Protection against TNF-Induced Lethal Shock by Soluble Guanylate Cyclase Inhibition Requires Functional Inducible Nitric Oxide Synthase

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Summary

Hypotension and shock observed in sepsis, SIRS, and tumor necrosis factor (TNF) or cytokine-based cancer treatment are the consequence of excessive nitric oxide (NO) production and subsequent soluble guanylate cyclase (sGC)-mediated vascular smooth muscle relaxation. We demonstrate here that, while NO synthase (NOS) inhibitors exacerbated toxicity, inhibitors of sGC activation protected against TNF-induced lethality, bradycardia, and hypotension. Importantly, sGC inhibition did not interfere with the antitumor activity of TNF. Using NOS inhibitors or iNOS-deficient animals, we furthermore observed that no protection against TNF toxicity could be obtained in the absence of NO. These data imply that iNOS- (and not eNOS-) derived NO is an endogenous protective molecule indispensable to survive a TNF challenge and exerting this beneficial effect via sGC-independent mechanisms.

Introduction

In the course of the last decade, tumor necrosis factor (TNF) has been attributed many diverse biological actions in both immunological, physiological, and pathophysiological processes. Originally this pleiotropic cytokine was identified as a pivotal systemic mediator of endotoxemic shock and tumor regression (Fiers, 1995). The remarkable antitumor potential has been successfully exploited in locoregional treatment (Lejeune et al., 1998) for which TNF was recently registrated as an anticancer drug, but its systemic use is still severely hampered by its shock-inducing properties. During clinical trials, hypotension was characterized as the dose-limiting factor of TNF toxicity (Alexander and Rosenberg, 1991).

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Nitric oxide (NO) is a very potent vasodilator generated by a family of enzymes called NO synthases (NOS). At least three different isoforms have been identified in mammalian cells: two constitutive isoenzymes designated endothelial (e) and neuronal (n) NOS, activated by an increase in intracellular Ca^{2+} and involved in the maintenance of normal physiologic processes; and one inducible isoenzyme, iNOS, which is Ca^{2+}-independent and transcriptionally induced by a variety of inflammatory stimuli. Induction of iNOS generally results in excessive NO production causing vascular hyporeactivity and the loss of vascular tone, hallmarks of refractory hypotension (Stuehr, 1999; Titheradge, 1999). In both septic shock patients (Geroulanos et al., 1992) and LPS- or TNF-induced animal models (Kilbourn et al., 1990; Thiemermann and Vane, 1990; Cobb et al., 1992), nonselective inhibitors of NOS were found to reverse the delayed hypotension but could not prevent organ damage or lethality, suggesting the existence of NO-mediated protective activities. To achieve a selective inhibition of the harmful effects of NO, most research has so far focused on the use of specific iNOS inhibitors in endotoxin-induced animal models, with varying results. Whereas in most studies systemic blood pressure was successfully restored (Hock et al., 1997; Wray et al., 1998), peripheral organ dysfunction was nevertheless not or only partially attenuated (Thiemermann et al., 1995; Gundersen et al., 1997; Wray et al., 1998). Therefore, we decided to look for selectivity among the effects rather than the production of NO. One of the main nitrosation targets of NO is the heme-iron present in soluble guanylate cyclase (sGC), thereby activating the enzyme resulting in cGMP accumulation and cGMP-dependent cardiovascular changes such as vascular relaxation, myocardial depression, and the inhibition of platelet aggregation and adhesion (Waldman and Murad, 1987; Wolkow, 1998).

Considering the cGMP-dependent nature of the NO-mediated cardiovascular effects, we decided to aim for the selective inhibition of cGMP-mediated activities using methylene blue (MB), a vital stain generally recognized as an inhibitor of sGC activation both in vitro and in vivo (Gruetter et al., 1981; Murad, 1986) and described to reverse hypotension in septic shock patients (Schneider et al., 1992; Preiser et al., 1995; Driscoll et al., 1996; Andresen et al., 1998), in anesthetized endotoxemic animals (Keaney et al., 1994; Paya et al., 1993), and in fecal peritonitis-induced shock (Galili et al., 1997), or hypotension elicited by mere infusion of NO donors in conscious rats (Salvemini et al., 1996). In view of its well-documented potential to restore blood pressure, we decided to evaluate MB for its use in the treatment of TNF-induced shock.

We here present evidence for MB to prevent lethality, cGMP accumulation, bradycardia, and hypotension in TNF-induced shock. The detrimental role of sGC was further documented using another inhibitor of sGC activation (LY83583) to protect or a cGMP phosphodiesterase inhibitor (zaprinast) to reverse MB protection. However, when the induction of NO was totally blocked via additional treatment with NOS inhibitors, MB protection was completely abolished. In addition, iNOS- (but not
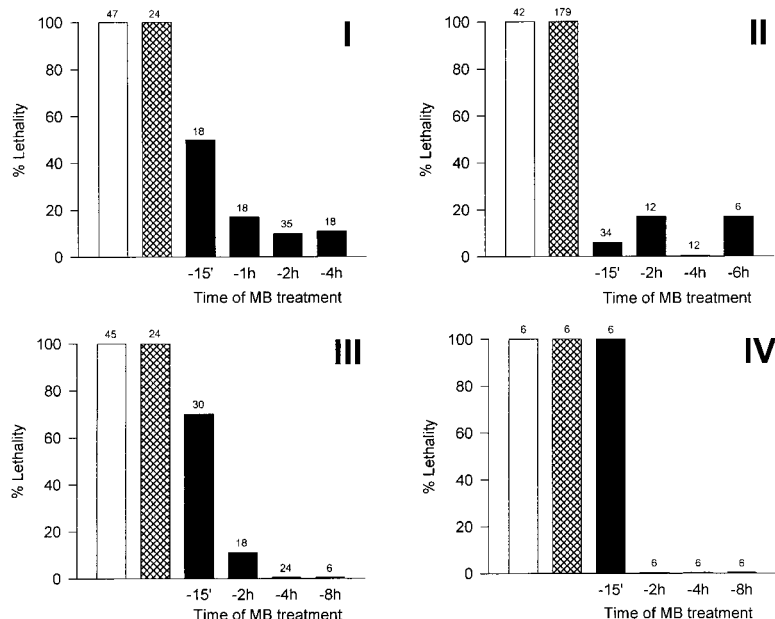
Figure 1. Lethality Induced by TNF in the Four Models

The total number of mice used is indicated above each bar. All mice were i.v. challenged with a 100% lethal dose of mTNF or hTNF, determined with the same lot of TNF and mice before the start of each individual experiment. Open bars represent control mice pretreated with glucose (six mice for each model) or PBS (the numbers indicated above the open bar minus six). Cross-hatched bars are mice that received 100 mg/kg L-NMMA, L-NAME, or L-NIO either 2 hr or 30 min before, together, or 5, 30, or 90 min after TNF. Independent of the NOS inhibitor used or the time point of its administration, mice could never be protected. Closed bars represent mice pretreated with MB at the time indicated below the bars. Lethality was scored up to 7 days after TNF challenge. Generally, various treatment groups were compared in one single experiment using five to seven mice per group. The results shown for models I, II, and III represent the combination of up to five independent experiments. Data for model IV come from one single experiment.

eNOS-) deficient mice could never be significantly protected. We therefore conclude a bivalent role for NO in TNF-induced toxicity: on the one hand mediating bradycardia, hypotension, and subsequent shock via sGC activation; on the other hand exerting a protective and cGMP-independent function, essential to survive. Contrary to the general assumption, the source for this protective NO is iNOS and not eNOS, prompting us to question the feasibility of selective iNOS inhibition as a strategy to provide protection against TNF-induced or -mediated diseases.

Results

MB and LY83583, but Not NOS Inhibitors, Protect against TNF-Induced Lethality

To investigate the role of NO and sGC in TNF-induced shock, we performed experiments in four different lethality models in mice. In model I, nonsensitized mice are i.v. challenged with a 100% lethal dose of mouse (m) TNF, typically ranging from 20 to 30 μg. In the other three models, human (h) TNF, which is normally not lethal in healthy mice except at extremely high doses (LD$_{50}$ $\approx$ 500 μg), is injected in sensitized mice. Mice are sensitized by the presence of an i.m. LLC tumor (model II), a BCG infection (model III), or repeated IL-12 injections (model IV). Sensitization models were described in detail before (Cauwels et al., 1995, 1996). Essentially, model III and IV may be regarded molecularly equivalent since BCG sensitization can be abrogated by anti-IL-12 treatment and both models were shown to depend on endogenous IFNγ for sensitization (Cauwels et al., 1995, 1996).

Treatment of mice with different NOS inhibitors (L-NAME, L-NMMA, or L-NIO) at various time points (before, together, or after TNF challenge) could not protect in any of the models used (Figure 1). Treatment with NOS inhibitors actually exacerbated rather than ameliorated TNF toxicity, in a sense that LD$_{100}$-challenged mice died earlier and an LD$_{25}$ TNF dose would become 100% lethal in combination with L-NAME (data not shown). However, when we used the harmless stain MB to block sGC

activation, mice were protected very efficiently against TNF-induced mortality in all four models (Figure 1).

Given the significant protection by MB, we next determined whether another inhibitor of sGC activation, LY83583 (Malta et al., 1988), had a similar effect against mTNF-induced mortality. As plotted in Figure 2, LY83583 proved as capable as MB to protect. To further substantiate the involvement of sGC-induced cGMP accumulation in TNF lethality, MB pretreatment was combined with multiple injections of zaprinast, a specific inhibitor of type V cGMP phosphodiesterase increasing cGMP concentrations (data not shown). Zaprinast was injected i.v. four times (together with, as well as 2, 5, and 8 hr after mTNF), a treatment not lethal as such but very efficient to abrogate MB protection (data not shown).

Effect of MB on TNF-Induced cGMP Production, Bradycardia, and Hypotension

The experiments using MB, LY83583, and zaprinast indicated that sGC is a pivotal detrimental mediator of TNF-induced lethality. To assure that MB is actually blocking
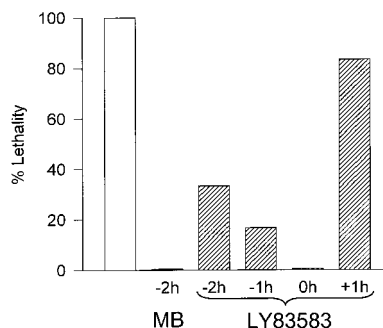

Figure 2. Protection by LY83583 as Compared to MB in Model I

All mice received a lethal dose of mTNF (25 μg), combined with 25% DMSO as a control (open bar), MB at -2 hr (closed bar), or LY83583 (diluted in 25% DMSO) at the time indicated beneath the hatched bars. Each bar represents a group of six mice. All mice were treated in the same experiment. Extra independent experiments (dose–response) revealed similar results.
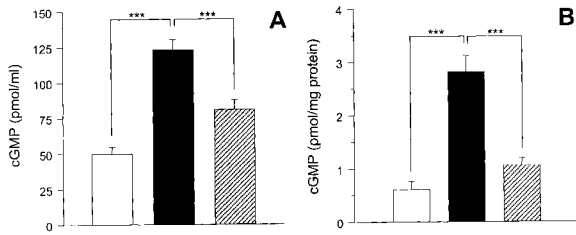
Figure 3. cGMP Accumulation in Plasma and Kidney Homogenates

Mice were injected with PBS (open bars), a lethal dose of 25 μg mTNF (closed bars), or MB + mTNF (hatched bars). Plasma (A) and kidneys (B) were collected 5 hr after mTNF challenge. Plotted are the means ± SEM of eight or nine mice coming from three separate experiments. ***p < 0.001 (one-way ANOVA Bonferroni test).

sGC activation, cGMP was measured in plasma and kidney homogenates. Mice showed increased cGMP concentrations peaking about 5 hr after mTNF injection, and this increase was downmodulated by MB pretreatment (Figure 3).

In the cardiovascular system, NO-induced cGMP accumulation in shock accounts for the relaxation of vascular smooth muscle cells and myocytes, causing vasodilatation and myocardial depression, respectively. To evaluate the effect of MB on TNF-induced hemodynamic changes, mean arterial blood pressure (MAP) and heart rate (HR) were monitored continuously in free-moving conscious mice. Both HR and MAP dropped drastically within 5 min of MB pretreatment, but recovered fast and were back to seminormal levels at the time of mTNF injection 2 hr later (Figures 4B and 4D). TNF lethality could invariably be predicted by a severe drop in HR starting only a couple of hours after the TNF challenge

(Figure 4A) but efficiently prevented by pretreatment with MB (Figure 4B). MAP data after TNF challenge showed an immediate transient drop in blood pressure, followed by the delayed hypotension. Nonlethal TNF doses caused minor hypotension (data not shown), whereas a lethal TNF injection resulted in severe hypotension (>40 mmHg MAP reduction) within the 10 hr observation period in 4/7 animals (Figure 4C). When mice were pretreated with MB, this severe hypotension was never observed (Figure 4D). All mice pretreated with MB survived the normally lethal TNF challenge, while all glucose-pretreated animals died within 20 hr after TNF injection. Overall, MAP and HR in MB + mTNF treated mice did not differ significantly from control mice treated with MB alone (data not shown).

Evidence for a Protective Role of NO in TNF-Induced Shock

To determine why sGC but not NOS inhibition can protect mice from a lethal TNF challenge (Figure 1), MB and L-NAME treatments were combined. MB protection was totally abrogated by L-NAME in models I and II, where the L-NAME injection was capable of reducing serum NO levels to below basal values (Figure 5). In models III and IV, however, L-NAME could not completely eliminate the systemic presence of NO (not even when L-NAME was injected twice at a 5-fold higher dose; data not shown) and MB could still provide 100% protection even in combination with L-NAME treatment (Figure 5). It should be noted that in these latter models the mere presence of a BCG infection or IL-12 sensitization caused a substantial presence of NO_x^- in circulation before TNF was administered (Figure 5, open bars). Taken together, these data imply that MB can no longer protect against TNF-induced lethality if NO is completely
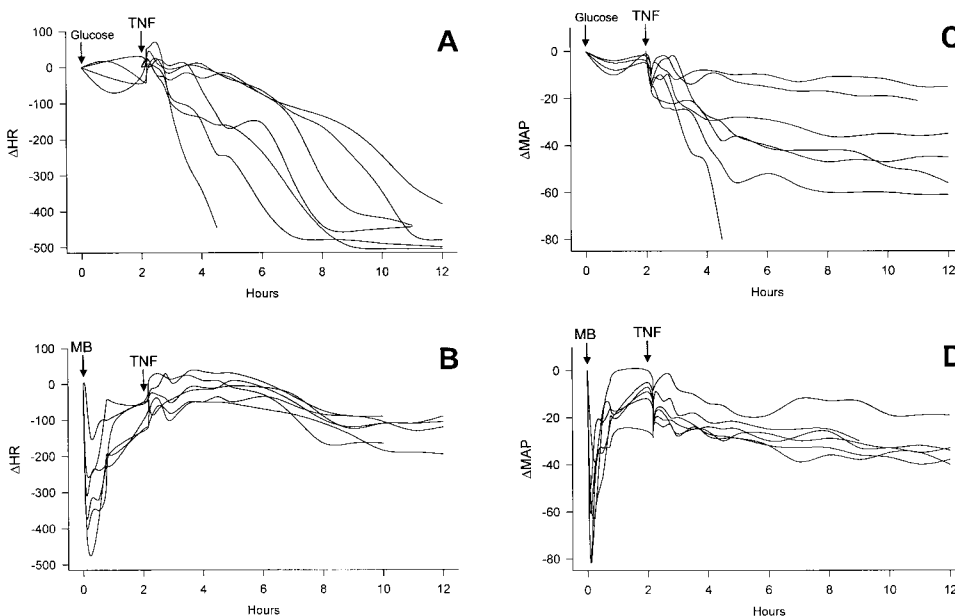

Figure 4. Individual Heart Rate Changes and MAP Changes

Heart rate (HR) (A and B) and MAP (C and D) changes are plotted as compared to baseline values at time 0 (typically varying between 650 and 750 beats per minute and 95 to 115 mmHg), immediately before the i.v. injection with 200 μl glucose as a vehicle (A and C) or 15 mg/kg MB (B and D). A lethal dose of 20 μg mTNF, or endotoxin-free PBS as a control, was administered i.v. 2 hr later. Mice were monitored continuously for 10–12 hr. All glucose-pretreated animals died within 20 hr after TNF challenge, whereas all MB-pretreated mice survived the otherwise lethal mTNF challenge.
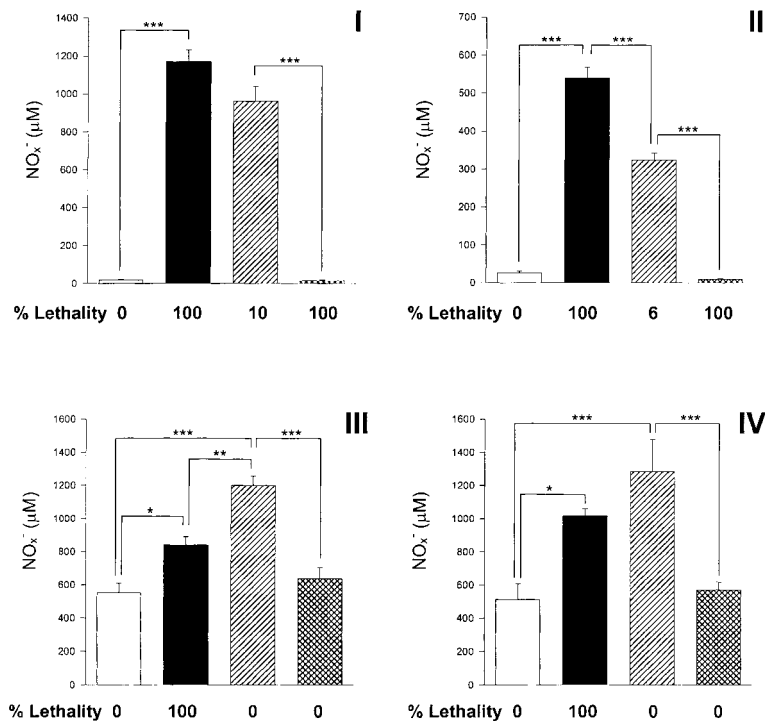
Figure 5. NO_x^- Determination as a Measure for NO Release

The four different lethality models (I, II, III, and IV) were used to measure NO_x^- ($NO_2^- + NO_3^-$) in serum obtained from mice i.v. injected with PBS (controls, open bars), TNF (closed bars), MB + TNF (hatched bars), or MB + TNF + L-NAME (cross-hatched bars). MB was given 2 hr before TNF, L-NAME 5 min afterward. Plotted are means ± SEM from at least six mice. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (one-way ANOVA Bonferroni test). Numbers underneath the bars represent the percent lethality in the respective treatment groups and models.

absent from the circulation, hinting toward the existence of an indispensable protective role for NO in TNF toxicity.

Identification of iNOS as the Source of the Protective NO

Considering the presumed protective role of NO in TNF toxicity, we examined the effect of MB and L-NAME in eNOS- and iNOS-deficient mice. NO was clearly induced following mTNF challenge in eNOS-deficient (Figure 6A),

but not in iNOS-deficient (Figure 6B), animals. Survival studies learned that eNOS-deficient mice behaved exactly like wild-type mice. In model I, MB protected completely against mTNF lethality and protection was abrogated by the extra treatment with L-NAME (Figure 6C), while, in model III, MB protection against TNF lethality could not be reversed by L-NAME (Figure 6D). In contrast to the results in the eNOS-deficient animals, iNOS-deficient mice could not significantly be protected by
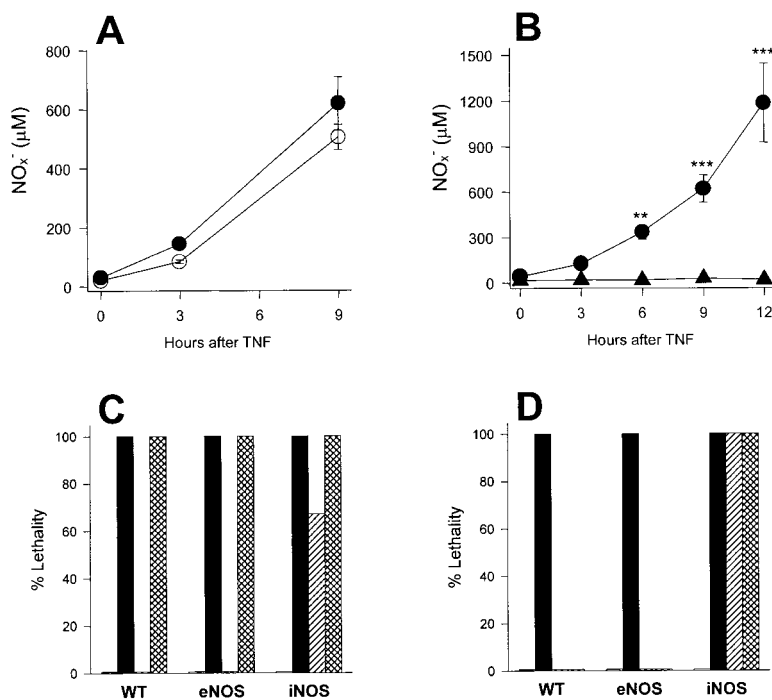

Figure 6. NO_x^- Measurement and Lethality of TNF in C57Bl/6 and eNOS- and iNOS-Deficient Mice

(A and B) To determine inducibility of NO, a lethal dose of 20 μg mTNF was i.v. injected in C57Bl/6 mice ([A and B], closed circle) or in eNOS-deficient mice ([A], open circle) or iNOS-deficient mice ([B], closed triangle). (A) and (B) represent separate experiments. Plotted are means ± SEM from groups of four to six mice. *** $p < 0.001$, ** $p < 0.01$ (one-way ANOVA Bonferroni test) comparing values of C57Bl/6 with NOS-deficient mice. When not indicated, NO_x^- levels were not statistically significant between wild-type or knockout animals.

(C and D) Mortality studies were performed in models I ([C], mTNF lethality) and III ([D], hTNF lethality in BCG-primed animals), where bars represent percent lethality after i.v. injection with PBS (open bars), TNF (closed bars), MB + TNF (hatched bars), or MB + TNF + L-NAME (cross-hatched bars) in either C57Bl/6 mice as wild-type (WT) controls or eNOS-deficient or iNOS-deficient mice as indicated below the respective groups of bars. MB was given 2 hr before TNF, L-NAME 5 min afterward. Each bar represents a group of 6–12 mice.
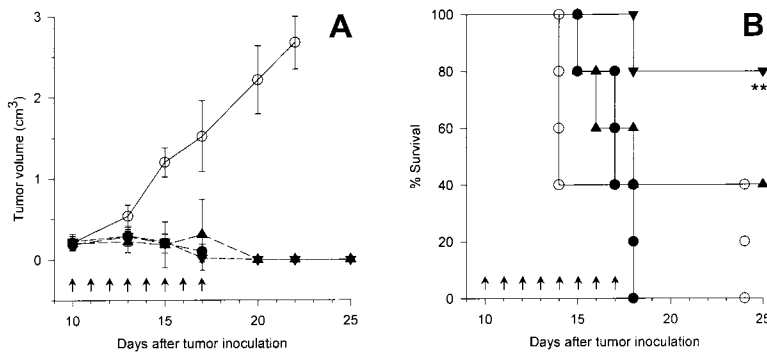
Figure 7. Effect of MB Pretreatment on Treatment of a B16BL6 Tumor with mTNF and mIFNγ as Described in the Experimental Procedures

Tumor volume (A) and survival (B) are plotted as a function of time (days after tumor inoculation). Each treatment group consisted of five animals. Daily antitumor treatment (arrows) was started on day 10 with PBS (open circle) or mTNF + mIFNγ (closed circle, triangle, inverted triangle). Every second (triangle) or third (inverted triangle) day, 15 mg/kg MB was injected 2 hr before mTNF + mIFNγ treatment. **p < 0.01 (logrank test), as compared to mTNF + mIFNγ without MB pretreatment (closed circle). In total, four independent experiments were performed using either 7.5, 10, or 15 mg/kg MB (in each individual experiment used daily, every other day, or every third day). Results from some of the other experiments are described in the Results.

MB pretreatment in model I (Figure 6C) and not at all in model III (Figure 6D). In addition, treatment with LY83583 could not protect iNOS-deficient mice against TNF mortality (data not shown). These results clearly prove that in the absence of iNOS MB or LY83583 fails to protect against TNF lethality, implicating iNOS as the source of protective NO in TNF toxicity.

MB Prevents Systemic Toxicity but Not the Antitumor Activity of TNF

TNF is a very potent antitumor agent, but its systemic use is seriously limited due to its shock-inducing properties. We have identified MB as a drug to prevent TNF-induced systemic toxicity and lethality. However, to be considered therapeutically valuable, we needed to verify whether MB interferes with the antitumor activity of TNF or not. For this purpose, we tested the effect of MB pretreatment on the antitumor efficacy of mTNF and mIFNγ against a syngeneic murine B16BL6 melanoma tumor. As described previously (Brouckaert et al., 1986), daily treatment of tumor-bearing mice with a combination of 10 μg mTNF and 5000 IU mIFNγ caused a complete regression of the tumor (Figure 7A) but proved toxic resulting in 100% lethality by the end of the treatment (Figure 7B). It should be noted that in contrast to the results obtained in the clinic, where the successful isolated limb perfusion treatments were achieved with a single high dose administration of TNF, regression and cure of murine tumors needs repetitive daily treatments with TNF. Since a daily administration of 15 mg/kg MB for 8 consecutive days caused toxicity as such, we decided to evaluate the effect of 15 mg/kg MB given every other day or every third day 2 hr before TNF + IFNγ. As is evident from Figure 7A, pretreatment with MB did not affect the antitumor efficacy of TNF + IFNγ at all, but afforded considerable protection against the shock-inducing effects of the antitumor treatment (Figure 7B) resulting in 40%–80% survival of the cured mice (injecting MB every other day or every third day, respectively). A lower dose of MB (10 mg/kg), used in a separate experiment, did not cause toxicity as such when administered daily and provided 80% protection against the TNF + IFNγ combination, or 60% protection when given every second or third day (data not shown). In still another experiment, the TNF + IFNγ treatment was preceded every third day by a combination of MB and the acute phase protein α₁-AGP previously reported to protect against TNF-induced shock (Libert et al., 1994). This protocol resulted in complete regression of the

tumor and 100% survival of the tumor-free mice (data not shown). Hence, we conclude that MB pretreatment impairs toxicity but not the antitumor potential of TNF, allowing a complete cure of tumor-bearing mice.

Discussion

In addition to the detrimental role of NO in shock-like diseases, described in a plethora of reports during the last decade, studies in animal models and clinical trials using NOS inhibitors suggest a beneficial role for NO as well. The disappointing results obtained with nonselective NOS inhibitors resulted in a quest for selective iNOS inhibitors, based upon the involvement of eNOS in the normal regulation of vascular tone and the upregulation of iNOS in inflammatory conditions. Still, discrepant results have been obtained using these iNOS-selective inhibitors in various endotoxic shock models. For that reason, we started to question the advantage of selective iNOS inhibitors as therapeutic agents and decided to aim for the selective inhibition of NO-mediated effects rather than NO production. So far, sGC is the only conclusively proven receptor for NO and is involved in many NO-mediated effects, most notably in the nervous and cardiovascular system. In the latter, NO-induced cGMP accumulation accounts for the relaxation of vascular smooth muscle cells, responsible for the excessive vasodilatation and hyporeactivity that are characteristics of hypotension in shock. Other cGMP-mediated cardiovascular effects include myocardial depression, causing cardiac dysfunction.

We present evidence here for MB, an inhibitor of sGC activation (Gruetter et al., 1981; Murad, 1986), to prevent TNF-induced mortality (Figure 1), cGMP accumulation (Figure 3), bradycardia, and hypotension (Figure 4) in mice. When used in combination with TNF and IFNγ as an antitumor treatment, it was clear that MB successfully attenuated TNF-associated toxicity but not its antitumor activity, thus broadening the systemic therapeutic potential of TNF (Figure 7). To further prove the involvement of sGC, LY83583, another archetypal inhibitor of sGC activation, was used to successfully protect mice against mTNF-induced lethality (Figure 2). However, both MB and LY83583 have been shown to produce superoxide in aqueous solutions and hence have been postulated to exert a significant part of their effect by inactivating NO via complex formation. Nevertheless, in

our experiments, total or partial inhibition of NO production via NOS inhibitors (Figures 1 and 5) or scavenging of NO via 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO; data not shown) could not mimic the protective effect of MB or LY83583, arguing against the elimination of NO to be the principal mechanism of MB- or LY83583-mediated protection. Moreover, combining the MB treatment with multiple injections of superoxide dismutase (SOD) or the cell-permeable SOD-mimetic Tempol, known to increase the bioavailability of NO in vitro (Zöllner et al., 1997) and in vivo (Schnackenberg et al., 1998), did not affect MB-provided protection at all (data not shown). This indicates again that MB does not simply protect via a superoxide-mediated mechanism. Furthermore, we actually demonstrated that MB prevents TNF-induced cGMP production in vivo (Figure 3) and that zaprinast, a cGMP phospodiesterase inhibitor that increases cGMP accumulation, abolished MB protection (data not shown). Taken together, these observations convincingly argue for inhibition of the activation of sGC to be the mechanism of protection by MB against TNF-induced mortality. Further evidence may come from studies using knockout mice, which are currently being constructed.

When evaluating the effect of MB on TNF-induced cardiovascular changes, we found that it very efficiently alleviated both bradycardia and hypotension. In septic shock patients, only a minor population shows reduced cardiac output, although it has been suggested that in patients with normal or increased cardiac output myocardial depression is actually masked by the ventricular dilation compensating for the extremely reduced ejection fraction (Parrillo et al., 1985). Later, TNF was identified to mediate endotoxin-induced myocardial depression (Tracey et al., 1986) and to be an important cardiodepressant factor in human sepsis and heart failure (Cain et al., 1998; Kubota et al., 1998). TNF by itself depresses both animal and human cardiac function in a dose-dependent and biphasic fashion (immediate via spingosine [Oral et al., 1997] and delayed via NO). Endogenous NO evidently causes the late myocardial depression via desensitization of the myofilament to Ca^{2+} (Finkel et al., 1992; Goldhaber et al., 1996), an effect proposed to be mediated via cGMP (Hare and Colucci, 1995; Vila-Petroff et al., 1999). Leakage of TNF during isolated limb perfusion was reported to cause distributive shock, in half of the patients combined with a severe cardiogenic shock (Eggimann et al., 1995). In our mouse experiments, mortality following TNF injection could invariably be predicted by a profound and sustained drop in HR starting 1–5 hr after TNF challenge (Figure 4A). When pretreated with MB, a normally lethal TNF dose causes the HR to diminish only mildly and transiently (Figure 4B), an effect we also observed in case of a nonlethal TNF injection (data not shown).

Excessive vasodilatation and vascular hyporeactivity with ensuing refractory hypotension have long been considered hallmarks of septic shock. In endotoxic animal models, it was proven that delayed hypotension in shock is mediated by NO exerting its vasorelaxatory properties mainly via the activation of sGC. Also in a hTNF-induced shock model in anesthetized dogs, NO-dependent hypotension was shown to develop following sublethal TNF injection (Kilbourn et al., 1990). In most phase I clinical TNF trials, hypotension reliably occurred and a severe drop of systolic blood pressure (>40 mmHg) was observed in 25% of the evaluable patients

(Alexander and Rosenberg, 1991). In 1992, a consensus committee defined sepsis-induced hypotension by a reduction in systolic blood pressure of 40 mmHg or more from baseline (Bone et al., 1992). In our mTNF toxicity model in conscious unrestrained mice, we observed that injection with TNF immediately causes a transient and minor hypotensive phase, followed by a delayed fall in blood pressure. In case of a lethal TNF challenge, severe hypotension (>40 mmHg drop) occurred in four out of seven animals within the 10 hr observation period (Figure 4C). When pretreated with MB, however, this severe delayed hypotension was never observed (Figure 4D). Hence, we conclude that in nonanesthetized mice a lethal TNF challenge is predictably accompanied by severe bradycardia and, in about half the cases, by profound hypotension within 10 hr after injection. Both cardiovascular changes are without exception efficiently prevented by a life-saving MB treatment.

In an attempt to understand why inhibition of sGC activation but not NO blockade could protect mice against TNF lethality, we combined MB treatment with L-NAME injection and found evidence for an indispensable protective role for NO in TNF toxicity, in addition to its sGC-mediated detrimental activities. Using eNOS- and iNOS-deficient mice, we could confirm this protective effect. Whereas eNOS-deficient animals behaved just like wild-type mice, iNOS-deficient mice could not be protected: not by MB (Figure 6C and 6D), not by LY83583 (data not shown), but also not by the acute phase protein α_1-AGP previously reported to protect against TNF-induced shock (Libert et al., 1994) (data not shown). Although the general assumption is for eNOS, rather than iNOS, to deliver protective NO, very recently evidence was presented for an obligatory role of induced NO in the cardioprotection afforded by the late phase of ischemic preconditioning (Guo et al., 1999) and as an endogenous neuroprotectant in a mouse and rat model of traumatic brain injury (Sinz et al., 1999). Also, in endotoxemic rats induced NO was proposed to serve as an endogenous antiapoptotic agent in the liver (Ou et al., 1997), and it has been postulated that depending on the insult induced NO can be either hepatotoxic or hepatoprotective (Li and Billiar, 1999). Here we present evidence for a life-saving function of NO in TNF-induced toxicity, identifying iNOS as its source. Since MB protects and downmodulates cGMP in our lethality models, we hypothesize that the protective effect of NO is independent of cGMP accumulation. What exactly the molecular target is for this beneficial NO remains to be elucidated. cGMP-independent effects of NO include the direct scavenging of superoxide anions, the S-nitrosylation of proteins such as caspases (Dimmeler et al., 1997) and NF-κB (Matthews et al., 1996), and the activation of cyclooxygenase (Salvemini et al., 1996). NO was also shown to interfere with apoptosis via mechanisms other than S-nitrosylation-mediated caspase inhibition, but the cGMP dependency of most of these effects remains controversial (Dimmeler and Zeiher, 1997).

Taken together, our results indicate that in conscious mice TNF exerts its lethal effects via sGC activation, invariably accompanied by severe bradycardia and in about half the cases by profound hypotension within 10 hr of observation. Pretreatment with MB can efficiently prevent TNF-induced cGMP accumulation, bradycardia, hypotension, and mortality but does not interfere with antitumor activities, thus broadening the therapeutic potential of systemic TNF as an anticancer treatment. In

addition, iNOS-derived NO exerts protective effects against TNF toxicity, arguing against the therapeutic use of iNOS-selective inhibitors in TNF-induced or -mediated pathologies such as septic shock, hepatotoxicity, inflammatory bowel diseases, or myocardial ischemia-reperfusion.

Experimental Procedures

Cytokines, Cell Lines, and Reagents

Recombinant hTNF, mTNF, and mIFNγ were produced in *Escherichia coli* and purified to homogeneity in our laboratory. The endotoxin content was <0.02 ng/mg, as assessed by a chromogenic *Limulus* amoebocyte lysate assay (Coatest; Kabivitrium, Stockholm, Sweden). Live BCG organisms were provided by the Institut Pasteur du Brabant (Brussels, Belgium), and recombinant mIL-12 was generously provided by Dr. M. Gately (Hoffmann-La Roche, Nutley, NJ). Murine LLC clone H61 and the B16BL6 melanoma subline were gifts from Dr. M. Mareel (University Hospital, Ghent, Belgium) by courtesy of Dr. G. Vaes and Dr. I. Fidler, respectively. Tumor cells were cultured in DMEM supplemented with 10% FCS, 50 U/ml penicillin G, 50 μg/ml streptomycin sulfate, and 2 mM L-glutamine. Before injection, cells were washed twice in PBS. NOS inhibitors L-NAME, L-NMMA, and L-NIO were purchased at Novabiochem and injected i.v. at a dose of 100 mg/kg unless stated otherwise. MB (Sterop Pharmacobel, Belgium) was purchased in glucose solution suitable for i.v. injection and was administered at a dose of 15 mg/kg unless mentioned otherwise. Glucose (Sterop Pharmacobel, Belgium) was used as a control. LY83583 (Biomol Research) was injected i.v. at 2.5 mg/kg in 25% DMSO. Zaprinast (Novabiochem) was administered i.v. at 30 mg/kg per injection. Injection solutions were diluted in endotoxin-free PBS, and, unless stated otherwise, injection volumes were 200 μl.

Mouse Models

Female C57Bl/6 mice (Charles River, Sulzfeld, Germany), eNOS-deficient mice (Huang et al., 1995), or iNOS-deficient mice (Laubach et al., 1995) (Jackson Laboratories) were used at the age of 7–12 weeks at the beginning of the experiment. The animals were housed in temperature-controlled, air-conditioned facilities with 12 hr dark/light cycles and food and water ad libitum. All experiments were approved by and performed according to the guidelines of the animal ethics committee from the Universities of Ghent, Belgium, and Maastricht, The Netherlands. Four different TNF lethality models were used. In model I, a lethal dose of mTNF is injected i.v. in healthy, nonsensitized mice. The sensitization models II, III, and IV were performed as described in detail before (Cauwels et al., 1995, 1996). Briefly, in model II mice bearing an i.m. LLCH61 tumor with a diameter of about 15 mm are challenged with 20 μg hTNF, while in model III mice are sensitized by a BCG infection 2 weeks before being challenged by a 10–15 μg hTNF injection. Model IV consists of a daily i.p. treatment with 1 μg rmIL-12 during 5 consecutive days, followed after a 2 day interval by a lethal hTNF challenge of 5–10 μg. All TNF injections shown are i.v. (200 μl, diluted in endotoxin-free PBS) and 100% lethal. The LD_{100} was determined with exactly the same lot of TNF and mice before the start of each individual experiment. Lethality is generally scored up to 7 days after TNF challenge.

cGMP Determination

cGMP concentrations were determined by EIA (Amersham Pharmacia Biotech, UK) after acetylation of the samples and according to the manufacturer's instructions. Plasma was collected via cardiac puncture using 7.5 mM EDTA as an anticoagulant and kept on ice before centrifugation and subsequent storage at −20°C. Organs were immediately immersed after excision in liquid nitrogen and stored at −20°C prior to homogenization and purification as suggested by the manufacturer. Kidney cGMP levels were plotted as picomoles cGMP per mg of TCA-precipitable protein solubilized with 1 N NaOH.

MAP and HR Measurements

It has been documented using eNOS-deficient mice that mere anesthesia influences NO-mediated blood pressure changes in mice (Ma et al., 1996). Hence, we chose to measure blood pressure in conscious mice. In a computerized tail–cuff method, however, very early after TNF challenge, pulse amplitude was reduced to values insufficient to use this technique, which left us with the method of permanent catheterization in mice. Mice were instrumented with catheters as described in detail previously (Janssen et al., 2000). In short, under ketamine (100 mg/kg i.m.) and xylazine (5 mg/kg s.c.) anesthesia, a heat-stretched piece of polyethylene (PE25) tubing (OD/ID 0.15/0.1 mm at the tip) was inserted (1.5 cm) into the right femoral artery and subcutaneously guided to the neck of the mouse. Here the catheter was fixed, extended, filled with heparinized saline (10 U/ml), and plugged. A venous catheter (silastic OD/ID 0.25/0.12 mm) was inserted into the right jugular vein and similarly guided and extended in the neck to allow i.v. injections of reagents (200 μl of glucose or MB 2 hr before mTNF or PBS challenge) without disturbing hemodynamics. After surgery, animals received i.p. 2 ml of a Ringer's solution to improve recovery as well as buprenorphine (2 mg/kg s.c.) as analgesic agent. The mice were kept at 30°C until fully recovered from the anesthesia and housed individually. Experiments were performed 3–6 days after surgery. On the day of the experiment, the arterial catheter was connected to a low-volume pressure transducer (micro-switch, model 156PC 156WL, Honeywell Inc., Amsterdam, the Netherlands) to record blood pressure. MAP and HR were measured continuously throughout the study. The preparation was allowed to stabilize for 50–90 min before the start of the experiment.

NO_x^- Measurement

Blood was collected via cardiac puncture, allowed to clot at 37°C, and subsequently centrifuged to obtain serum. Sera were half diluted with 5.10^9 CFU/ml *Pseudomonas oleovorans* suspension (reducing nitrate to nitrite) and incubated at 37°C for 2 hr. After removal of the bacteria by centrifugation, the supernatant was one-third diluted with Griess reagent (Green et al., 1982) and serum proteins were precipitated by addition of 10% TCA. The absorbance of the supernatant was measured at 540 nm. Total NO_x^- concentrations were calculated based on a nitrate standard curve.

Antitumor Treatment

C57Bl/6 mice were inoculated s.c. with 6.10^5 B16BL6 melanoma cells on day 0. Paralesional treatment with 10 μg mTNF and 5000 IU mIFNγ started on day 10, when the tumor inoculum formed a palpable tumor. Tumor-bearing mice were thus treated for 8 consecutive days. Tumor size was assessed every 2–3 days and expressed as $0.4 \times a^2 \times b$, with a being the smaller and b the larger diameter of the tumor. Deaths were monitored daily. When mentioned, MB was administered i.v. daily, every second day, or every third day, each time 2 hr before the TNF + IFNγ treatment.

Statistics

Significance of change in cGMP or NO_x^- levels was examined using a one-way ANOVA Bonferroni test (with comparison of all pairs). For survival studies in Figure 7, statistical significance was evaluated using a logrank test.

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