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Maleylated-BSA Enhances Production of Nitric Oxide from Macrophages

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Maleylated-bovine serum albumin (maleyl-BSA) elicits transcription and secretion of a number of proinflammatory genes via ligation of the low-affinity scavenger receptor (SR) on macrophages. We now demonstrate that while neither maleyl-BSA, nor interferon-γ (INF-γ) alone induce nitric oxide (NO) production, when combined they promote release of NO from murine peritoneal macrophages. This effect was blocked by treatment with oxidized-low density lipoprotein. Maleyl-BSA activated NF-κB dimers capable of binding the NF-κBd sequence unique to the iNOS promoter, but this failed to induce significant new transcription or accumulation of iNOS mRNA. The combination of maleyl-BSA and IFN-γ failed to demonstrate synergy at the transcriptional or mRNA levels, as these levels were comparable to those elicited by IFN-γ alone. These studies suggest that the synergy in NO production between maleyl-BSA and IFN-γ occurs after the accumulation of iNOS-specific mRNA, possibly at the translational or post-translational level.

Scavenger receptors (SR) were first described by Brown and Goldstein in 1983 when they observed that macrophages do not take up native lipoproteins extensively, but do take up these proteins if they are covalently modified by reactions such as acetylation or maleylation (1). It is now recognized that macrophages express at least three distinct receptors for modified lipoproteins, as determined by surface binding studies (2-5). Two of these SR bind acetylated-low density lipoprotein (acetyl-LDL) and maleylated-bovine serum albumin (maleyl-BSA) at high and low affinity respectively; while the third binds oxidized-LDL (ox-LDL) (6).

Maleylated proteins induce a complex set of effects in macrophages, which in part resemble the effects of lipopolysaccharide (LPS). Specifically, binding of maleyl-BSA results in the accumulation of message for and secretion of several proinflammatory gene products such as tumor necrosis factor-α (TNF-α), interleukin-1, MCP-1, and GRO (3). Direct cytotoxicity of tumor cells can also be elicited in some types of primed macrophages by maleyl-BSA (3,7). By contrast, message accumulation and surface expression of MHC class II genes is suppressed (8,9). These effects arise from ligation of the low-affinity SR rather than the high-affinity SR (3).

Because nitric oxide (NO) is an important proinflammatory mediator induced in macrophages by LPS, we performed studies to determine whether maleyl-BSA induces the release of NO by macrophages. We here present evidence that maleyl-BSA in combination with interferon-γ (IFN-γ) results in synergistic release of NO. Like LPS, maleyl-BSA initiates activation of NF-κB dimers which bind to the specific NF-κBd sequence found in the iNOS gene promoter (10). Maleyl-BSA does not, however, initiate significant transcription of the iNOS gene or significant accumulation of specific mRNA.

EXPERIMENTAL PROCEDURES

Materials. Tissue culture media were purchased from MediaTech (Washington, DC) and fetal bovine serum from HyClone Laboratories (Logan, UT). All tissue culture reagents contained less than 0.125 ng/ml endotoxin (LPS), as quantified by the *Limulus* amoebocyte assay supplied by Associates of Cape Cod, Inc. (Woods Hole, MA). Dupont NEN Research Products (Boston, MA) was the source of all radiolabeled chemicals. rIFN-γ was a gift of Schering-Plough (Kenilworth, NJ); LPS from *Escherichia coli* 026:B6 was purchased from Difco (Detroit, MI).

Cell culture. Specific pathogen-free inbred C57B1/6J mice (6 to 8 weeks old) were purchased from Charles River Breeding Laboratories (Raleigh, NC). Thioglycolate-elicited macrophages were ob-

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Abbreviations: TNF-α, tumor necrosis factor-α; LPS, bacterial lipopolysaccharide; IFN-γ, interferon-γ; maleyl-BSA, maleylated-bovine serum albumin; LDL, low density lipoprotein; ox-LDL, oxidized LDL; acetyl-LDL, acetylated LDL; SR, scavenger receptor; iNOS, inducible nitric oxide synthase; nitric oxide, NO.

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tained and cultured as previously reported (11). All macrophage cultures were routinely found to contain greater than 98% macrophages, as determined by Giemsa stain or by histochemical assay for nonspecific esterase. After 16–24 h, the macrophages were treated as indicated.

Isolation and modification of LDL. LDL was isolated from human plasma according to published techniques (12,13). Acetylation and oxidation were performed as previously described (14). All lipoprotein preparations were tested for electrophoretic mobility, protein and cholesterol content, and endotoxin level.

Maleyl-BSA. Maleyl-BSA was prepared as previously described (14). Briefly, maleic anhydride was recrystallized from chloroform prior to reaction. Bovine serum albumin (Sigma, St. Louis. MO) in 0.2 M $Na_2B_4O_7$, pH 8.5, was reacted with excess recrystallized maleic anhydride and the pH was maintained at 8.5 with sodium carbonate. The mixture was dialyzed overnight against 0.1 N NH_4HCO_3, then was dialyzed 48 h against frequent changes of distilled water and was lyophilized. Trinitrobenzene sulfonic acid tritration demonstrated that greater than 90% of lysine groups were maleylated (14). The maleyl-BSA was dissolved in Hank's Balanced Salt Solution and was stored at −20°C until use. All lots were tested for LPS contamination prior to use.

NO analysis. Macrophages were cultured and treated overnight (18 h) in 96-well plates. At the end of treatment, 100 μl of medium was transferred to a new plate. One hundred μl of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naptyl ethylenediamine dihydrochloride, 1.5% phosphoric acid) were added, and the plate was read in a molecular Devices (Menlo Park, CA) plate reader at a wavelength of 570 nm. Each experiment was performed at least in triplicate.

Northern blot preparations and analysis. The iNOS cDNA was a generous gift of Carl Nathan. γ-actin and iNOS probes were prepared, purified (50 ng), labeled, and Northern blots were done as described previously (11). Films were scanned with a Molecular Dynamics Phosphorimager (Sunnyvale, CA). To ensure that equivalent amounts of RNA were blotted to each lane the blots were rehybridized with the probe for γ-actin and the results normalized to γ-actin. All Northern blots were done at least 3 times.

Nuclear transcription run-on assay. Nuclei were isolated from treated macrophages by detergent lysis and frozen in liquid nitrogen until time of assay. The nuclei were later thawed and mixed with reaction buffer containing nucleotides plus [α-^{32}P]UTP. They were then treated with DNase I and proteinase K to digest all DNA and protein. The RNA was then extracted, precipitated and hybridized to the cDNA probe for iNOS immobilized on nitrocellulose (15). The blots were analyzed with a Molecular Dynamics Phosphoimager and done in triplicate.

Electrophoretic mobility shift assay. Nuclear extracts were prepared, protein quantified, and oligonucleotides prepared as previously described (16). Each experiment used 2.5×10^7 macrophages/treatment. DNA binding proteins present in the nuclear extracts were analyzed using 3 μgs of protein to bind the synthetic oligonucleotide (5′TGGGACTCTCCCTTTGGGAA3′, NF-κBd) (10). Data represent the results of at least 3 experiments.

RESULTS

Effects of maleyl-BSA on production of nitric oxide. The production of NO by peritoneal macrophages was minimally affected by an 18 h treatment with either maleyl-BSA or IFN-γ (Fig. 1A). When these two stimulants were combined, NO levels increased significantly to ~50% of the maximum response, obtained by treatment with IFN-γ and LPS. A dose response curve for maleyl-BSA, in the presence of 2 units/ml IFN-γ was constructed (Fig. 1B). The maximum effect was achieved at concentrations just above 1 mg/ml (14.5 μM); the EC_{50} was ~0.75 mg/ml of maleyl-BSA. These concentrations are comparable to the reported K_d of the low-affinity SR for acetyl-LDL/maleyl-BSA (K_d=4.9 μM) (3). The addition of ox-LDL reduced the production of NO to near control levels. Previously MTT assay demonstrated that the concentrations of maleyl-BSA used here are not toxic to macrophages over the treatment times employed here (14).

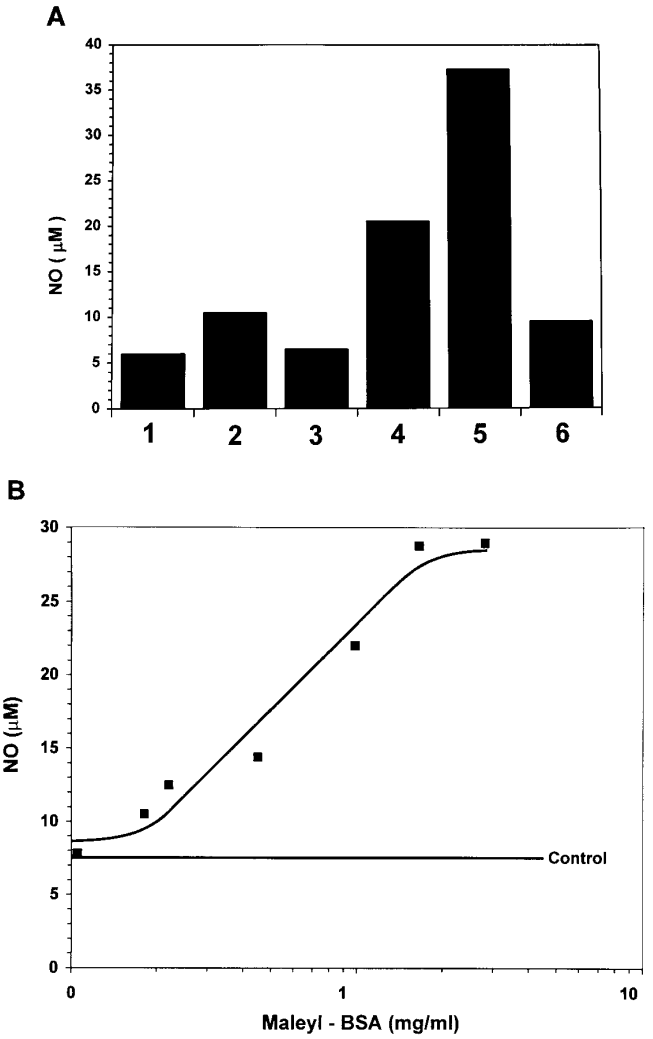

FIG. 1. Panel A. The ability of maleyl-BSA and IFN-γ to induce the production of NO during an 18 h treatment was determined. Lane 1, untreated; lane 2, maleyl-BSA; lane 3, IFN-γ; lane 4, IFN-γ/maleyl-BSA; lane 5, IFN-γ/LPS; lane 6, IFN-γ/maleyl-BSA/ox-LDL. The [maleyl-BSA], 1 mg/ml; [IFN-γ], 2 units/ml; [ox-LDL], 100 μg/ml; [LPS], 10 ng/ml. Panel B. A dose response curve for maleyl-BSA, in the presence of 2 units/ml IFN-γ, was constructed. Maximum response was at concentrations above 1 mg/ml, with an EC_{50} of about 0.7 mg/ml.

Effects of maleyl-BSA on iNOS mRNA. In order to asses accumulation of mRNA specific for the iNOS

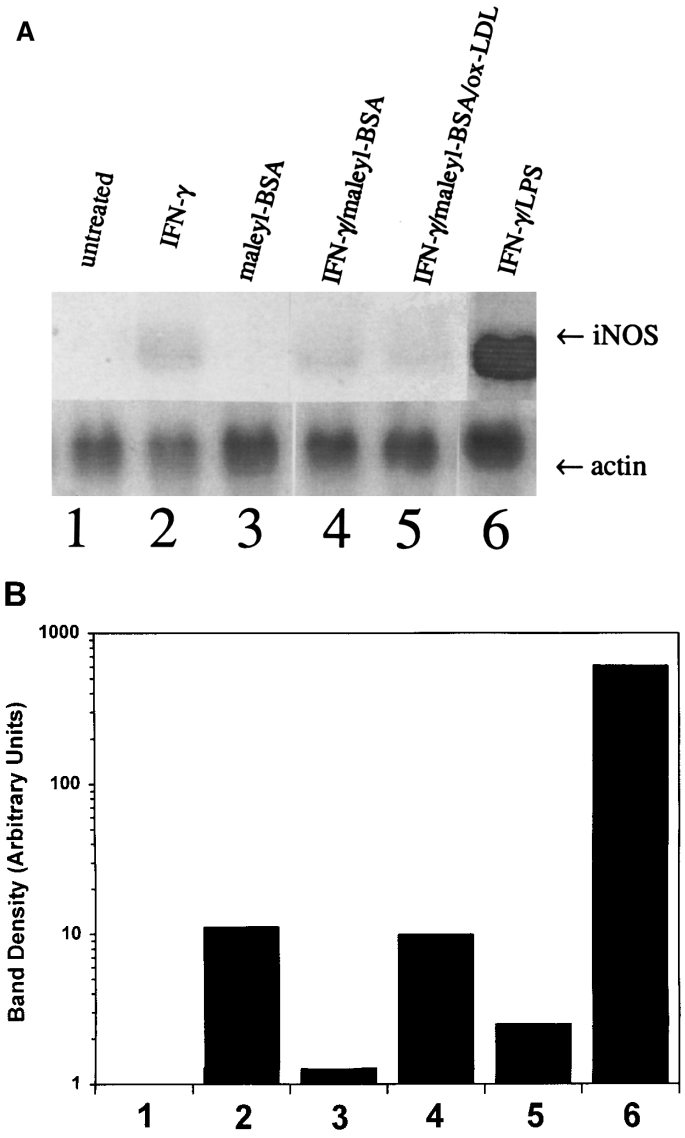

FIG. 2. Panel A. Northern analysis of total cellular RNA for the iNOS gene. Hybridization with the iNOS probe shows the presence of iNOS mRNA after treatment with IFN-γ, IFN-γ and maleyl-BSA, and IFN-γ and LPS, but not after treatment with maleyl-BSA alone. Lane 1, untreated; lane 2, IFN-γ; lane 3, maleyl-BSA; lane 4, INF-γ/maleyl-BSA; lane 5, IFN-γ/maleyl-BSA/ox-LDL; lane 6, IFN-γ/LPS. The concentrations of maleyl-BSA, IFN-γ, and LPS employed were identical to those in Fig. 1. The treatment time was 24 h. Panel B. Densitometry of iNOS nothern analysis normalized to actin. Untreated = 1; IFN-γ = 9.97; maleyl-BSA = 1.28; IFN-γ/maleyl-BSA = 9.43; IFN-γ/maleyl-BSA/ox-LDL = 2.3; IFN-γ/LPS = 624.

gene, Northern analyses were performed on total macrophage RNA. Maximum levels of mRNA specific for iNOS were induced by treatment with LPS/IFN-γ (Fig. 2A, lane 6; Fig. 2B). Only marginally detectable increases in production of mRNA specific for iNOS was observed when the cells were treated with maleyl-BSA alone. The combination of maleyl-BSA plus IFN-γ did not increase the levels of mRNA above that seen for IFN-γ alone. Additions of ox-LDL to IFN-γ/maleyl-BSA treated cells reduced the level of iNOS mRNA.

Effects of maleyl-BSA on transcription of iNOS gene. Treatment with maleyl-BSA or maleyl-BSA/IFN-γ resulted in a slight increase in new transcription of iNOS mRNA in run-on assays; the levels were ~4% of those seen in cells treated with LPS/IFN-γ (Fig. 3).

Effects of maleyl-BSA on activation of NF-κB. The electrophoretic mobility shift assay was used to examine the activation of NF-κB proteins. Specifically, a labeled oligonucleotide probe, identical to the unique NF-κBd site in the iNOS promoter was employed (10). The intensity of the upper band on these gel shifts was increased when the macrophages were treated with maleyl-BSA alone or IFN-γ/maleyl-BSA (Fig. 4, lanes 2 and 4, respectively). IFN-γ, by itself, did not induce binding to the NF-κBd oligonucleotide (Fig. 4, lane 3). In untreated control samples, constitutive presence of the band was noted, but at lesser densities (Fig. 4, lane 1). LPS, when applied to the macrophages, also induced

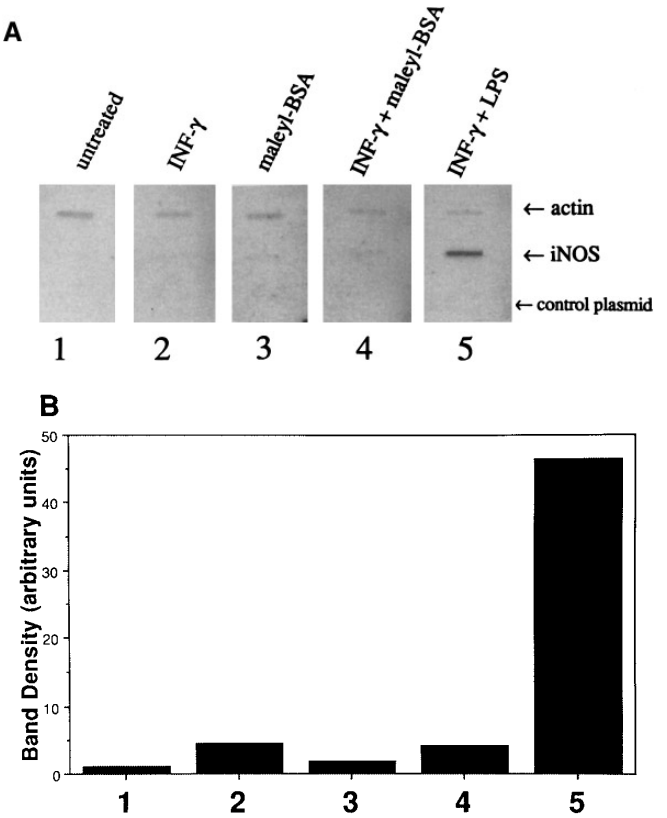

FIG. 3. Panel A. Nuclear transcription run-on assay for new iNOS message transcription. Lane 1, untreated; lane 2, IFN-γ; lane 3, maleyl-BSA; lane 4, IFN-γ/maleyl-BSA; lane 5, IFN-γ/LPS. The concentrations of maleyl-BSA, IFN-γ, and LPS employed were identical to those in Fig. 1. The treatment time 20 h. Panel B. Densitometry analysis nuclear transcription run-on assay for new iNOS message transcription normalized to actin. Untreated = 1; IFN-γ = 4.57; maleyl-BSA = 1.86; IFN-γ/maleyl-BSA = 4.12; IFN-γ/LPS = 46.5.

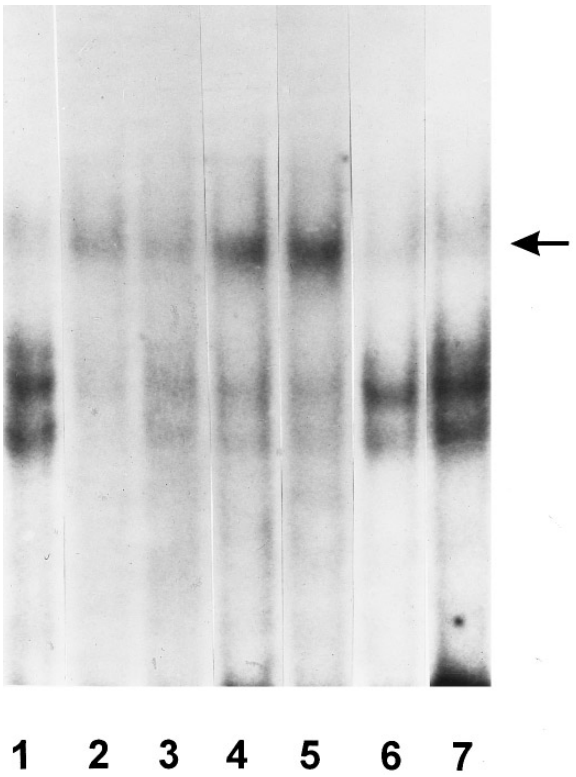

FIG. 4. EMSA gel using the NF-κBd probe. Three bands present in all samples with the two lower bands being most prominent in control samples. The upper band (arrow), while constitutively present in controls, is enhanced by treatment with a known NF-κB activator (lane 5, IFN-γ/LPS). This band is also enhanced by treatment with maleyl-BSA and maleyl-BSA/IFN-γ (lanes 2 and 4, respectively), but not IFN-γ (lane 3). Lanes 6 and 7 demonstrate the ability of ox-LDL to inhibit the activation of NF-κB elicited by treatment with maleyl-BSA and maleyl-BSA/IFN-γ. The concentrations of maleyl-BSA, IFN-γ, LPS, and ox-LDL employed were identical to those in Fig.1. The treatment time was 1 h.

binding to the NF-κB probe (data not shown) as previously found in a macrophage-like cell line (10). Treatment of the macrophages with ox-LDL, under conditions previously documented to inhibit LPS-mediated binding to a TNF-α promoter NF-κB probe (16), suppressed enhancement of the upper band induced by maleyl-BSA or maleyl-BSA/IFN-γ treatment (Fig. 4, lanes 6 and 7, respectively).

DISCUSSION

Maleyl-BSA, by binding to the low-affinity SR on macrophages (3), induces multiple gene and functional effects (7). Previously maleyl-BSA was found to induce pertussis toxin-insensitive hydrolysis of PIP_2, spikes in$[Ca^{2+}]$i, activation of PLA-2, activation of NF-κB protein binding to the TNF-α promoter probe, and transcription of the TNF-α gene (14). Here, we report that maleyl-BSA acts synergistically with IFN-γ to initiate the production of NO in macrophages.

NO is produced by nitric oxide synthase, which exists in three isoforms; neural (nNOS), endotheil (eNOS), and inducible (iNOS) (17). The neural and endothelial forms are constitutive, but production of NO by macrophages requires newly synthesized iNOS which is produced by transcription of the iNOS gene (17). Production of NO from macrophages is induced by LPS or by IFN-γ and synergistically by the combination; transcription is also synergistically enhanced by IFN-γ plus LPS (18).

The promoter region of the murine iNOS gene contains at least 22 sequences that could serve as binding sites for various transcription factors. Among these sites are 2 NF-κB sequences: 1) NF-κBu (nucleotides −971 to −962) which is identical to the site in the interleukin-6 promoter; and 2) NK-κBd (nucleotides −85 to −76) which is unique to the iNOS promoter. The first 1749 bp fragment upstream of the transcription start site also contains 10 copies of the IFN-γ response element. Although the lower one-third of the fragment when transfected into RAW Z647 macrophage-like cell line confers inducibility by LPS, only the entire fragment confers synergistic inducibilty by IFN-γ and LPS (18). Subsequent studies showed that the NF-κBd site is necessary for inducibility by LPS (10). Release of NO is further regulated in that IFN-γ appears to increase stability of message (19).

Our findings in terms of increased production of NO from macrophages with maleyl-BSA and IFN-γ is similar to the synergy observed by many laboratories in regard to production induced by IFN-γ and LPS. We further found that maleyl-BSA increases the binding of NF-κB to the NF-κBd site (Fig. 3). This is consistent with previous studies demonstrating that maleyl-BSA also induces hydrolysis of PIP_2, binding of NF-κB to a sequence from the TNF-α promoter, and transcription of the TNF-α gene (14). Finally, ox-LDL interferes with activation of NF-κB for binding to the NF-κBd probe. These findings are similar to previous results were ox-LDL was found to inhibit LPS-induced binding of NF-κB to an NF-κB site in the TNF-α promoter (16).

The present studies, however, document distinct differences in the effects of malyel-BSA versus LPS on the transcription of the iNOS gene. Maleyl-BSA alone dose not induce significant iNOS transcription, and the previously reported synergistic effects of IFN-γ are not observed (18). Maleyl-BSA appears to potentiate production of NO by acting after the accumulation of mRNA specific for iNOS, since the increases in production considerably out-pace any increases in message accumulation (compare Fig. 1 and Fig. 2B). Although maleyl-BSA does activate binding to the specific NF-κBd sequence found in the promoter of macrophage iNOS, maleyl-BSA or maleyl-BSA/IFN-γ does not initiate significant transcription when compared to LPS/IFN-γ. The present data suggest that binding of NF-κB to the iNOS promoter *in vivo* when induced by ma-

leyl-BSA may be qualitatively or quantitatively insufficient to activate significant transcription of the gene. Maleyl-BSA may well act translationally or post-translationally to increase production of NO. The synergy observed between IFN-γ and maleyl-BSA on the output of NO may thus result from their synergistic actions at this level(s) (20).

In summary, we have demonstrated that maleyl-BSA activates NF-κB, only marginally increases transcription of the iNOS gene and does not increase NO production. By contrast, maleyl-BSA in the presence of IFN-γ induces significant production of NO. The synergy does not result from increased transcription or accumulation of iNOS mRNA, suggesting another level of regulation, possibly translational or post-translational where IFN-γ may also act.

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