Heat shock proteins and macrophage resistance to the toxic effects of nitric oxide

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Nitric oxide (NO) functions as a pathophysiological mediator in mammalian tissues. Activated macrophages produce NO as a non-specific immune response directed against invading bacteria or micro-organisms. The same macrophages that initiate the production of NO also can be toxically affected by NO. Incubation of RAW 264.7 macrophages with lipopolysaccharide (LPS) and/or interferon-γ (INF-γ) induced the formation of NO by the activation of a cytokine-inducible NO synthase (NOS). The viability of these macrophages was inversely correlated with the formation of nitrite, a final NO-oxidation product measurable in the incubation medium. The addition of an NOS inhibitor, N^G-monomethyl-L-arginine, diminished NO formation and preserved cell viability in a dose- and time-dependent fashion. Treatment of macrophages with ten cycles of non-lethal doses of LPS and INF-γ, each followed by subculturing of the surviving cells, resulted in cell resistance to the NO toxic insult induced by LPS and INF-γ. These resistant macrophages showed a 2-fold increase in the expression of the constitutive heat shock protein (HSC 70) which is known to be involved in protecting cells against the action of various metabolic insults. Our results establish a link between cell resistance to the toxic effects of NO, and the expression of heat shock proteins in RAW 264.7 macrophages.

INTRODUCTION

Activated macrophages can generate large amounts of nitric oxide (NO) from L-arginine by the action of inducible NO synthase (iNOS) [1,2]. NO is a cellular mediator of multiple biological functions [3], including macrophage-mediated cytotoxicity, neurotransmission and smooth-muscle relaxation [4]. Previous studies show that macrophages activated by cytotoxic substances cause inhibition of DNA synthesis, mitochondrial respiration and aconitase activity in tumour target cells. The activity of NO synthase (NOS) in macrophages induced by lipopolysaccharide (LPS) and interferon-γ (INF-γ) correlates inversely with the life span of these macrophages in culture [5]. The cell death caused by NO is thought to be apoptotic [6].

Tumour necrosis factor (TNF), which produces cytolytic effects, initiates cellular signals similar to those observed in macrophages incubated with LPS [7]. These intracellular signals include formation of TNF-α and interleukin 1β. When these same agents are experimentally applied to the cells, they all enhance induction of NOS [8]. Such enhancement is also observed with INF-γ [9] and phorbol esters [8], indicating that independent pathways affect NOS in macrophages.

The highly conserved inducible heat shock proteins (HSPs) have been implicated in cellular protective mechanisms against a variety of injuries, including TNF-α cytotoxicity. Cells that constitutively overexpress HSPs are also resistant to a variety of oxidizing agents and to heat [10]. HSPs function as molecular chaperones and are essential to cell survival under stressful conditions. Upon stress, HSPs move from the cytoplasm into the nucleus; their protective effect against oxidative injury may result from protection against oxidant-induced DNA damage. Below we report our investigation of the possible relationship of NO-induced oxidative stress to production of constitutive or inducible HSPs. Macrophage resistance to increased synthesis of NO induced by LPS and/or INF-γ was associated with higher levels of constitutive HSPs production in these cells.

EXPERIMENTAL

Materials

Rat macrophage cell line RAW 264.7 (RAW) was obtained from the American Type Tissue Collection (Rockville, MD, U.S.A.) and grown at 37 °C, under 5% CO_2, in RPMI medium 1640 (Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratory, Logan, UT, U.S.A.), 1% L-glutamine (JRH Biosciences, Lenexa, KS, U.S.A.) and 1% PNS antibiotic mixture (penicillin and streptomycin) (JRH Biosciences, Lenexa, KS, U.S.A.). Sodium nitroprusside (SNP), sulphamilamide, naphthylethylenediamine dihydrochloride and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium)-buffered substrate tablets were from Sigma Chemical Co., St. Louis, MO, U.S.A.; *S*-nitroso-*N*-acetylpenicillamine was from Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A.; N^G-monomethyl-L-arginine (NMMA) was from Burroughs Wellcome Co., Beckenham, U.K. TNF and INF-γ were from Boehringer–Mannheim, Indianapolis, IN, U.S.A. and Gemzyme, Cambridge, MA, U.S.A. respectively. *Escherichia coli* LPS was from List Biological Laboratories, Campbell, CA, U.S.A.; HSP 70, inducible and constitutive forms, were from StressGen, Victoria, British Columbia, Canada. Anti-(rat IgG), anti-(mouse IgG), and anti-(rabbit IgG) antibodies were from Jackson Immune

Abbreviations used: NO, nitric oxide; NOS, NO synthase; iNOS, inducible NOS; LPS, lipopolysaccharide; INF-γ, interferon-γ; HSP, heat shock protein; HSC 70, constitutive HSP; HSP 70, inducible HSP; NMMA, N^G-monomethyl-L-arginine; TNF, tumour necrosis factor; SNP, sodium nitroprusside; PEG, poly(ethylene glycol); BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium.

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Research Laboratory, West Grove, PA, U.S.A. Plates for SDS/7.5 %-PAGE were from Daiichi Pure Chemicals Co., Tokyo, Japan.

Treatment of RAW 264.7 cells

To induce NO production in the cell line, RAW 264.7 cells were incubated for different periods of time in fresh medium containing 0.1–10000 units/ml of INF-γ and/or 0.01–1000 μg/ml LPS. The time course of LPS- and/or INF-γ-induced effects on cell viability and nitrite production was studied at 2, 4, 8, 16, 24 and 48 h. The cellular response toward exogenously applied NO was studied using SNP at 50, 100, 200, 400 and 800 μM. The effects of TNF-α alone or in combination with LPS/INF-γ on cell viability and nitrite production were studied at 10, 100, 1000 and 10000 units/ml. In addition, the inhibition of LPS/INF-γ-induced activation of iNOS in the macrophages was studied using NMMA (0.5, 2.5, 5.0 and 7.5 mM). Expression of the inducible or constitutive forms of HSPs was studied both in control cells and cells treated with LPS and INF-γ.

Repetitive treatment of RAW 267.4 cells with LPS and INF-γ

RAW 264.7 macrophages were induced in medium containing 100 units/ml of INF-γ and 10 μg/ml of LPS. After 24 h, the old medium was removed and fresh medium was added; the surviving cells (viability 5–10 % in the culture) were allowed to grow for 2 to 3 weeks, until the viability of the cell culture was again almost at control levels (> 80 %). The same procedure was repeated ten times with the surviving cells, which were always derived from the former round of treatment. We observed that after three rounds of treatment, the viability of the new cells obtained after the LPS and INF-γ 24-h induction was higher than the viability of the original parent macrophages. This viability increased dramatically after ten rounds of treatment with LPS and INF-γ (see the Results section).

Nitrite analysis

Nitrite produces a chromophore with Griess reagent, absorbing at 543 nm, which can then be quantified spectrophotometrically [11] using an automated colorimetric procedure. Briefly, 50 μl of cell culture medium was added to each well of a 96-well plate. After the addition of 50 μl of Griess reagent (1 % sulphamilamide and 0.1 % naphthylethylenediamine dihydrochloride in 2 % phosphoric acid), the plate was left shaking for 10 min at room temperature. The A_{340} was measured using a Bio-Rad model 3550 microplate reader (Richmond, CA, U.S.A.). The nitrite concentrations were calculated by comparison with standard solutions of sodium nitrite produced in culture medium.

Western-blot analysis

Cells were washed with cold PBS, lysed in lysis buffer (50 mM Tris/HCl, 1 mM EDTA, 10 μM PMSF and 10 μl/ml leupeptin), and resuspended on ice for 10 min. The cell suspension was frozen quickly on solid CO_2, lysed mechanically, and centrifuged (1400 rev./min) for 20 min. Then, 4 × sample buffer was added to the supernatant and heated at 95 °C for 8 min. Lysates were subjected to electrophoresis through SDS/7.5 %-PAGE, and proteins were electrophoretically transferred to a nitrocellulose filter. Filters were incubated overnight at 4 °C in blocking buffer [50 mM Tris/HCl, 150 mM NaCl, 1 mg/ml poly(ethylene glycol) (PEG) 2000, 3 % BSA]. Then the filters were cut into two pieces (just above the 80 kDa marker) and incubated for 2 h at room temperature in washing buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mg/ml PEG 2000, 1 mg/ml BSA) containing monoclonal antibodies. The lower part of the immunoblot was incubated with a 1:5000 dilution of rat monoclonal antibody specific for HSC 70 (the constitutive form) or a 1:1000 dilution of mouse monoclonal antibody specific for HSP 70 (the inducible form). The upper part of the immunoblot was incubated with a dilution of 1:500 of rabbit monoclonal 4195 antibody obtained against the N-terminal fragment of mouse macrophage iNOS [12]. After three 10-min washes in washing buffer, the filters were incubated for 1 h at room temperature in washing buffer containing a 1:2000 dilution of alkaline phosphatase-conjugated anti-(rat IgG), anti-(mouse IgG), or anti-(rabbit IgG). After three 10-min washes in washing buffer, the filters were exposed for 2 min to alkaline phosphatase developing buffer (100 mM NaCl, 100 mM Tris base, 5 mM MgCl, pH 9.5) and developed using BCIP/NBT-buffered substrate tablets. The quantitative analysis of the protein bands was performed using a Vilbert Lourmat system, which measures the amount of stained proteins, giving arbitrary units.

Cell viability

Culture viability was measured by calculating the percentage of macrophages alive after dyeing the cells with Trypan Blue solution.

Statistical analysis

The data were analysed using one-way analysis of variance and Duncan's Multiple Range test. Statistical differences between the cell lines were also analysed using the Student's t-test. The accepted level of statistical significance was $P < 0.05$.

RESULTS

Effect of LPS and INF-γ treatment on macrophages

In RAW 264.7 macrophages, LPS and INF-γ, alone or in combination, induced time-dependent production of the stable NO-oxidation product nitrite, which was assayed in the cell supernatant using the Griess reaction (Figure 1A). With increasing concentrations of nitrite, cell viability, assayed by Trypan Blue exclusion, steadily decreased (Figure 1B). Significant differences between control and treated cells were already observed by 4 h and were much more evident after incubations of 24 and 48 h (Figures 1A and 1B). The NOS inhibitor NMMA (5 mM), applied together with LPS and INF-γ, reduced nitrite production significantly. During a 24-h incubation period with the activating agents, nitrite accumulation amounted to 40–50 μM; the addition of NMMA brought these values down to concentrations around 20 μM (18.50 ± 2.34; $n = 5$). Under similar experimental conditions, cell viability increased from values around 20 % (viability assayed after 24 h in the presence of LPS and INF-γ) to about 70 % (viability assayed after 24 h in the presence of LPS, INF-γ, and NMMA). Although NMMA did not totally block nitrite generation, it very efficiently hindered nitrite formation and increased cell viability dramatically.

Viability of RAW 267.4 macrophages after repetitive treatment with LPS and INF-γ

Our experiments showed that RAW 264.7 macrophages gained resistance to the effects of LPS and INF-γ when repeatedly treated with these two agents (see the Experimental section). After ten rounds of treatment the cells were resistant to LPS- and INF-γ-induced cell death. Results of a comparative study of the LPS and INF-γ stimulation in the original cells (RAW) and in

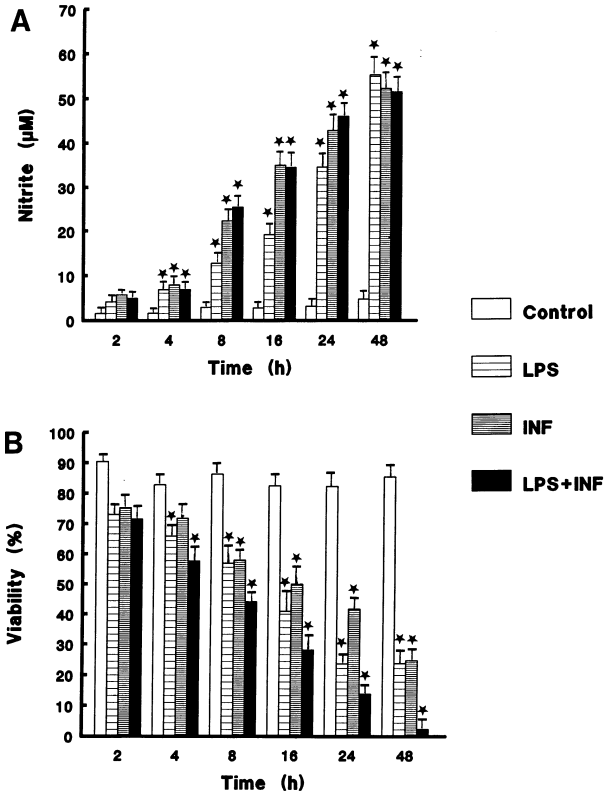

Figure 1 Effects of LPS (10 μg/ml) or INF-γ (100 units/ml) or both on (A) production of nitrite and (B) cell viability in RAW 264.7 macrophages after treatment for 2, 4, 8, 16, 24 and 48 h

Each bar represents mean $\pm$ S.E.M. of five independent experiments. The data were analysed with one-way analysis of variance and Duncan's Multiple Range test ($^*P < 0.05$).

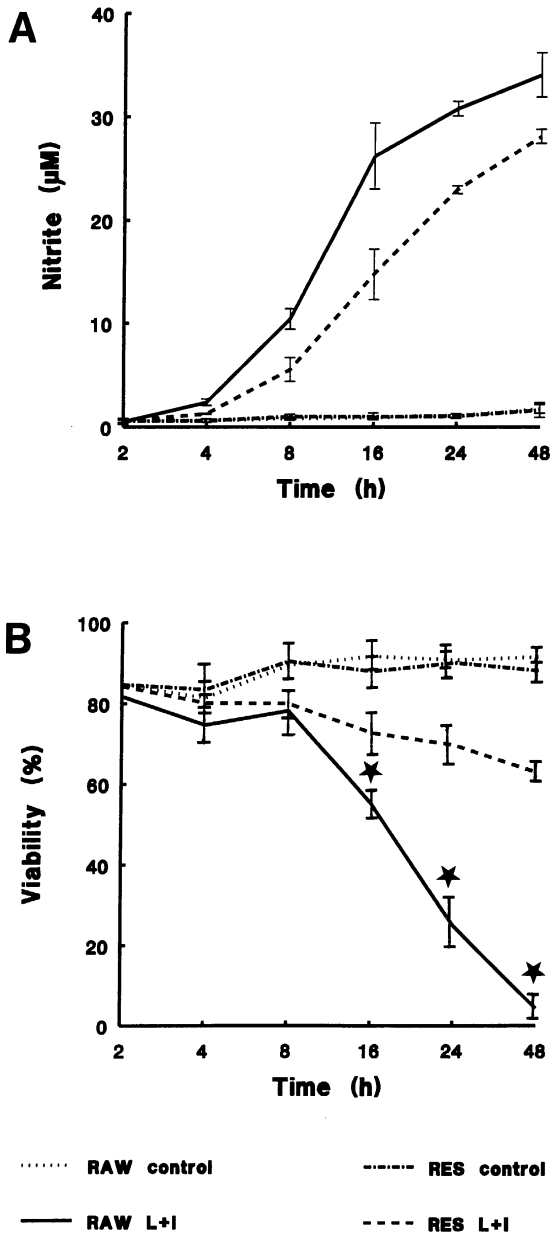

Figure 2 Time course of the effect of LPS (L, 10 μg/ml) and IFN-γ (I, 100 units/ml) on (A) the production of nitrite and (B) cell viability in RAW 264.7 (RAW) macrophages and resistant (RES) macrophages after treatment for 2, 4, 8, 16, 24 and 48 h

Each time-point represents mean $\pm$ S. E. M. of five independent experiments. Statistical analysis of the data as in Figure 1.

the resistant (RES) cells are shown in Figure 2. Stimulation with a combination of LPS and INF-γ revealed dramatic differences in the viability of these two sets of cells (Figure 2B). However, the stimulation of RES and RAW cells with LPS and INF-γ for times of up to 48 h resulted in no statistically significant differences in the ability of these cells to produce nitrite (Figure 2A). Survival of RAW cells was significantly lower than survival of RES cells (Figure 2B). These differences were most dramatic between 16 and 48 h in the presence of LPS and INF-γ (Figure 2).

To definitively demonstrate that RES cells are still able to produce comparable levels of nitrite, we chose increasing concentrations of LPS and INF-γ and measured nitrite generation and cell viability after a 24-h incubation period (Figure 3). The unstimulated cells produced insignificant amounts of nitrite and their cell viability was nearly 90%. Incubating cells with a combination of increasing concentrations of LPS and INF-γ resulted in massive nitrite accumulation in RAW cells, as detected in the cell culture supernatant after 24 h (Figure 3A). Although RES cells formed less nitrite when compared with RAW cells, the difference never reached statistically significant values (Figure 3A). Despite the similarity in nitrite accumulation levels, RES and RAW cells showed dramatic differences in cell viability. RAW cells treated with increasing concentrations of LPS and INF-γ responded with steadily decreasing cell viability, whereas 60–80% of the RES cells stayed alive (Figure 3B).

Effect of SNP on macrophage viability

After establishing that the endogenous NO production was associated with a loss of cell viability, we explored the cellular response to exogenously applied NO. SNP, a commonly used NO donor, releases NO spontaneously in a thiol-catalysed reaction. Incubation of RAW macrophages with increasing concentrations of SNP for 24 h reduced cell viability. Cell viability started to decrease at concentrations of 200 μM SNP (results not shown), and more severe damage occurred at 800 μM SNP (Table 1). At the highest SNP concentration used (800 μM),

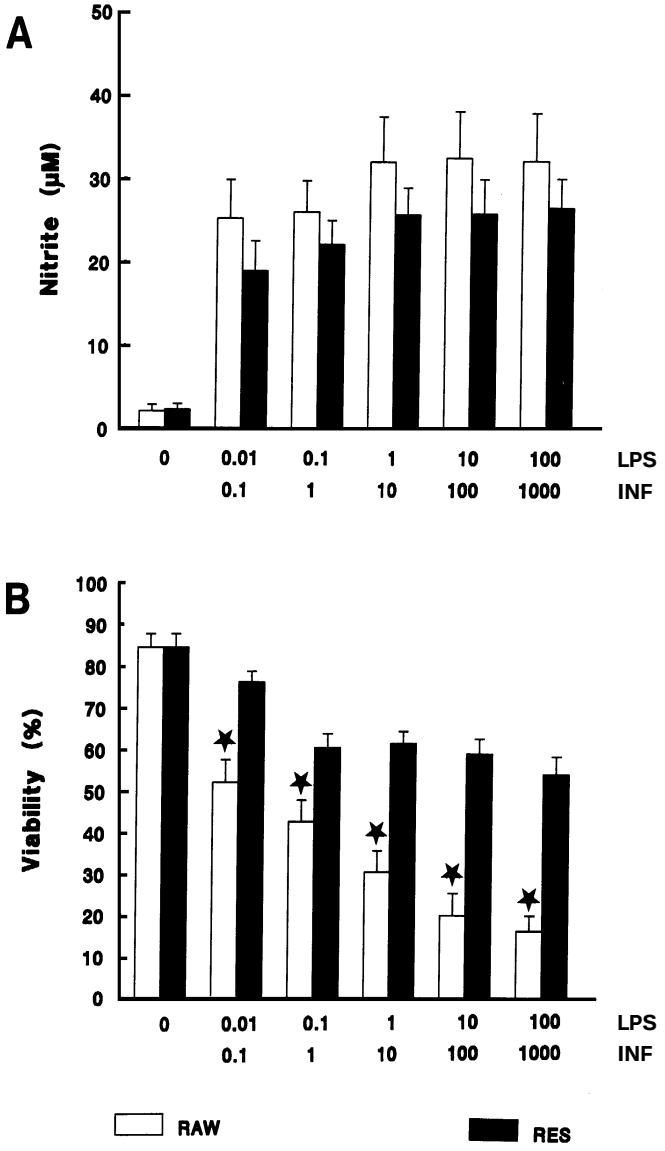

Figure 3 Dose–response curves of LPS and INF-γ induced effects on (A) the production of nitrite and (B) cell viability in RAW 264.7 (RAW) and resistant (RES) macrophages after treatment for 24 h

Each bar represents mean ± S.E.M. of five independent experiments. Units: LPS, μg/ml; INF, units/ml. Statistical analysis of the data as in Figure 1.

cell viability decreased less in the RES cells than in the RAW cells (Table 1). Examination of nitrite formation by RAW and RES cells after SNP application revealed no significant differences. Therefore, differences in the thiol-catalysed decomposition of SNP was ruled out as a possible explanation for variations in the cellular response of RAW versus RES macrophages (Table 1).

Effect of TNF-α on macrophages

To determine whether alterations in the release of other mediators like TNF-α confer resistance to macrophage cells, we exogenously added TNF-α to both cell types. TNF-α by itself was not toxic to RAW or RES cells. Moreover, added in combination with LPS and INF-γ, TNF-α did not alter nitrite generation or cell viability in RAW or RES cells (results not shown).

Table 1 Effects of SNP on cell viability and nitrite production in RAW 264.7 (RAW) and resistant (RES) macrophage cell lines after 24 h induction with LPS and IFN-γ

	Cell type	Cell viability (%)	Nitrite production (μM)
Control	RAW	92.9 ± 1.7	2.00 ± 0.76
	RES	87.5 ± 4.5	2.16 ± 0.74
0.8 mM SNP	RAW	40.1 ± 1.3*	40.16 ± 3.01
	RES	55.9 ± 1.7	41.33 ± 3.20

* Statistically significant ($P < 0.05$; one-way analysis of variance and Duncan's Multiple Range test) difference between RAW and RES cell lines ($n = 5$).

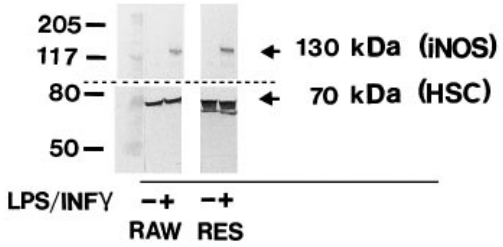

Figure 4 Western blot using antibodies against iNOS and HSC

RAW 264.7 (RAW) macrophages and resistant (RES) macrophages were treated without and with LPS (10 μg/ml) and INF-γ (100 units/ml). Proteins were separated on SDS/PAGE and electrophoretically transferred to nitrocellulose filters. The upper part of the filter was incubated with an antibody against mouse macrophage iNOS, and the lower part of the filter was incubated with antibodies against HSC.

Relationship of HSPs and macrophage resistance

In the quest for a possible explanation for the acquired resistance of the macrophages after several rounds of stimulation with LPS and INF-γ, we directed our attention to the possible role of HSPs. Macrophages express the constitutive form of the HSP with a molecular mass of 70 kDa (HSC 70). Western-blot analysis revealed the presence of this protein in both RAW and RES macrophages. However, RES cells expressed the protein in much higher quantities and, in addition, showed immunoreactivity associated with two different proteins (Figure 4). The quantitative analysis showed that the relative intensity of the HSC 70 bands in control and in LPS/INF-induced RAW cells were 48861 and 46790 respectively. In the RES cells the intensity of the bands was roughly doubled if compared with RAW cells both in control and in induced cells, i.e. 87361 and 86821 respectively. The lower protein band stained in RES cells (Figure 4) was excluded during the quantification.

Activation of both cell types with LPS and INF-γ did not result in expression of the inducible form of the heat shock protein, HSP 70 (results not shown), nor did the agonists alter the expression of the constitutive HSC 70 protein (Figure 4). When the upper portions of the same blots were probed with an antibody directed against iNOS, no differences in the level of this enzyme in RAW and RES cells were observed (Figure 4). Under resting conditions, RAW and RES cells did not express iNOS (Figure 4). However, stimulation with LPS and INF-γ led to the appearance of a massive immunoreactive iNOS protein in both RAW and RES cells. The relative intensity of the iNOS in RAW

cells is 6674 and in RES cells 8807. This is consistent with the generation of nitrite in stimulated RAW and RES macrophages, which gave indistinguishable responses.

DISCUSSION

Macrophages are versatile mammalian cells that take part in the non-specific immune defence against invading bacteria, micro-organisms and pathogens. Among various responses, activated macrophages induce cytokine-inducible NOS, generating large amounts of NO for several days [13]. Both the cytotoxic and cytostatic activities of activated macrophages may involve the production of NO and/or NO-oxidation products, such as peroxynitrite, resulting from the combination of NO with superoxide anion. Macrophage activation is easily achieved *in vitro* by incubating cells with a combination of LPS and INF-γ. Using this technique, we found that one response of RAW 264.7 macrophages, the generation of nitrite (a final NO-oxidation product), is dose- and time-dependent. In agreement with this, macrophages express iNOS [14], which has a molecular mass of 130 kDa on reducing SDS/PAGE gels. NO is known as an intra- and inter-cellular messenger that acts preferentially on distinct haem proteins, iron–sulphur groups of proteins, and protein thiol groups. These protein modification responses explain the physiological effects and some of the cytotoxic activity of NO.

Our results demonstrate that RAW 264.7 macrophages are themselves a target for LPS- and/or INF-γ-mediated cytotoxicity. Results of experiments in which the NOS inhibitor NMMA or exogenous NO-releasing compounds were added suggest that the cytotoxic effects directly relate, at least in part, to NO formation. Similar observations have been reported for endothelial cell damage [15]. Our results are supported by other observations showing that the life span of macrophages inversely correlates with NO production [6]. Although the exact mechanism of NO's cytotoxic effect is at present unclear, NO might play a role in apoptotic cell death [16]. The susceptibility of different cells to NO-mediated toxic insult varies dramatically, and the biochemical events associated with cell resistance toward NO remain unclear.

In the present study we produced a resistant macrophage cell line, which we refer to as RES macrophages. RES cells were derived from RAW 264.7 macrophages after repeated cycles of stimulation with LPS and INF-γ followed by subculture of surviving cells. These resistant cells still produced high concentrations of nitrite without initiating the apoptotic pathway. These cells obviously acquired resistance to NO-mediated toxic insult, as indicated by our finding that exogenously applied NO, released from SNP, was less toxic to RES macrophages than it was to the original RAW macrophages. The more pronounced difference in survival rate between RAW and RES cells when NO was endogenously generated, compared with when NO was produced exogenously, may be explained by the fact that NO is released from SNP at a higher rate than it is formed endogenously. Moreover, the simultaneous release of CN^- from SNP may activate other cytotoxic mechanisms as well. RES and RAW cells are comparable in their ability to respond to exogenous stimuli such as LPS and INF-γ and to synthesize proteins like iNOS; they are also comparable in their ability to decompose SNP. Moreover, there was no difference between RES and RAW macrophages in the response to exogenously applied TNF-α. Of greatest interest, however, is the fact that RES cells were resistant to the toxic effects of NO.

Searching for a plausible mechanism to explain the acquired resistance of RES cells toward NO, we considered HSPs, which are involved in one of the best-characterized responses of eukaryotic cells to noxious stimuli or stress [17,18]. Under physiological conditions, several HSPs function as molecular chaperones, that is they mediate the folding and assembly of other polypeptides and help direct them to their correct intracellular locations. Under conditions of cellular stress, HSPs may stabilize denatured proteins and allow for their subsequent refolding or disposal. The induction of HSPs in response to immune and inflammatory conditions as well as chemically induced stress has been extensively reported [10,19]. Through HSP induction, mainly correlated with HSP 70 expression, cells are protected from various toxic insults like TNF-induced toxicity or metabolic stress [20,21]. It is interesting that chemical-stress-induced heat-shock responses elicited by sodium arsenite and cadmium chloride increase the synthesis of the constitutive form of HSPs in HA-1 or CHO cells [22,23]. A similar mechanism may underlie the 2-fold increase in the expression of the constitutive HSC 70 that we observed in RES cells. Obviously, the amount of HSC 70 in these cells is not sufficient to protect them against the lethal metabolic alterations brought about by LPS and INF-γ, because under those conditions RAW cells are still sensitive to NO-mediated cell death. Only in the presence of an increased level of HSC 70, acquired during the progressive cyclic treatment of cells with LPS and INF-γ, did the cells acquire resistance to an otherwise lethal challenge by LPS and INF-γ.

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