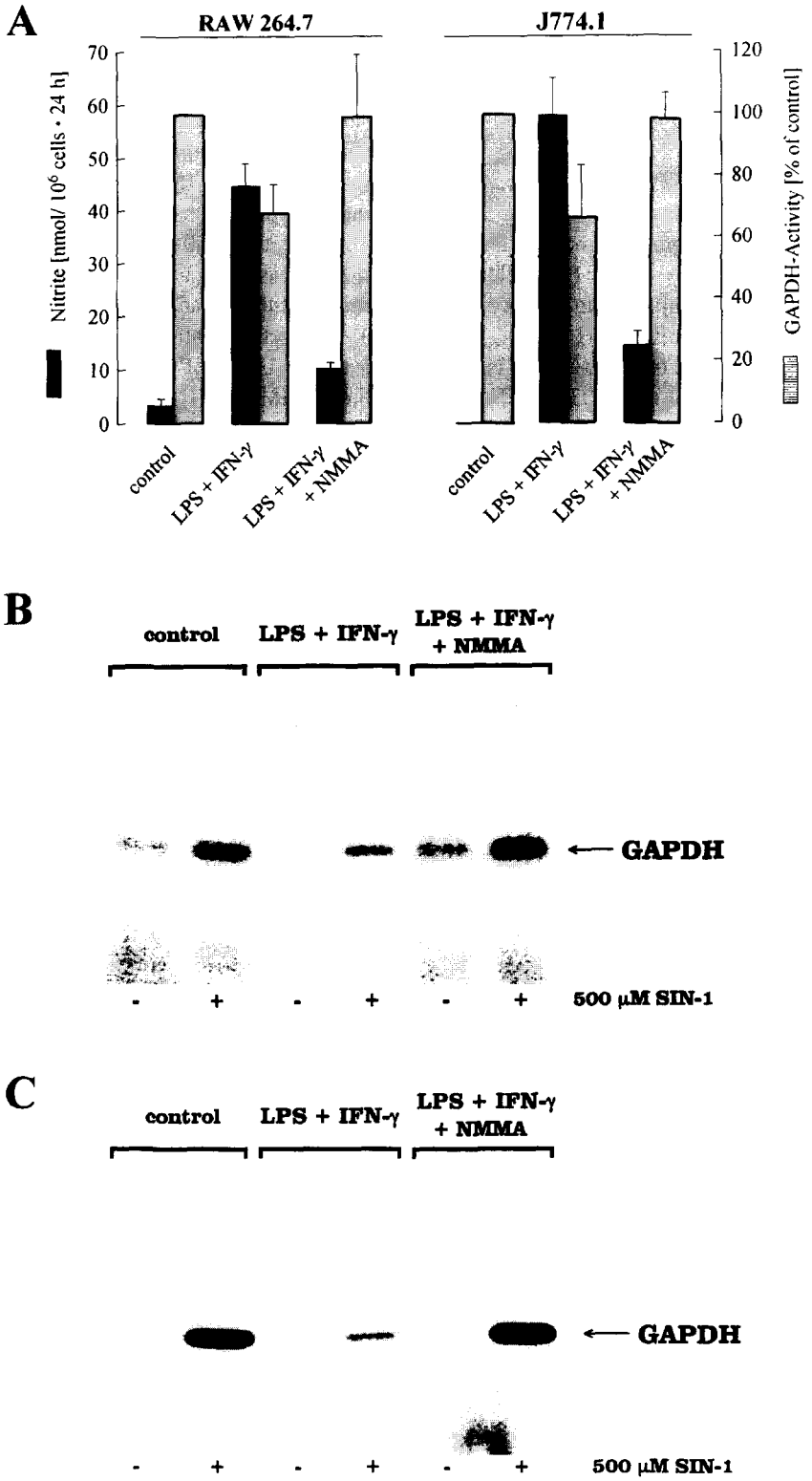

Fig. 1. Modification of GAPDH following endogenous NO production. RAW 264.7 and J774.1 macrophages (8×10^6 cells/8 ml) were incubated in complete RPMI for 24 h with 10 µg/ml lipopolysaccharide (LPS), 100 U/ml IFN-γ, and 1 mM N^G-monomethyl-L-arginine (NMMA) as indicated or remained as controls. A: GAPDH activity was measured in the cytosolic fraction while nitrite accumulation was determined in the culture supernatant as described in Materials and methods. Data are means ± S.D. of four or five independent determinations. The 'differential radioactive labelling method' was applied to detect covalent NAD^+-dependent modification of GAPDH in the cytosolic fractions of RAW 264.7 (B) and J774.1 (C) macrophages. The covalent ^{32}P-NAD^+-dependent protein modification and detection of radioactivity using the phosphor image system was outlined in Materials and methods. The gel reveals a molecular weight range of 25–60 kDa. Representative gels from four independent experiments are shown for each cell line.

the activity measured in the cell lysate. Cells were lysed by adding phosphate buffered saline (PBS)/0.2% Triton X-100.

2.10. Lactate determination

For these measurements, L-lactate was enzymatically oxidized to pyruvate using lactate dehydrogenase while NAD^+ was stoichiometrically reduced to NADH. To quantitate lactate dehydrogenation, pyruvate was removed as a pyruvate-hydrazone complex, while protons were neutralized with alkali (Bergmeyer, 1974).

For lactate determinations, 1×10^6 cells were incubated in 1 ml complete RPMI supplemented with the substances indicated. Culture supernatants were collected at the time points given in Table 2. Prior to lactate determination, culture supernatants were deproteinized with an equal volume of 1 N perchloric acid and centrifuged for 5 min at $10000 \times g$, followed by neutralization using 1/5 volume 1.2 M $KHCO_3$/1.44 M KOH, and centrifugation. Reactions were performed in a total volume of 3 ml containing 0.43 M glycine, 0.34 M hydrazine, pH 9.0, 2.75 mM NAD^+ and 200 μl of the treated culture supernatant. Assays were started by addition of 18 U/ml lactate dehydrogenase followed by a 30 min incubation at 37°C. The increase in NADH following the absorbance at 340 nm was used to calculate the amount of lactate being produced.

2.11. Statistics

Each experiment was performed at least three times and statistical analysis was done with the two-tailed Student's t test.

3. Results

3.1. Modulation of GAPDH and cell viability by endogenous NO

GAPDH and aconitase are two enzymes indirectly involved in energy production. Their modulation by endogenously and exogenously supplied NO was studied in the mouse macrophage cell lines, RAW 264.7 and J774.1. Both cell lines responded with a marked nitrite accumulation in the cell supernatant when challenged with 10 μg/ml lipopolysaccharide and 100 U/ml IFN-γ (Fig. 1A). Formation of the Griess reactive material was significantly reduced in the presence of the NO synthase inhibitor, N^G-monomethyl-L-arginine. The J774.1 macrophages produced more nitrite than did RAW cells. Individual values amounted to roughly 60 nmol nitrite/10^6 cells $\times$ 24 h versus 45 nmol nitrite/10^6 cells $\times$ 24 h, respectively. To investigate GAPDH modification under these conditions, we stimulated cells with lipopolysaccharide and IFN-γ for 24 h followed by activity determination (Fig. 1A), quantification of the covalent NAD^+-dependent protein modification (Fig. 1B and Fig. 1C), and measurement of GAPDH. GAPDH activity was decreased by roughly 40% in RAW and J774 cells. Inhibition was reversed in the presence of the NO synthase inhibitor N^G-monomethyl-L-arginine. In search of an explanation, we investigated a possible NAD^+-dependent posttranslational modification of the enzyme. As radiolabelled NAD^+ is not membrane permeable, we used an indirect method described for bacterial toxin-catalyzed ADP ribosylation reactions (Just et al., 1992). The assay termed 'differential radioactive labelling method' is based on the difference between the endogenously modified protein, which is less susceptible to further modification using ^{32}P-NAD^+ in the cytosolic fraction of treated cells. Assuming NO-induced, NAD^+-dependent covalent GAPDH modification as the underlying mechanism for decreased enzyme activity, the differential radioactive labelling method was carried out on the cytosolic fraction of control versus lipopolysaccharide/IFN-γ-treated cells in the presence of maximal concentrations of the NO donor, 3-morpholinosydnonimine (SIN-1). We observed a low basal radioactive modification of GAPDH in the absence of the NO donor, SIN-1, in control cytosol of both cell lines (Fig. 1B and Fig. 1C). Addition of SIN-1 resulted in a marked incorporation of radioactivity from ^{32}P-NAD^+ into GAPDH. When measuring incorporation of radioactivity in the cytosol of cells stimulated with lipopolysaccharide and IFN-γ, we detected no basal auto-modification of the protein, and only a marginal stimulatory effect of SIN-1. This was evident for RAW 264.7 (Fig. 1B) and J774.1 cells (Fig. 1C). Stimulating cells with lipopolysaccharide and IFN-γ in the presence of N^G-monomethyl-L-arginine (1 mM) restored SIN-1-stimulated radioactive GAPDH labelling, comparable to the controls. Excluding decreased protein synthesis and/or increased degradation of GAPDH as interfering signals, we determined the amount of GAPDH protein. Western blot analysis revealed a slightly decreased GAPDH in the cytosol of stimulated cells versus controls (interassay differences < 10%, data not shown).

Table 1
Inhibition of cell viability by endogenous NO production

	RAW 264.7	J774.1
	% control	% control
Control	100	100
LPS + IFN-γ	45.24 ± 3.66 [a]	58.22 ± 3.48 [a]
LPS + IFN-γ + NMMA	85.52 ± 2.12 [b]	79.42 ± 4.65 [c]

RAW 264.7 and J774.1 macrophages (10^6 cells/ml) were incubated for 24 h with 10 μg/ml lipopolysaccharide (LPS), 100 U/ml IFN-γ and 1 mM N^G-monomethyl-L-arginine (NMMA) as indicated or remained as controls. Cell viability was measured with the MTT cytotoxicity assay as described in Materials and methods. Data are the means ± S.E.M. from five separate experiments. [a] $P < 0.001$ versus control incubations, [b] $P < 0.001$ and [c] $P < 0.02$ versus stimulated cells without NMMA.

Paying attention to possible cytotoxic effects as a result of excessive NO formation we studied cell viability employing the MTT assay, basically addressing mitochondrial succinate dehydrogenase activity. As shown in Table 1, addition of lipopolysaccharide/IFN-γ decreased the MTT-reducing ability in RAW and J774 macrophages. N^G-Monomethyl-L-arginine largely restored the mitochondrial MTT-reducing potency. RAW cells responded with roughly 50% reduced MTT transformation whereas J774.1 macrophages showed only 40% reduction, although producing more nitrite (Fig. 1).

3.2. Modification of GAPDH by sodium nitroprusside

To directly address GAPDH modulation by NO under cellular conditions, excluding any possible interference with lipopolysaccharide/IFN-γ signalling, we performed experiments with the NO donor, sodium nitroprusside. Sodium nitroprusside incubated for 24 h caused a concentration-dependent inhibition of GAPDH activity in RAW 264.7 and J774.1 cells which was paralleled by nitrite accumulation (Fig. 2A). GAPDH activity was more efficiently blocked in RAW macrophages, which degraded sodium nitroprusside more potently as judged by higher nitrite values. Using the 'differential radioactive labelling method' we probed for NAD^+-dependent covalent GAPDH modification under these conditions. In RAW 264.7 macrophages (Fig. 2B), basal as well as SIN-1 stimulated radioactive labelling of GAPDH decreased dose dependently in cytosol obtained from sodium nitroprusside-pretreated cells. Sodium nitroprusside (500 μM) applied to cells for 24 h totally blocked the incorporation of ^{32}P-NAD^+ into GAPDH following SIN-1 addition in the post-labelling assay. Cytosolic fractions of J774.1 macrophages were labelled adequately (Fig. 2C). Nitrite formation, GAPDH inhibition, and incorporation of ra-

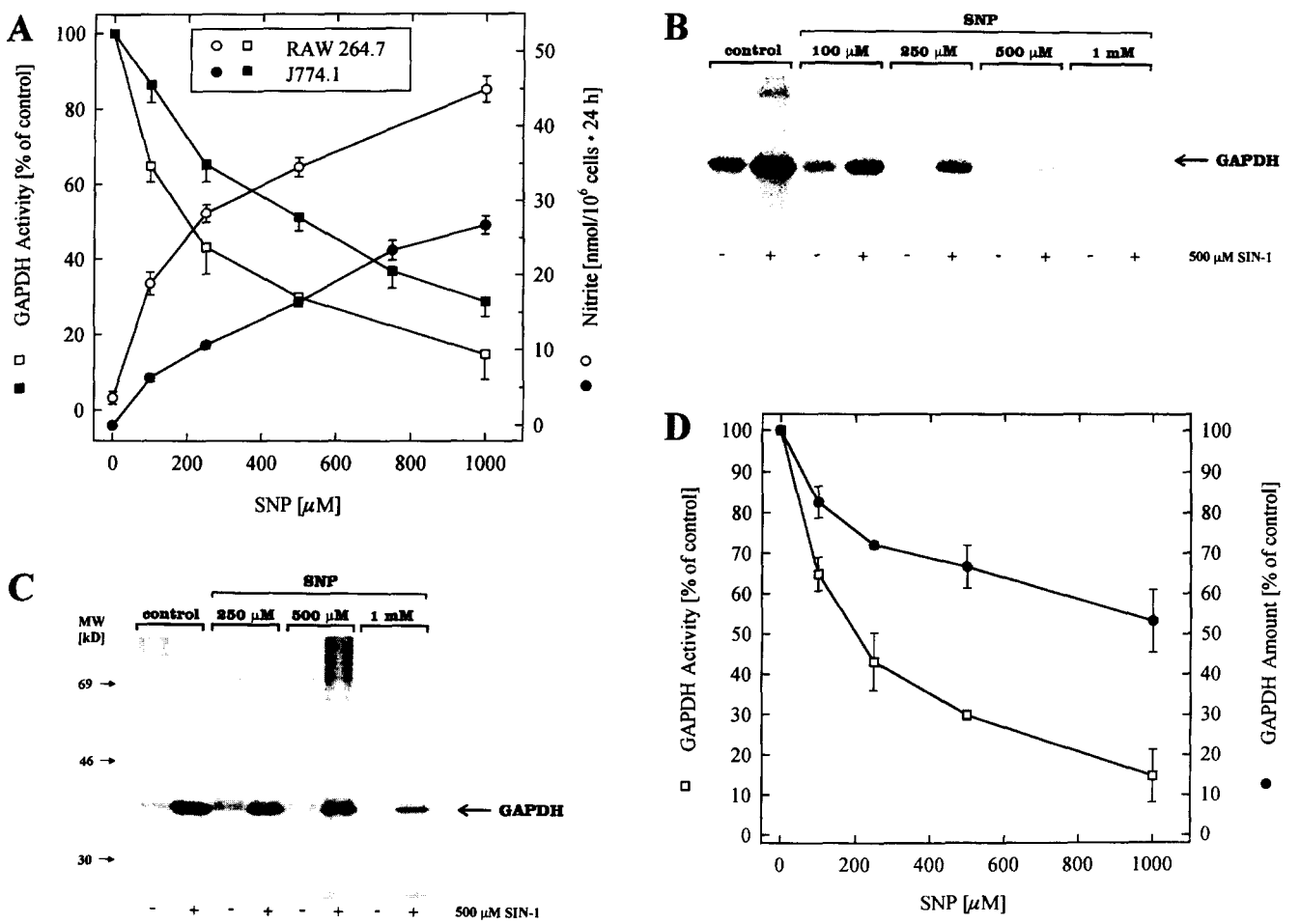

Fig. 2. Sodium nitroprusside induced GAPDH modification. Macrophages (8×10^6/8 ml) were treated for 24 h with the sodium nitroprusside (SNP) concentrations indicated. GAPDH activity was measured in the cytosolic fractions and nitrite was determined in cell-free culture supernatants (A). Data are means ± S.E.M. of five separate experiments. The 'differential radioactive labelling method' was applied in parallel to the cytosolic fractions of RAW 264.7 (B) and J774.1 (C) cells. Graph B reveals a molecular weight range of about 27–50 kDa. Other details are as in Fig. 1. Representative results from five different experiments are shown. D: Western blot analysis of GAPDH in cytosol of RAW 264.7 macrophages treated for 24 h with sodium nitroprusside as indicated. Western blots were performed as described in Materials and methods. The data are the means ± S.E.M. of four different experiments.

dioactivity using the 'differential labelling technique' were similar, although showing a different sensitivity compared to RAW cells.

3.3. Redistribution of GAPDH in response to sodium nitroprusside

Considering GAPDH inhibition and sodium nitroprusside-mediated protein modification in RAW 264.7 macrophages, we concentrated on the compartmentalization of GAPDH. A polyclonal antibody raised against GAPDH detected a single 36 kDa protein band, showing an identical mobility to purified GAPDH (Fig. 3). In Fig. 2D we analyzed GAPDH activity and GAPDH amount in the cytosol of sodium nitroprusside-treated RAW cells. Antibody-detectable GAPDH was quantitated by Western blot analysis using the phosphor image system and is expressed relative to controls (100% value). Higher sodium nitroprusside concentrations decreased the amount of cytosolic GAPDH with a more dramatic effect on enzyme activity. Extending these experiments, we tested for increased degradation or an altered distribution of the enzyme. Blocking protein biosynthesis by incubating RAW

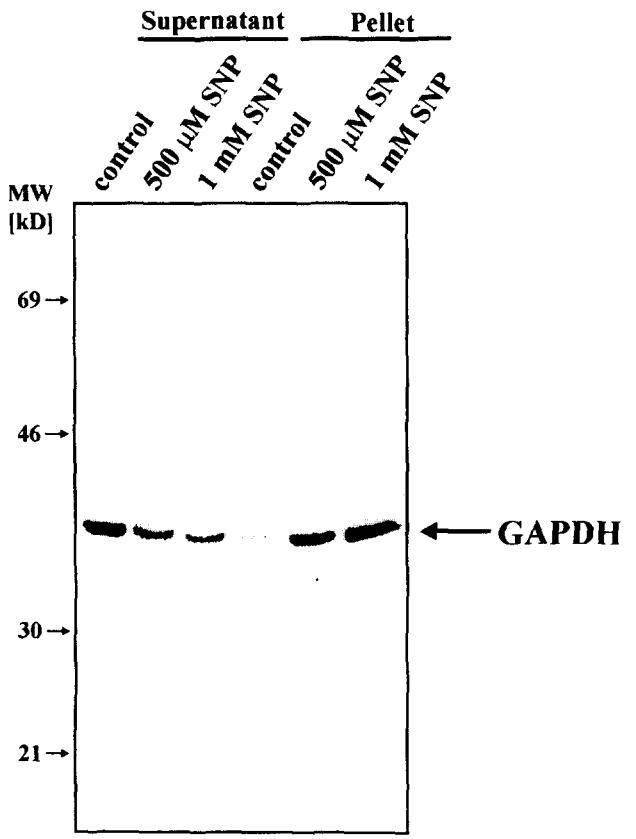

Fig. 3. Redistribution of GAPDH in response to sodium nitroprusside. RAW 264.7 macrophages were treated for 24 h with sodium nitroprusside (SNP) as indicated, followed by separation into cell supernatant and cell pellet. Western blot analyses of GAPDH were performed as described in Materials and methods. The blot is representative of four individual experiments.

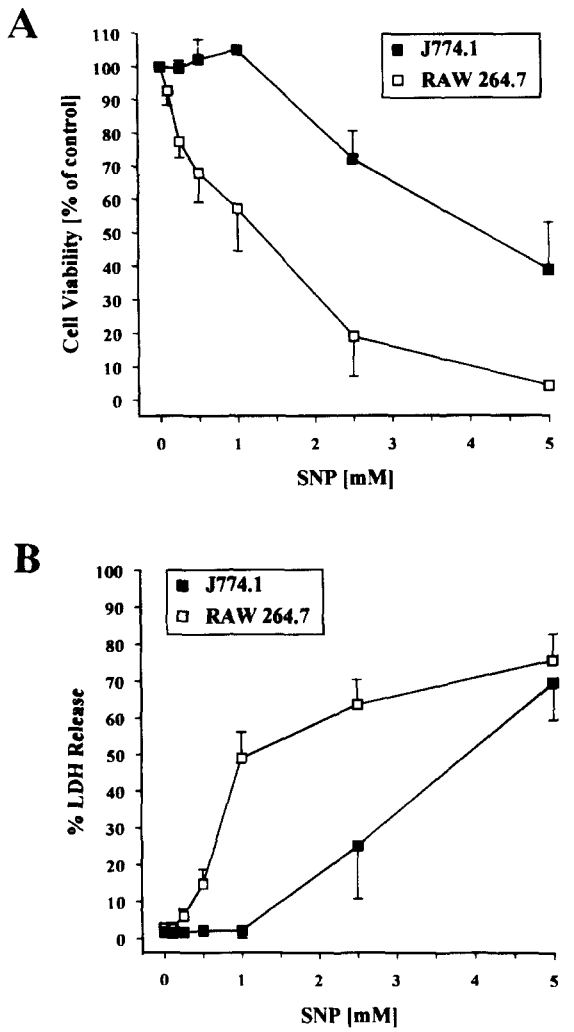

Fig. 4. Cytotoxicity of sodium nitroprusside in RAW 264.7 versus J774.1 macrophages. Cells were incubated for 24 h with sodium nitroprusside (SNP) and cell viability was determined with the MTT cytotoxicity assay (A) or quantitated by LDH release (B) as described in Materials and methods. Results are means ± S.D. from four individual experiments.

macrophages for 24 h with 10 μM cycloheximide followed by Western blot analysis revealed a protein half-life of GAPDH exceeding 24 h. Next we probed for the GAPDH amount using the cytosolic fraction (supernatant) and the cell pellet following RAW cell rupture and ultracentrifugation. Control cells contain GAPDH immunoreactivity exclusively in the 100 000 × *g* supernatant (Fig. 3). Sodium nitroprusside treatment produced a shift of GAPDH immunoreactivity from the cytosol to the cell pellet (nuclear and membrane fraction). Notably, there was no enzymatic activity in the pellet of control or sodium nitroprusside-treated cells. Comparable data were obtained from J774.1 macrophages (data not shown).

3.4. Inhibition of cell viability by sodium nitroprusside

To correlate exogenous sodium nitroprusside application and cell viability we assayed for MTT reduction (Fig.

Table 2
Modulation of lactate accumulation by sodium nitroprusside

	18–24 h μmol/10^6 cells	24–26 h μmol/10^6 cell
Control	3.07±0.18	1.74±0.08
500 μM sodium nitroprusside	3.89±0.27	2.09±0.08 [a]
1 mM sodium nitroprusside	3.70±0.17 [a]	1.99±0.08 [a]
1.5 mM sodium nitroprusside	3.60±0.33 [a]	1.84±0.12
2 mM sodium nitroprusside	3.59±0.32 [a]	1.27±0.12 [b]
10 μM iodoacetate	0.18±0.06 [b]	

J774.1 macrophages (10^6 cells) were incubated in 1 ml complete RPMI supplemented with the substances indicated. Culture supernatants were collected between 18–24 h and 24–26 h. Lactate was determined as described in Materials and methods. Data are the means ± S.E.M. from at least three independent experiments. [a] $P \leq 0.05$ versus control and [b] $P \leq 0.01$ versus control, respectively.

4A). RAW macrophages responded to sodium nitroprusside with a proportional loss of cell viability. Half maximal effects occurred at 1 mM sodium nitroprusside with substantial inhibition (≥ 80%) at 2–3 mM of the NO donor. In contrast, J774.1 macrophages were less susceptible. They remained unresponsive to sodium nitroprusside up to 1 mM, although GAPDH at the same time showed 70% inhibition (Fig. 2A). With the LDH release assay we obtained comparable results to the MTT assay during NO intoxication (Fig. 4). LDH release was dramatically different in both cell lines. In response to 1 mM sodium nitroprusside, the RAW cells showed 50% LDH release, while J774.1 macrophages still remained intact.

3.5. Modulation of lactate accumulation by sodium nitroprusside

Further experiments searched for a possible correlation between NO generation and toxicity, concentrating on glycolysis as one energy delivering pathway. We tested whether GAPDH becomes rate-limiting in J774 macrophages under conditions of massive NO generation determining lactate production. Lactate accumulation was measured in the time period between 18 h and 24 h after addition of up to 2 mM sodium nitroprusside (Table 2). Unexpectedly, sodium nitroprusside caused increased rather than decreased lactate formation, whereas 10 μM iodoacetate, a suggested GAPDH blocker was inhibitory. Accumulating lactate in the time period between 24–26 h after sodium nitroprusside addition again gave higher values compared to the controls. Exceptionally, maximal inhibition of GAPDH at 2 mM sodium nitroprusside (data not shown) led to decreased lactate accumulation during the 24–26 h incubation period.

3.6. Inhibition of aconitase activity by NO

The non heme-iron-sulfur containing aconitase activity seems relevant for NO toxicity. Aconitase activity dose dependently decreased in RAW 264.7 cells incubated with sodium nitroprusside for 24 h (Fig. 5A) Complete inhibition was achieved with 250 μM of the NO-releasing compound. Enzyme inhibition was also noticed after NO production in response to lipopolysaccharide or lipopolysaccharide and IFN-γ. Reversibility by N^G-monomethyl-L-arginine (Fig. 5B) referred to endogenous NO generation. The inhibitory effect of NO on aconitase activity was comparable with J774.1 macrophages. Stimulation with lipopolysaccharide or lipopolysaccharide and IFN-γ dramatically decreased enzyme activity, an effect which was reversed by N^G-monomethyl-L-arginine (Fig. 6). The NO donor sodium nitroprusside (250 μM) caused strong inhi-

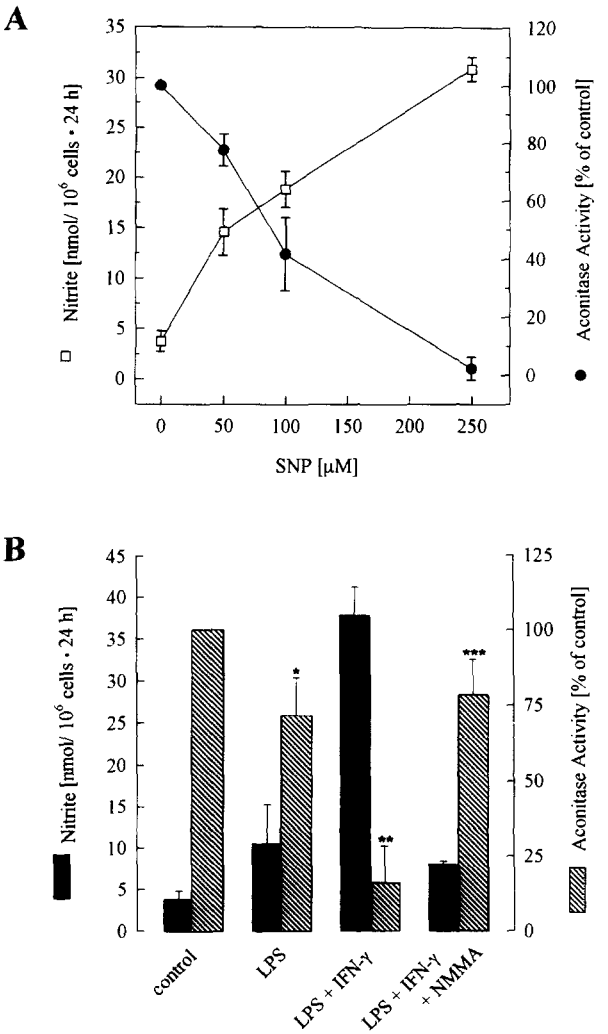

Fig. 5. Inhibition of RAW cell aconitase in response to nitric oxide. Cells (4×10^6) were treated for 24 h with different sodium nitroprusside (SNP) concentrations (A) or 10 μg/ml lipopolysaccharide (LPS), 10 U/ml IFN-γ, and 1 mM N^G-monomethyl-L-arginine (NMMA) as indicated (B). Aconitase enzyme activity was determined spectrophotometrically and the results are expressed as percent of controls. Nitrite was measured with the Griess reaction as described in Materials and methods. Values are means ± S.D. of five individual experiments. * $P < 0.01$ and ** $P < 0.001$ versus control incubations, *** $P < 0.001$ versus stimulated cells without N^G-monomethyl-L-arginine (NMMA).

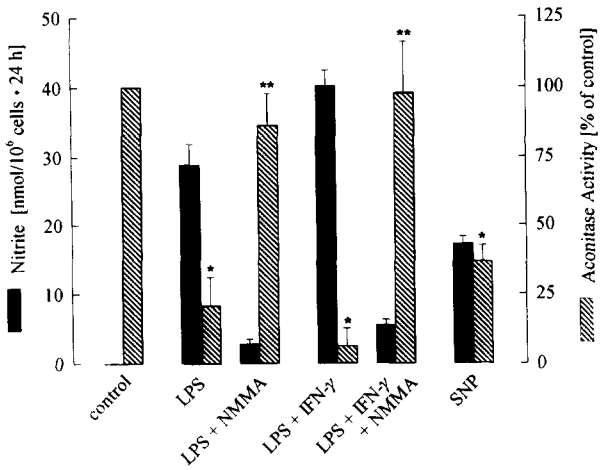

Fig. 6. Nitric oxide inhibits aconitase activity in J774.1 cells. Macrophages were treated for 24 h with 10 μg/ml lipopolysaccharide (LPS), 10 U/ml interferon-γ (IFN-γ), 1 mM N^G-monomethyl-L-arginine (NMMA), and 250 μM sodium nitroprusside (SNP) as indicated. For details see Fig. 6. Data are means ± S.E.M., $n = 4$. * $P < 0.01$ versus control incubations, ** $P < 0.01$ versus stimulated cells without NMMA.

bition of aconitase, although cell viability was not altered at concentrations up to 1 mM sodium nitroprusside (Fig. 4).

4. Discussion

4.1. Nitric oxide and macrophage cell viability

Severe clinical symptoms like insulin-dependent diabetes mellitus (IDDM) (Nathan, 1992) endotoxic or septic shock conditions (Moncada et al., 1991) are related to massive endogenous NO generation, resulting in elevated plasma nitrite values. Activated macrophages are the premier source of NO, as one component of the non-specific immune defense system involved in tumor, bacteria, and protozoa cell killing. A likely endpoint of toxicity seems to be interference with cellular energy-producing steps (Zhang et al., 1994; Radons et al., 1994). So far mainly highly NO-sensitive cells such as pancreatic β cells have been studied to elucidate potential cytotoxic mechanisms of the molecule. Murine peritoneal macrophages responded to lipopolysaccharide/IFN-γ treatment with induction of the inducible type NO synthase and concomitant NO-dependent apoptotic cell death (Albina et al., 1993; Sarih et al., 1993). In our study we focussed on toxic actions of the radical or related species using two NO-producing monocytic macrophage cell lines, RAW 264.7 and J774.1 cells. Our interest concentrated on GAPDH and aconitase modification in relation to toxic mechanisms of nitric oxide.

Mitochondrial activity, assayed as MTT reduction and N^G-monomethyl-L-arginine inhibition experiments implies an inverse correlation between endogenous NO formation in response to lipopolysaccharide/IFN-γ and decreased cell viability (Table 1). This is supported by a dose-response curve for inhibition of MTT reduction or LDH release in response to the NO donor sodium nitroprusside (Fig. 4). Consistently, J774 cells are less responsive towards NO. This is most obvious at a concentration of 1 mM sodium nitroprusside, with 50% of RAW cells responding (MTT assay and LDH release), while J774 cells still remain intact. With the assumption that nitrite values reflect cellular conversion of sodium nitroprusside (Fig. 2A), variations in the metabolic activity of the two cell lines may partially account for the differences observed. Conversely, J774 cells produce more nitrite in response to lipopolysaccharide (Fig. 5 vs. Fig. 6) or the combination of lipopolysaccharide/IFN-γ (Fig. 1) but show minor inhibition in the MTT assay. Obviously, a macrophage specific cellular response to exogenously supplied NO donors and NO synthase inducers determines the vulnerability of cells to a potential toxic insult by NO. This is well shown in the cerebellum with a specific response of NO-producing and NO target cells (Dawson and Snyder, 1994).

Lipopolysaccharide/IFN-γ activated macrophages respond with increased production of not only NO but also reactive oxygen species (O_2^-). The diffusion-controlled reaction of superoxide with NO results in the formation of peroxynitrite ($ONOO^-$), a potentially highly toxic molecule (Stamler, 1994). Assuming that antioxidants like *N*-acetylcysteine did not affect macrophage cell death (Meßmer and Brüne, 1996) the participation of peroxynitrite-mediated oxidative events is not plausible.

4.2. GAPDH as a target of nitric oxide

Biochemical alterations induced by NO include modification of the glycolytic enzyme, GAPDH and the Krebs cycle enzyme, aconitase. Both enzymes are suppressed in response to exogenous or endogenous NO formation (Figs. 1 and 5). A strict sodium nitroprusside dose dependence and reversing effects of N^G-monomethyl-L-arginine support this notion. Decreased GAPDH enzyme activity is paralleled by protein modification which hinders ^{32}P-NAD^+-dependent modification of the enzyme in the cytosol of pretreated cells. The involvement of endogenous NO formation is substantiated by N^G-monomethyl-L-arginine application. ^{32}P-NAD^+ labelling of two proteins, particularly a p43 and a p39 in brain was found similarly decreased in homogenates from stimulated hippocampus at preconvulsive stage II and stage V of dentate gyrus kindling compared to controls (Vezzani et al., 1994). In brain, the effect probably reflects enhanced endogenous ADP ribosylation of the proteins, depending on the progressive activation of the NMDA receptors and generation of nitric oxide. However, other mechanisms blocking access of ^{32}P-NAD^+ to the protein active site thiol, cys-149, may explain the decreased labelling. One could envision oxidative modification of the cysteine residue, following super-

oxide, hydrogen peroxide, or increased $ONOO^-$ production (Hyslop et al., 1988; Beckman et al., 1990). Furthermore, protein *S*-thiolation as reported for GAPDH in response to oxidative cell activation might block cys-149 (Ravichandran et al., 1994). Although the 'differential radioactive labelling method' does not allow one to determine the exact nature of the modified active site thiol, it conclusively establishes a modification of GAPDH due to NO action.

Sodium nitroprusside also causes GAPDH translocation from the cytosol to the plasma membrane (Fig. 3). GAPDH associated with membrane structures has been demonstrated for erythrocytes (Harris and Winzor, 1990), glial (Daum et al., 1988), and synaptic membranes (Walsh and Kuruc, 1992). Translocation of GAPDH in relation to a vitamin E diet has been reported for rhesus monkey erythrocytes (Shapiro and Mott, 1982). Altered intracellular protein distribution may influence enzyme function, especially as GAPDH has other functions beside its role in glycolysis. GAPDH is associated with synaptic vesicles (Schläfer et al., 1994), binds to microtubules (Huitorel and Pantaloni, 1985), serves as a transfer RNA binding protein, and participates in nuclear RNA export (Singh and Green, 1993). In the nucleus, GAPDH is recognized as a DNA binding protein (Ronai, 1993), is involved in transcription (Morgenegg et al., 1986), and exhibits uracil DNA glycosylase activity (Meyer-Siegler et al., 1991). Signalling pathways related to these alternative functions so far remain unknown. However, transducing mechanisms are required to achieve translocation and/or a specialized compartmentalization of the protein. Our results may indicate a NO-related function. This is in some agreement with the proposal that a free radical-mediated mechanism may function similarly (Shapiro and Mott, 1982).

4.3. GAPDH and aconitase as potential targets for cell injury

When establishing the relative impact of GAPDH inhibition and translocation in relation to its glycolytic activity, we probed for lactate production during NO intoxication. Unexpectedly, lactate production under conditions of maximal NO formation was increased rather than decreased (Table 2). Assuming strong inhibition of aconitase under these conditions, an increased flux through glycolysis resulting in more lactate production (Pasteur effect) is plausible in order to maintain cellular ATP. Co-cultivation of activated macrophages with tumor cells was reported to cause inhibition of aconitase in both NO-generating and NO target cells (Moncada et al., 1991, for references). Interestingly, cytotoxic injured target cells showed an absolute requirement for glycolyzable hexose in order to survive mitochondrial injury in response to macrophage activation (Granger et al., 1980). This relates to a compensatory glycolytic pathway as a result of Fe-S enzyme inhibition. Although these early observations have not been related to NO action, they can now be attributed to macrophage NO formation. In our experiments, profound suppression of GAPDH coincided with decreased lactate accumulation only in concert with a profound loss of cell viability. Obviously, despite GAPDH inhibition, the enzyme is not rate limiting during NO intoxication. Similar observations have been reported for erythrocytes, where profound GAPDH inhibition ($\geq$ 90%) did not alter glucose metabolism (Maretzki et al., 1989). Accordingly, other enzymes such as phosphofructokinase control glycolysis. However, inhibition of GAPDH and decreased lactate production as a result of oxidant-mediated cell injury is known (Shapiro and Mott, 1982).

The Krebs cycle produces reduced pyridine-nucleotide coenzymes coupled to mitochondrial respiration. This links substrate oxidation to energy production. Aconitase, an iron-sulfur protein, catalyzing the conversion of citrate to isocitrate as one step of the cycle is susceptible to NO-mediated inhibition. Although two isoforms exist (the cytosolic enzyme is recognized as the iron-responsive element-binding protein) there is agreement that enzymatic properties and iron-sulfur centers are rather similar. Earlier detailed studies showed NO-mediated inhibition of aconitase enzyme activity (Moncada et al., 1991, for references; Stadler et al., 1991). Our results substantiate these reports demonstrating inhibition of aconitase due to endogenously generated or exogenously supplied NO. Complete inhibition of total aconitase activity is achieved with 250 μM of the NO-releasing compound, sodium nitroprusside (Fig. 5). Obviously, iron-sulfur enzymes are much more sensitive to NO compared to GAPDH. Despite pronounced inhibition of aconitase, RAW cell viability is only moderately ($\leq$ 20% inhibition of MTT reduction and less than 10% LDH release) affected, whereas J774 macrophage viability is not altered at all. It must be concluded that inhibition of aconitase or other iron-sulfur enzymes with a similar sensitivity to NO is not a major cause of cell death in macrophages. Macrophage cytotoxic actions on tumor cells are linked to Fe-S enzyme inhibition (Drapier and Hibbs, 1986). EPR complexes consistent with nitrosylated Fe-S complexes have been detected in NO-producing macrophages (Lancaster and Hibbs, 1990) and macrophage-injured target cells (Drapier et al., 1991). Obviously, for macrophages, neither partial inhibition of GAPDH nor substantial suppression of aconitase or similar responsive Fe-S enzymes are a major cause of cell death. Alternative NO-associated mechanisms like DNA damage may operate in parallel as a toxic pathway. As suggested for neuronal cells, NO-mediated death is a likely consequence of energy depletion (Zhang et al., 1994). On the contrary, in macrophages which are specialized for massive NO formation, inhibition of energy-producing steps is an unlikely death-signalling pathway. GAPDH, a previleged NO target in macrophages may either signal alternative functions beside its glycolytic activity or may represent a sink for excessive NO due to its abundant cytosolic appearance.

Protein translocation may again be relevant for alternative protein activities with the involvement of NO deserving further investigation.

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