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Induction of nitric oxide synthase in L929 cells by tumour-necrosis factor α is prevented by inhibitors of poly(ADP-ribose) polymerase

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The fibroblast cell line L929 contains a constitutively expressed NO synthase (EC 1.14.29.–) activity, which can be increased about 10-fold by tumour-necrosis factor α (TNF-α). Activities of the constitutive and the inducible enzymes are tetrahydrobiopterin-independent and can be inhibited by L-N^G-nitroarginine. Induction of NO synthase by TNF-α was prevented by inhibitors of poly(ADP-ribose) polymerase, namely nicotinamide, 3-methoxybenzamide and 3-aminobenzamide. TNF-α did not lead to an increase in ADP-ribosyltransferase activity nor to a change in the pattern of ADP-ribosylated proteins. The inhibitors were only active during the first 4–5 h after exposure to TNF-α and they were found to suppress synthesis of protein, DNA and RNA. These data suggest that the inhibitors prevent induction of NO synthase by interference with RNA and protein synthesis. It is not yet known which reactions of these biosynthetic processes are affected by the inhibitors.

INTRODUCTION

Although the cytolytic ability of tumour-necrosis factor α (TNF-α) has been well documented [1–3], little is known about the molecular mechanisms underlying TNF-α-induced lysis. The reactive nitrogen intermediates, including NO, NO_2^- and NO_3^-, may be involved in TNF-α-mediated cytolysis. Here, we show that TNF-α induces NO and NO_2^- production in L929 cells and that the cells contain a constitutive and a TNF-α-inducible nitric oxide synthase (NO synthase). The existence of at least two distinct types of NO synthases has recently been shown in a wide variety of tissues [4].

There have been several reports suggesting that ADP-ribosylation plays a role in the tumoricidal activities of TNF-α. According to Agarwal *et al.* [5], TNF-α caused a 4-fold increase in ADP-ribosylation in L929 cells, and inhibitors of ADP-ribosylation prevented TNF-α-mediated cytotoxicity. Lichtenstein *et al.* [6] reported that, depending on the target studied, the inhibitors either decreased or increased TNF-α-mediated cytotoxicity. Here, we present evidence that treatment with TNF-α is not associated with an increased ADP-ribosyltransferase activity or a change in the pattern of ADP-ribosylated proteins. We show that the inhibitors mainly act by inhibiting synthesis of RNA, DNA and protein.

MATERIALS AND METHODS

Reagents

Mouse recombinant TNF-α was purchased from Genzyme (Boston, MA, U.S.A.). The specific activity was 4×10^7 units/mg of protein where 1 unit is defined as the amount of TNF-α required to mediate half-maximal cytotoxicity of L929 cells. L-N^G-Nitroarginine (L-NNA) was obtained from Serva (Heidelberg, Germany) and (6*R*)-5,6,7,8-tetrahydrobiopterin (THBP) from Dr. Schirck's Laboratory (Jona, Switzerland). L-[2,3-^{3}H]Arginine (specific radioactivity 58 Ci/mmol) and L-[^{14}C]citrulline (specific radioactivity 50 mCi/mmol) were supplied by NEN-Dupont (Dreieich, Germany). [^{32}P]NAD$^+$ (specific radioactivity 1000 Ci/mmol) was purchased from Amersham–Buchler (Braunschweig, Germany).

Cell line

The mouse fibroblast cell line L929 was maintained in RPMI 1640 medium (Gibco, Eggenstein, Germany) supplemented with 2 mM-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), 60 μM-2-mercaptoethanol and 10% (v/v) inactivated fetal calf serum (FCS). Cell viability was determined by Trypan Blue exclusion and by measuring lactate dehydrogenase activity in supernatants.

Determination of NO_2^- production in L929 cells

L929 cells (2.5×10^6/ml) were suspended in 2 ml of RPMI 1640 (free of Phenol Red) with 5% (v/v) FCS and incubated for 24 h at 37 °C. Supernatants were collected and the NO_2^- concentration was determined by diazotization as described [7].

Preparation of cytosol from L929 cells

L929 cells were removed from the culture dishes with a rubber wiper and were suspended in ice-cold Hepes buffer (15 mM; pH 7.5) containing sucrose (0.32 M). The cells were washed three times and finally suspended in Hepes buffer (without sucrose) containing pepstatin A, antipain, chymostatin and leupeptin (each 10 mg/l). The cells (5×10^6/0.5 ml) were disintegrated by sonication (3 × 10 s bursts; 100 W). The homogenate was centrifuged (100000 *g*, 1 h) to obtain the cytosolic (supernatant; 2–3 mg of protein/ml) and particulate (pellet; 1–2 mg of protein/ml) fractions, which were divided into portions and stored at −70 °C. Protein concentration was determined as described by Bradford [8].

Measurement of NO synthase activity by activation of purified soluble guanylate cyclase

NO synthase activity in the cytosol was determined by L-arginine- (0.3 mM) and NADPH- (0.1 mM) dependent activation of soluble guanylate cyclase (GC) purified from bovine lung [9]. In short, cytosol (0.03 mg of protein/ml) was incubated (30 min;

Abbreviations used: TNF-α, tumour-necrosis factor α; L-NNA, L-N^G-nitroarginine; THBP, tetrahydrobiopterin; FCS, fetal calf serum; GC, guanylate cyclase.

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37 °C) in a final volume of 0.1 ml with GC (1 μg/ml), [α-^{32}P]GTP (0.1 μM; 0.2 μCi), cyclic GMP (0.1 mM), $MgCl_2$ (4 mM), EGTA (0.1 mM), glutathione (2 mM), Hepes (15 mM; pH 7.5), phosphocreatine (3.5 mM), creatine kinase (4.8 units), bovine γ-globulin (0.1 mg/ml) and 3-isobutyl-1-methylxanthine (0.5 mM) in the presence of L-arginine, NADPH and THBP (100 nM). In some experiments, the effect of poly(ADP-ribose) polymerase inhibitors on GC activity in the presence or absence of cytosol or a pharmacological NO donor (sodium nitroprusside) was tested. GC activity was calculated from the amount of cyclic [^{32}P]GMP formed as described previously [10].

Measurement of L-[^{3}H]citrulline formation [11]

For measurement of NO synthase activity by formation of L-citrulline, the cytosol was incubated with L-[^{3}H]arginine (0.1 mM; 0.05 μCi) at 37 °C, in the absence of soluble GC and [α-^{32}P]GTP. L-Citrulline (0.1 mM) was added to ensure a reproducible recovery of L-[^{3}H]citrulline. After 1 h, 0.9 ml of ice-cold stop solution (EDTA, 2 mM; sodium acetate, 20 mM, pH 5.5; L-citrulline, 0.1 mM) was added to the incubation mixtures (0.1 ml). The mixture was loaded on to a strongly acidic cation-exchange column (0.8 cm × 1 cm; Bio-Rad AG-5 DW-X8) pre-equilibrated with 1 M-NaOH (2 × 0.5 ml) and water (2 × 3 ml). The flow-through and eluate (1 ml of water) were collected, thoroughly mixed with 8 ml of Szintigel (Rotiszint ecoplus, Roth, Karlsruhe, Germany) and ^{3}H was quantified by using a β-counter. The recovery of L-[^{14}C]citrulline by this procedure was 80%, as determined by incubation of L-[^{14}C]citrulline in the absence of L-[^{3}H]arginine. The assay is linear with time for up to 1 h (data taken at 10 min, 30 min, 1 h). Its linearity with protein concentration was tested and confirmed with 0.05, 0.1 and 0.2 mg of cytosolic protein/ml.

Measurement of protein synthesis

L929 cells (1×10^5/ml) were incubated at 37 °C in leucine-deficient RPMI medium in the presence of L-[4,5-^{3}H]leucine (2 μCi/ml; specific radioactivity 58 Ci/mmol). After 3 h, the cells were diluted with 2 ml of ice-cold 0.9% NaCl, and protein was precipitated with 2 ml of 25% (w/v) trichloroacetic acid after the addition of 250 μl of 0.1% (w/v) BSA. After reprecipitation, the pellets were collected on Whatman GF/C glass-fibre filters and washed with 25% trichloroacetic acid. Filters were counted for radioactivity by liquid-scintillation spectrometry.

ADP-ribosylation assay

Enzymic determination. ADP-ribosylation was carried out as described [5,12]. Briefly, L929 cells (2×10^7/ml) were sonicated (3 × 30 s, 40 W), in an ice-cold permeabilizing buffer, containing 10 mM-Tris/HCl, pH 7.8, 1 mM-EDTA, 4 mM-$MgCl_2$ and 30 mM-2-mercaptoethanol. Samples of the homogenate in a final volume of 50 μl of permeabilizing buffer were added to 25 μl of an ADP-ribosylation reaction mixture which contained 100 mM-Tris/HCl, pH 7.8, 120 mM-$MgCl_2$, 50 μM-[^{32}P]NAD (specific radioactivity 1000 Ci/mmol; 0.45 μCi/assay) 0.1 mM-GTP, 0.1 mM-ATP and 0.1% Lubrol.

After incubation for various times at 30 °C, the reaction was terminated by adding 250 μl of BSA (1 mg/ml) followed by 2 ml of 25% (w/v) trichloroacetic acid. After reprecipitation, the pellets were collected on Whatman GF/C glass-fibre filters. The filters were washed and counted for radioactivity by liquid-scintillation spectrometry.

PAGE and autoradiography. ADP-ribosylation was carried out with cytosolic supernatants of L929 cells as described above. The ADP-ribosylation mixture contained 1 μM-[^{32}P]NAD (specific radioactivity 1000 Ci/mmol; 15 μCi/assay). The reactions were terminated by precipitating proteins with methanol [13]. The pellets were resuspended in SDS/PAGE sample buffer and boiled for 3 min. Proteins were separated by SDS/PAGE with a 10% acrylamide gel [14] and the gels were subjected to autoradiography.

Assay for RNA and DNA synthesis

Fibroblasts were cultured for 48 h at a cell density of 5×10^5/ml in 0.25 ml samples/well in flat-bottomed microtitre plates. Before harvesting, cultures were pulsed for 24 h by the addition of 0.6 μCi of [^{3}H]uridine or [^{3}H]thymidine.

RESULTS

NO_2^- formation in L929 fibroblasts

When L929 cells were incubated for 24 h in the absence of any stimulus they produced NO_2^-. The production was linear with respect to the cell concentration (Table 1). When stimulated with TNF-α, NO_2^- production increased in a dose-dependent manner. L-NNA (1 mM) inhibited NO_2^- production in stimulated and unstimulated cells by 93% and 75% respectively.

Time course of NO_2^- induction by TNF-α

Fig. 1 shows the time course of NO_2^- production on stimulation with 500 units of TNF-α. TNF-α caused a marked increase in

Table 1. Dependence of NO_2^- production on cell number and TNF-α concentration

L929 cells were incubated in the presence or absence of TNF-α. After 24 h, supernatants were assayed for NO_2^- production as described in the Materials and methods section. Values are means ± S.D. from one of three experiments performed in triplicate.

Cell number (× 10^7/well)	TNF-α (units/well)	NO_2^- production (nmol/well)
0.5	–	7.6±0.7
1	–	11.7±0.5
2	–	22.9±0.4
0.5	100	12.5±0.2
1	100	41.5±1.4
2	100	75.0±2.1
0.5	500	24.3±2.2
1	500	67.0±1.8
2	500	135.7±1.8

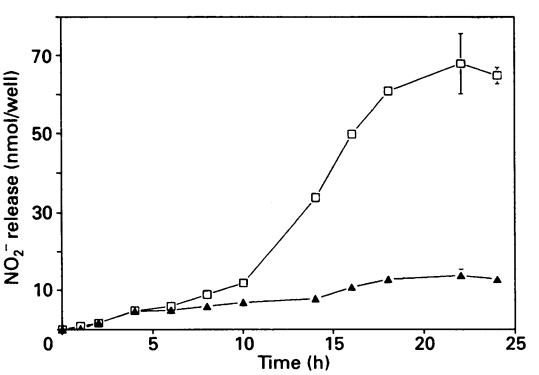

Fig. 1. Time course of NO_2^- formation in L929 fibroblasts

Cells (2.5×10^6/ml) were incubated in the presence (□) or absence (▲) of TNF-α (500 units/ml) for different periods of time. Supernatants were assayed for NO_2^- formation. The results are means of triplicates from one of three similar experiments. Standard errors between triplicates were less than 2% of the mean.

NO_2^- release which reached its maximum after 24 h of incubation. For the first 10 h the increase in NO_2^- release, however, was minimal. In the absence of TNF-α, NO_2^- accumulation remained at a low level throughout the whole incubation time.

Effect of poly(ADP-ribose) polymerase inhibitors and cycloheximide on NO_2^- formation

Fibroblasts were incubated with 500 units of TNF-α for 24 h in the presence of various concentrations of the three poly(ADP-ribose) polymerase inhibitors nicotinamide, 3-methoxybenzamide or 3-aminobenzamide. All three compounds tested induced inhibition of NO_2^- formation in a dose-dependent manner (Fig. 2). The most potent inhibitor turned out to be 3-methoxybenzamide, which at a concentration of 10 mM nearly completely prevented NO_2^- formation. Three times as much nicotinamide and 3-aminobenzamide was necessary to abolish NO_2^- release. To test whether the protein-synthesis inhibitor cycloheximide affected NO_2^- production similarly in stimulated and unstimulated cells, we incubated the cells for 24 h in the presence of different concentrations of cycloheximide and measured NO_2^- production in the supernatant. As can be seen from Table 2, cycloheximide had no effect on NO_2^- production in unstimulated cells, whereas it inhibited TNF-α-induced NO_2^- production to basal levels similar to those measured in supernatants of unstimulated cells.

Time course of suppression of NO_2^- formation by nicotinamide

Fibroblasts were incubated in the presence of TNF-α (500 units/ml) for 24 h, and nicotinamide (30 mM) was added at various times after addition of TNF-α. Maximal inhibition of NO_2^- formation was observed when nicotinamide was added at the same time or 1 h later than TNF-α (Table 3). The inhibition gradually declined if the time before addition of nicotinamide was increased. After 10 h, nicotinamide no longer affected NO_2^- formation.

NO synthase in L929 cytosol: cofactors

In the presence of L-arginine and NADPH, the cytosol from unstimulated L929 cells activated purified soluble GC in an L-NNA-inhibitable manner (Table 4). In accordance with the significant NO_2^- release from unstimulated cells, this finding suggests the existence of a constitutive NO synthase in L929 cells. This enzyme was not activated by THBP (Table 4), as reported for other constitutive NO synthases [15,16]. Similar findings were obtained with NO synthase preparations partially purified by ADP–agarose affinity chromatography (results not shown). The cytosolic NO synthase activity was increased 5-fold in L929 cells incubated with TNF-α (500 units/ml; Table 4). The induced NO synthase activity was not affected by THBP (Table 4).

NO synthase in L929 cytosol: effect of poly(ADP-ribose) polymerase inhibitors

To test whether inhibitors of poly(ADP-ribose) polymerase directly inhibit TNF-α-induced NO synthase activity, nicotinamide, 3-aminobenzamide and 3-methoxybenzamide were added to the incubation mixtures containing L929 cytosol and purified GC. Activation of purified GC by cytosol from control and TNF-α-stimulated L929 cells was inhibited by the three compounds (Table 5), 3-methoxybenzamide being more effective than the two other inhibitors. Since GC activation by the pharmacological NO donor sodium nitroprusside was also inhibited, these compounds certainly interfere with activation of soluble GC by NO. To avoid this interference, NO synthase activity was determined by measuring formation of L-[^{3}H]citrulline from L-[^{3}H]arginine. Cytosol (0.1 mg/ml) from TNF-α-stimulated L929 cells generated 6.6 nmol of L-[^{3}H]citrulline/h per mg (Table 5) which was completely inhibited by 1 mM-L-NNA (not shown). The three poly(ADP-ribose) polymerase inhibitors did not affect NO synthase even when used at higher concentrations than those in the GC-activation assay.

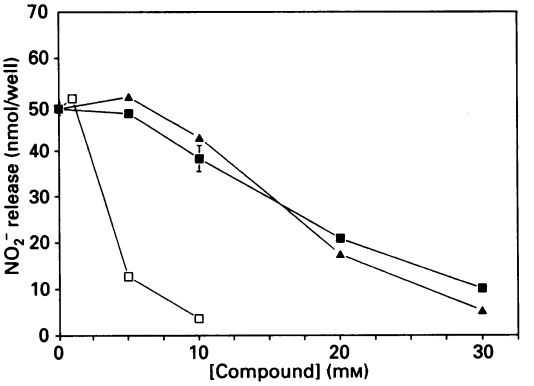

Fig. 2. Effect of nicotinamide, 3-methoxybenzamide and 3-aminobenzamide on TNF-α-induced NO_2^- release in L929 fibroblasts

Cells (2.5×10^6/ml) were incubated in the presence of TNF-α (500 units) and in the presence or absence of various concentrations of nicotinamide (▲), 3-methoxybenzamide (□) and 3-aminobenzamide (■). After 24 h, supernatants were collected and assayed for NO_2^- formation. The results are means ± S.D. from one of three experiments performed in triplicate.

Table 2. Effect of cycloheximide on NO_2^- release in stimulated and unstimulated L929 cells

NO_2^- formation was measured in supernatants of L929 cells (2.5×10^6/ml) incubated with or without TNF-α (500 units/ml) for 24 h in the presence or absence of cycloheximide. Values are means ± S.D. from one of three experiments performed in triplicate.

Additions	NO_2^- release (nmol/well)	
	−TNF-α	+TNF-α
None	8.1 ± 0.6	56.8 ± 0.3
Cycloheximide		
10 μg	6.8 ± 0.4	7.0 ± 0.4
1 μg	9.6 ± 0.4	6.9 ± 0.2
0.1 μg	5.8 ± 0.9	12.5 ± 0.1

Table 3. Time course of suppression of NO_2^- formation by nicotinamide

L929 cells (2.5×10^6/ml) were incubated in the presence of TNF-α (500 units/ml) for 24 h. After different times, nicotinamide (30 mM) was added and, after 24 h, NO_2^- was measured in the supernatant. The values are means ± S.D. from one of three experiments performed in triplicate.

Time of addition of nicotinamide (h)	NO_2^- formation (nmol/well)
0	8.8 ± 0.3
1	8.1 ± 0.6
2	14.4 ± 0.1
3	18.7 ± 0.4
4	25.9 ± 1.1
5	33.5 ± 0.4
10	44.3 ± 0.5
24	48.7 ± 0.1

Table 4. Effect of THBP on NO synthase in cytosol from untreated (control) and TNF-α-treated L929 cells

The activity of purified soluble GC was determined in the presence of cytosol (0.03 mg/ml) from untreated (control) and TNF-α-treated (TNF-α) L929 fibroblasts. THBP (100 nM) and L-NNA (1 mM) were present during incubations as indicated. Mean values ± S.E.M. from $n = 3$ different L929 batches tested in triplicate. *Significantly different ($P < 0.05$; Student's t test) from incubations without additions.

Additions	Cytosol-induced GC activation (nmol/min per mg)	
	Control cytosol	TNF-α cytosol
None	100 ± 5	534 ± 26
THBP	93 ± 18	490 ± 13
THBP/L-NNA	28 ± 7*	97 ± 23*

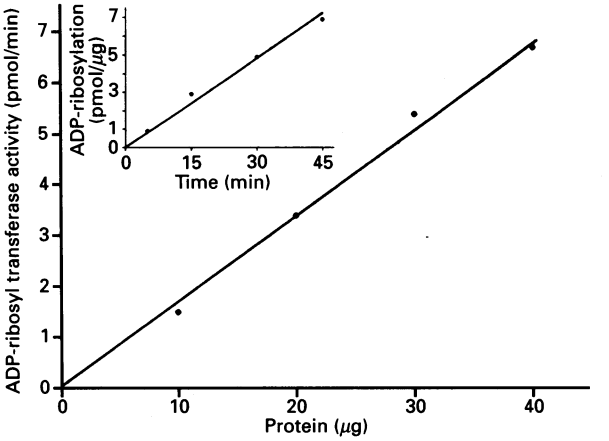

Fig. 3. Concentration-dependence of ADP-ribosyltransferase activity in L929 fibroblasts

ADP-ribosylation was carried out for 30 min with the indicated amounts of protein in the presence of 50 μM-[^{32}P]NAD$^+$. The inset shows the time course of ADP-ribosylation in permeabilized fibroblasts (20 μg of protein/assay).

Effect of TNF-α on ADP-ribosyltransferase activity in L929 cells

Fig. 3 shows the time course and protein-concentration-dependence of ADP-ribosyltransferase in L929 cells. The protein-concentration-dependence was linear up to 40 μg of protein/assay and the amount of ADP-ribosylated substrate formed was linear as a function of time for 45 min (Fig. 3, inset). It has previously been shown that the addition of 4000 units of rabbit TNF-α/ml to L929 cells caused an increase in ADP-ribosylation that reached a maximum about 24 h after stimulation [5]. When incubating L929 cells for 24 h in the presence of 500 units of TNF-α/ml, we did not observe any change in ADP-ribosyltransferase activity. Enzyme activities in controls and TNF-α-treated cells were 0.157 ± 0.028 and 0.158 ± 0.031 pmol/min per μg of protein (means ± S.D.; $n = 3$) respectively.

Although enzyme activities did not change, it cannot be excluded that specific proteins were ADP-ribosylated in response to TNF-α. We therefore carried out an SDS/PAGE of cytosolic proteins after incubation of cytosols with [α-^{32}P]NAD$^+$. Fig. 4 shows the autoradiographs obtained from cytosols of controls and stimulated cells. There were no differences between the two ADP-ribosylation patterns. The addition of the NO-generating compound sodium nitroprusside (200 μM) to the assay mixture resulted in an increased labelling of a 39 kDa protein band. The enhanced ADP-ribosylation of this protein by sodium nitroprusside has previously been shown to occur in a number of other tissues [17].

Inhibition of synthesis of protein, RNA and DNA by inhibitors of poly(ADP-ribose) polymerase

Protein synthesis in L929 cells was determined by measuring [^{3}H]leucine incorporation into acid-insoluble protein after an incubation time of 3 h. As can be seen from Table 6, the inhibitors nicotinamide, 3-aminobenzamide and 3-methoxybenzamide inhibit protein synthesis in a concentration-dependent manner. Of all three inhibitors tested, 3-methoxybenzamide suppressed protein synthesis most effectively.

Not only protein but also RNA and DNA synthesis were

Table 5. Effect of inhibitors of poly(ADP-ribose) polymerase on purified soluble GC and on L-[^{3}H]citrulline formation in cytosol from TNF-α-treated and untreated L929 cells

The stimulation (-fold increase) of the basal activity of purified soluble GC by sodium nitroprusside (0.1 mM) or by cytosol (50 μg of protein/ml) from untreated (control) or TNF-α-treated (500 units/ml) L929 fibroblasts was measured. Numbers in parentheses indicate percentage of activity compared with that measured in the absence of inhibitors. L-[^{3}H]Citrulline formation from L-[^{3}H]arginine by cytosol (0.1 mg/ml) from TNF-α-stimulated L929 cells was measured as described in the Materials and methods section. It was completely inhibited by 1 mM-L-NNA (not shown). Results are mean values ± S.E.M. ($n = 3$); n.d., not determined.

Inhibitors	Inhibitor concn. (mM)	Increase in GC activity above basal by:			L-[^{3}H]Citrulline formation in TNF-α cytosol (nmol/h per mg)
		Sodium nitroprusside	Control cytosol	TNF-α cytosol	
None	–	39 ± 2 (100)	4.5 ± 0.6 (100)	50 ± 5 (100)	7.5 ± 0.1
Nicotinamide	5	35 ± 1 (90)	4.9 ± 0.2 (109)	43 ± 4 (86)	n.d.
	10	17 ± 1 (44)	2.4 ± 0.2 (53)	25 ± 3 (50)	n.d.
	20	n.d.	n.d.	n.d.	6.4 ± 0.2
3-Aminobenzamide	5	39 ± 2 (100)	5.5 ± 0.3 (122)	41 ± 4 (82)	n.d.
	10	18 ± 1 (46)	2.8 ± 0.1 (62)	23 ± 4 (46)	n.d.
	20	n.d.	n.d.	n.d.	6.5 ± 0.2
3-Methoxybenzamide	5	10 ± 1 (26)	0.6 ± 0.1 (13)	37 ± 4 (74)	n.d.
	10	n.d.	n.d.	n.d.	6.7 ± 0.1

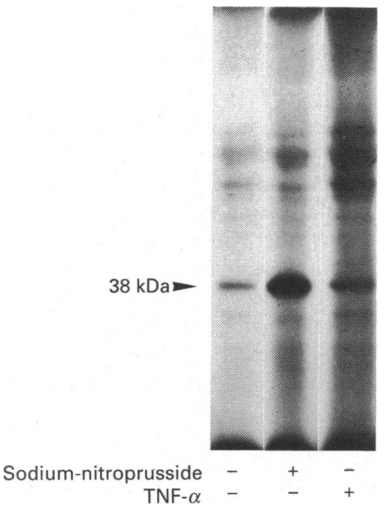

Fig. 4. Effect of TNF-α and sodium nitroprusside on ADP-ribosylation of cytosolic proteins from L929 fibroblasts

Fibroblasts (2.5×10^6/ml) were incubated in the presence or absence of TNF-α (500 units/ml) or sodium nitroprusside (200 μM). After 24 h, cytosolic supernatants were prepared and incubated with [α-^{32}P]NAD$^+$ for 30 min. Proteins were separated on SDS/PAGE (10% gels) as described. The autoradiograph is representative of three experiments.

Table 6. Effect of poly(ADP-ribose) polymerase inhibitors on protein synthesis in L929 fibroblasts

L929 cells (2.5 ± 10^6/ml) were incubated in the presence of TNF-α (500 units/ml) and [^{3}H]leucine (2 μCi/ml). After 3 h, incorporation of [^{3}H]leucine into proteins was measured. Values are means ± S.D. from one of three experiments performed in triplicate.

Additions	Concn. (mM)	10^{-3} × Incorporation of [^{3}H]leucine (c.p.m.)
None	–	3.9 ± 0.2
Nicotinamide	30	1.3 ± 0.2
	20	2.4 ± 0.6
	10	3.7 ± 0.6
3-Aminobenzamide	30	1.7 ± 0.2
	20	3.0 ± 0.2
	10	3.8 ± 0.3
3-Methoxybenzamide	10	0.6 ± 0.2
	5	3.0 ± 0.1

inhibited by the three compounds (Table 7). Whereas treatment with 10 mM-3-methoxybenzamide resulted in an almost complete prevention of [^{3}H]uridine and [^{3}H]thymidine incorporation, 10 mM-nicotinamide and 10 mM-3-aminobenzamide hardly influenced RNA synthesis and even stimulated DNA synthesis. This may be due to progressive metabolic conversion of the inhibitors into NAD or other stimulating compounds. For shorter incubation times (24 h), this effect was not seen. The concentration needed to prevent RNA and DNA synthesis was found to be 30 mM.

Effect of poly(ADP-ribose) polymerase inhibitors on cell viability

As seen in Table 8, none of the inhibitors affected cell viability, whereas treatment with nicotinic acid led to a decrease in viable cells. Nicotinic acid was used as a nicotinamide analogue which does not inhibit poly(ADP-ribose) polymerase activity [18].

Measuring lactate dehydrogenase activity in the supernatants of the treated cells showed that enzyme activity slightly increased (1.5–2-fold) only when the highest concentrations of the inhibitors were used. Control values ranged between 0.22 and 1.59 mmol/min per litre. No enhanced release of lactate dehydrogenase was observed when the cells were exposed to nicotinic acid. pH measurements carried out in supernatants of cells treated for 24 h with the compounds listed in Table 8 showed that none of the compounds affected the pH values which ranged between 7.7 and 7.8.

Table 7. Effect of poly(ADP-ribose) inhibitors on [^{3}H]uridine and [^{3}H]thymidine uptake in L929 fibroblasts

L929 cells (2.5×10^6/ml) were incubated for 48 h in the absence or presence of the inhibitors; 24 h before harvesting cultures were given 0.625 μCi of [^{3}H]uridine or [^{3}H]thymidine per well. Results are means ± S.D. of triplicate assays.

Addition	Concn. (mM)	Incorporation of: 10^{-3} × [^{3}H]Uridine (c.p.m.)	10^{-3} × [^{3}H]Thymidine (c.p.m.)
None		42.0 ± 5.4	148.2 ± 23.7
Nicotinamide	30	6.7 ± 1.0	23.1 ± 4.9
	20	25.5 ± 3.0	114.8 ± 8.9
	10	68.2 ± 5.8	246.4 ± 23.6
3-Aminobenzamide	30	3.4 ± 0.2	11.7 ± 2.1
	20	23.3 ± 4.5	152.7 ± 13.4
	10	53.6 ± 3.7	190.0 ± 20.7
3-Methoxybenzamide	10	8.6 ± 0.6	0.22 ± 0.04
	5	20.4 ± 1.0	53.3 ± 14.4

Table 8. Effect of inhibitors of poly(ADP-ribose) polymerase and nicotinic acid on cell viability of TNF-α treated cells

L929 cells (2.5×10^6/ml) were incubated with TNF-α (500 units/ml) in the absence or presence of inhibitors and nicotinic acid. After 24 h, cell viability was determined by Trypan Blue exclusion. In the absence of TNF-α, cell viability was 68 ± 4%. Values are means ± S.D. ($n = 3$).

Addition	Concn. (mM)	Viable cells (%)
None	–	62 ± 5
3-Aminobenzamide	30	70 ± 3
	20	77 ± 4
	10	71 ± 12
Nicotinamide	30	72 ± 3
	20	79 ± 10
	10	79 ± 7
3-Methoxybenzamide	10	70 ± 12
	5	70 ± 8
Nicotinic acid	30	30 ± 12
	20	35 ± 13
	10	46 ± 1

DISCUSSION

Here we show that stimulating L929 cells with TNF-α leads to an increase in NO_2^- production which can be prevented by inhibitors of poly(ADP-ribose) polymerase (EC 2.4.2.30). Ac-

cording to Agarwal *et al.* [5], these inhibitors prevent TNF-α-mediated cytotoxicity in L929 cells. In contrast with the findings of Agarwal *et al.* [5] who used 800 units of recombinant human TNF-α/ml in the presence or 4000 units of rat TNF-α/ml in the absence of actinomycin D to induce cytotoxicity, we did not observe an increase in total ADP-ribosylation activity when stimulating the cells with 500 units of TNF-α/ml. Neither did the pattern of ADP-ribosylated proteins change in response to TNF-α. In agreement with a recent report [17], we showed that NO generated from sodium nitroprusside enhanced ADP-ribosylation of a 39 kDa protein. The reasons that an enhanced labelling of this protein was not detected in TNF-α-stimulated cells may be that (a) the intracellular NO concentrations produced by TNF-α were not high enough, (b) the 39 kDa protein was ADP-ribosylated resulting in a diminished availability for subsequent ADP-ribosylation with radioactive NAD or (c) NO may be involved in short-term reversible regulations not detected by us. However, when TNF-α was added together with actinomycin D (1 μg/ml) to the cells, ADP-ribosylation rose from 0.15 ± 0.02 to 0.26 ± 0.03 pmol/min per μg of protein ($n = 3$). The TNF-α-mediated cytotoxicity measured by staining with Crystal Violet [19] also increased from $10 \pm 3\%$ in the absence to $70 \pm 10\%$ (means ± S.D.; $n = 3$) in the presence of actinomycin D. It thus seems that ADP-ribosylation increases concomitantly with progressing cell death. Increased ADP-ribosylation most probably results from stimulation of poly(ADP-ribose) polymerase, an enzyme known to participate in DNA repair. DNA repair occurs after DNA breakage [20] which in turn can be induced by high amounts of TNF-α [21]. The fact that the inhibitors prevent biological responses such as NO_2^- formation in the absence of increased ADP ribosylation and that they are only active if present during the first 4–5 h after addition of TNF-α led us to assume that they act by inhibiting NO synthase induction, which precedes NO_2^- release [12,22]. Indeed, we found that the inhibitors suppressed protein synthesis and that these compounds also inhibited RNA and DNA synthesis. In a previous paper, we described similar effects of the inhibitors on lipopolysaccharide-stimulated macrophages [12]. It can be ruled out that the inhibitors act via general toxic mechanisms. It is more likely that they inhibit ADP-ribosylating enzymes which may be involved in these biosynthetic processes. We can rule out direct inhibition of NO synthase activity, since poly(ADP-ribose) polymerase inhibitors did not affect L-[^{3}H]citrulline formation in cytosol from TNF-α-stimulated cells. L-Citrulline is generated concomitantly with NO from L-arginine. Interestingly, poly(ADP-ribose) polymerase inhibitors prevented activation of purified GC by NO generated either by sodium nitroprusside or by NO synthase. This could be interpreted as scavenging of NO, which decreases the amount of NO detected by GC.

The production of NO_2^- in L929 fibroblasts also occurs in the absence of TNF-α, which is indicative of a constitutive NO synthase. In fact, the presence of a constitutive NO synthase in L929 cell cytosol could be demonstrated by L-arginine-dependent activation of a purified soluble GC, which is activated by NO in smooth-muscle cells and platelets [23]. Unexpectedly, this NO synthase did not depend on THBP as reported for other NO synthases [15,16].

NO_2^- release and NO synthase activity were increased several-fold by TNF-α in a concentration-dependent fashion, following a typical time course similar to that known from induction of NO synthases in other cells [7,22]. The TNF-α-induced NO_2^- release was prevented by cycloheximide, whereas the constitutive NO_2^- release was not affected, demonstrating the requirement of protein synthesis for the induced enzyme only. The induced NO synthase, as well as the constitutive enzyme, were not affected by THBP. The insensitivity of NO synthase to THBP could be due to saturation with endogenous THBP. However, partial purification of the enzyme by ADP–agarose affinity chromatography did not result in a significant dependence of NO formation on THBP, though this purification step largely increased the effect of exogenous THBP on other NO synthases [24,25]. In summary, the finding of inhibition of NO synthases by L929 cells by inhibitors of poly(ADP-ribose) polymerase which occurs in the absence of increased ADP-ribosyltransferase activity is interesting because the compounds do not inhibit NO synthases directly, but most probably act by interfering with synthesis of RNA and protein.

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