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RAPID COMMUNICATION

In Vivo Inhibition of Nitric Oxide Synthase Gene Expression by Curcumin, a Cancer Preventive Natural Product with Anti-Inflammatory Properties

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ABSTRACT. Curcumin is a naturally occurring, dietary polyphenolic phytochemical that is under preclinical trial evaluation for cancer preventive drug development and whose working pharmacological actions include anti-inflammation. With respect to inflammation, *in vitro*, it inhibits the activation of free radical-activated transcription factors, such as nuclear factor κB (NFκB) and AP-1, and reduces the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), and interleukin-8. Inducible nitric oxide synthase (iNOS) is an inflammation-induced enzyme that catalyzes the production of nitric oxide (NO), a molecule that may lead to carcinogenesis. Here, we report that in *ex vivo* cultured BALB/c mouse peritoneal macrophages, 1–20 μM of curcumin reduced the production of iNOS mRNA in a concentration-dependent manner. Furthermore, we demonstrated that, *in vivo*, two oral treatments of 0.5 mL of a 10-μM solution of curcumin (92 ng/g of body weight) reduced iNOS mRNA expression in the livers of lipopolysaccharide(LPS)-injected mice by 50–70%. Although many hold that curcumin needs to be given at dosages that are unattainable through diet to produce an *in vivo* effect, we were able to obtain potency at nanomoles per gram of body weight. This efficacy is associated with two modifications in our preparation and feeding regimen: 1) an aqueous solution of curcumin was prepared by initially dissolving the compound in 0.5 N NaOH and then immediately diluting it in PBS; and 2) mice were fed curcumin at dusk after fasting. Inhibition was not observed in mice that were fed *ad lib.*, suggesting that food intake may interfere with the absorption of curcumin. BIOCHEM PHARMACOL **55**;12:1955–1962, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. curcumin; *in vitro* and *in vivo* effects; anti-inflammation; inducible nitric oxide synthase

At inflammation sites, such as the liver, TNFα[‖], IL-1β, and IFNγ induce the production of iNOS. iNOS is expressed in many cell types, and it catalyzes the production of NO·, a reactive free radical that mediates signal transduction and destroys invading pathogens, but it may also damage host tissues. NO· has been implicated as playing a role in many pathological conditions, including allergic airway diseases, pneumonitis, a certain form (carrageenan-induced) of arthritis, vasculitis, acute rejection of allograft, and toxic shock syndrome [1].

Curcumin is contained in the rhizome of the plant *Curcuma longa* Linn. It is a naturally occurring, polyphenolic phytochemical under preclinical trial for cancer chemopreventive drug development, whose working pharmacological actions include anti-inflammatory activities [2, 3]. We have shown previously that curcumin reduces the production of TNFα and IL-1β and inhibits the activation of NFκB, a transcription factor that regulates the expression of many genes related to inflammation [4]. In addition, we have found that curcumin inhibits the production of NO· by mouse peritoneal macrophages, as indicated by the decreased accumulation of nitrite [5]. TNFα and IL-1β are essential for optimal induction of iNOS gene expression in many human and murine cells, and Xie *et al.* [6] and Murphy *et al.* [7] have shown that NFκB is essential for the transcription of iNOS in murine macrophages. Therefore, expanding upon these studies, we analyzed the effect of curcumin on iNOS mRNA expression. We now report a concentration-dependent response in *ex vivo* cultured macrophages, and we found that curcumin, when given orally, inhibited iNOS gene expression in mouse liver.

There is presently in cancer prevention studies much controversy on the bioavailability of curcumin as an oral chemopreventive agent against cancer, mainly because in some of the carcinogenesis studies, non-physiological levels

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‖ *Abbreviations:* GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFNγ, interferon-γ; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; NFκB, nuclear factor κB; RT-PCR, reverse transcription–polymerase chain reaction; and TNFα, tumor necrosis factor-α.

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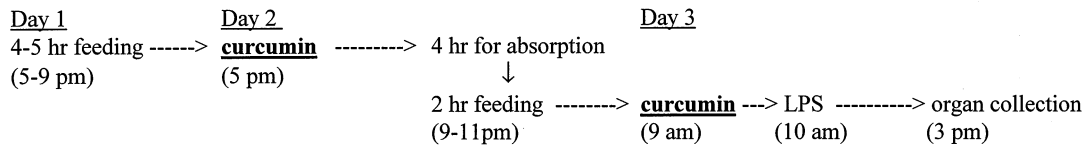

FIG. 1. Schedule for feeding and curcumin treatments.

of isolated compounds need to be administered to animals. Huang *et al.* [8] and Rao *et al.* [9] have observed that curcumin has to be given at 0.2 to 5% (w/w) of the daily diet, a concentration that is not likely to be adopted by most individuals in order to protect against colon cancer. However, there are also many reports which showed that when given at 10–200 mg/kg i.p. or by gavage, curcumin produced various biological effects (such as reduction of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced tumor formation in rat mammary tissue and attenuation of croton oil-induced edema) in many organs (including lung, mammary gland, bone marrow, peritoneum, lens, ear, and paws) [10–19]. In the present study, using an inflammation model, we found that *in vivo* efficacy can be obtained at nanomoles/gram, a level that is likely attainable through oral administration. This report may provide insight into the importance of the methods of administration on *in vivo* efficacy of dietary chemopreventive agents, in general.

MATERIALS AND METHODS

Animals

Adult BALB/c mice weighing between 20 and 30 g were used. They were purchased from Jackson Laboratories, maintained in micro-isolators with autoclaved bedding and cages, and fed autoclaved food pellets and deionized water. Although the mice were not germfree, sterile procedures were used in handling these animals so as to prevent unintentional introduction of microbes that could activate iNOS production.

Ex Vivo *Assays*

Normal nonelicited peritoneal cells were obtained from the mice by aspiration. The cells were cultured at 10^6/mL in RPMI-1640 medium as previously described [5]. To the cultures, 100 ng/mL of LPS from *Salmonella typhosa* (Difco Laboratories), 10 U/mL of recombinant IFNγ (a gift from Dr. S. Pestka, University of Medicine and Dentistry of New Jersey), and commercial grade curcumin (70% curcumin, 30% demethoxycurcumin and bisdemethoxycurcumin; Fluka) were added simultaneously. Curcumin was dissolved as a 100-mM solution in acetone and then diluted to the desired concentration in RPMI-1640 medium containing 0.1% acetone. The cultures were incubated for 4–5 hr; then total RNA was isolated with a Purescript RNA isolation kit (Gentra).

In Vivo *Assays*

Adult BALB/c mice were placed on a restricted, once a day, diet and given curcumin and LPS according to the regimen illustrated in Fig. 1. The feeding schedule mimics the natural biorhythm and feeding habits of wild mice, feeding at dusk. The experimental group was given two doses of 0.5 mL of 10 μM (5 nmol) of curcumin solution orally by gavage and 0.5 μg/g of body weight of LPS intravenously. The positive control group was given LPS but no curcumin. The negative control group was given curcumin in the same manner as the experimental group but no LPS. The curcumin solution was freshly prepared as a 100 mM of solution in 0.5 N of NaOH, and then diluted immediately to 10 μM using a 10-fold dilution scheme. At 5–6 hr after LPS treatment, the animals were killed. Their livers were collected, and total RNA was isolated with Tri-Reagent (Molecular Research Center) and stored at $-70°$ until used.

RT-PCR

For first strand cDNA synthesis, RNA from each sample was reverse-transcribed using 100 U MMLV reverse transcriptase, 20 U RNase inhibitor, 0.6 mM of dNTP, and 0.4 μM of oligo(dT_{16}). Three hundred nanograms of RNA from peritoneal cells or 3 μg of RNA from organs was used in each RT reaction. Then PCR analyses were performed on the aliquots of the cDNA preparations for detecting iNOS and GAPDH gene expression. The reactions occurred in a 50-μL volume with 10 mM of Tris–HCl, pH 8.3, 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.3 mM of dNTP, 2 U of *Taq* DNA polymerase, and 50 pmol of 5′ and 3′ primers. For iNOS, competitive-based PCR analyses were performed to determine the concentrations of cDNA semi-quantitatively. The conditions for the latter PCR reactions were similar to that of the former, except that 1.25×10^{-4} amol of iNOS competitive template, designed to amplify at similar efficiency as the target iNOS cDNA, was added. Murine iNOS and GAPDH 5′ and 3′ primers and competitive fragments (MIMIC) were purchased from Clontech [20, 21]. The PCR products were separated on 1.6% agarose gels and were stained with SYBR Green I dye (Molecular Probes); the intensities of the bands were determined with the Molecular Dynamics Storm fluorescence scanning system.

Citrulline Assay

Murine iNOS, 100,000 *g* fraction, was purchased from Cayman. Enzyme inhibition studies for iNOS were con-

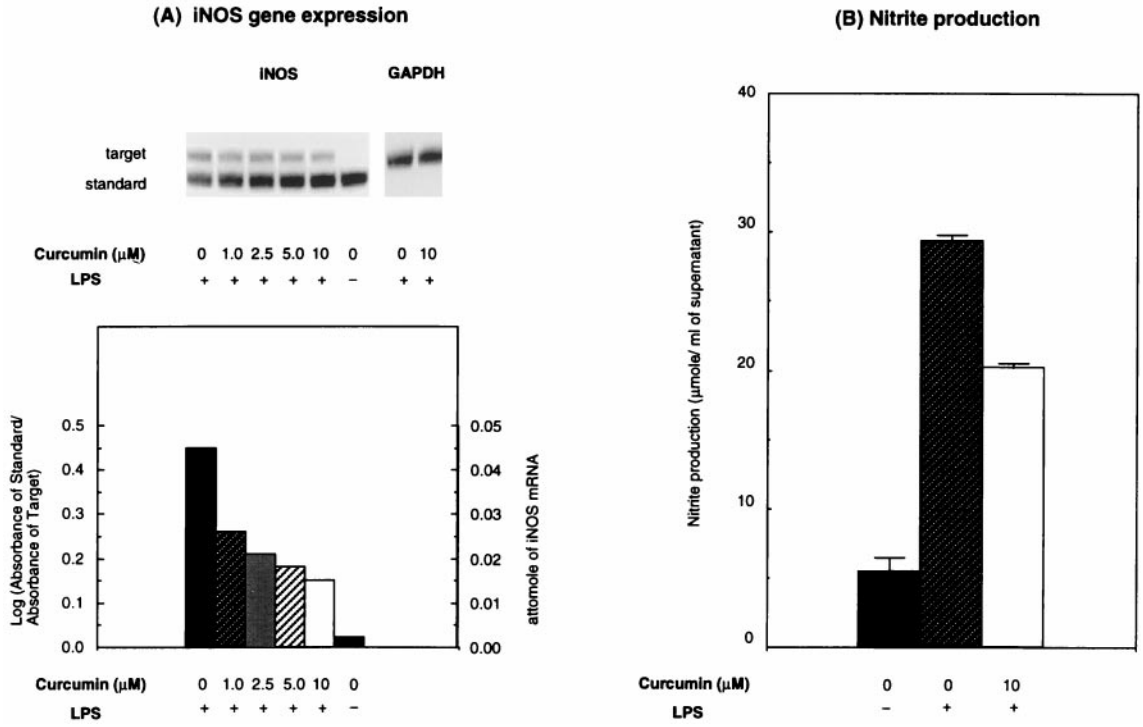

FIG. 2. Effect of curcumin on iNOS mRNA expression and nitrite production in *ex vivo* stimulated peritoneal cells. (A) Peritoneal cells were cultured in the presence and absence of LPS (0.1 μg/mL), IFNγ (10 U/mL), and curcumin (at the indicated concentrations). After 5 hr of incubation, RNA was extracted, and first strand cDNA was synthesized. Competitive based PCR was performed to determine the amount of iNOS cDNA, as described in the text. Noncompetitive PCR was performed to determine the amount of GAPDH cDNA. The reactions were carried out for 23 cycles. The amplified products were run on a 1.6% agarose gel, the gels were stained, and the intensity of the bands was determined by scanning. For iNOS cDNA, the concentrations were determined from the ratio of the products from the experimental samples (target) and the competitive cDNA template standard. The ratio of iNOS to GADPH products was calculated. The inset panel on the left shows the iNOS PCR products from the peritoneal cell RNA extract (target) and the competitive iNOS template (standard). The inset panel on the right shows the RT–PCR product of GADPH from the samples that were treated with 0 or 10 μM of curcumin. The presented data were representative of three similar experiments. (B) In an identical cellular arrangement, the culture supernatants from the samples that were treated with 0 or 10 μM of curcumin were collected after 24 hr of incubation, and the amount of nitrite was determined by the Griess reaction. The values are means ± SD, N = 3.

ducted in 50 mM of HEPES buffer (pH 7.4) containing 1 mM of calcium chloride, 1 mM of magnesium acetate, 100 mM of NADPH, and 1.35 mM of [^{3}H]arginine (DuPont/ NEN). Curcumin was added at the indicated concentrations, and reactions were terminated by the addition of AG 50WX8 resin (Bio-Rad) in HEPES-EDTA buffer (pH 5.5). Enzyme activity was measured by monitoring the conversion of [^{3}H]arginine to [^{3}H]citrulline [22]. Reactions were allowed to occur at 37° for 10–20 min, because time-course analyses showed that the reactions were linear under these conditions. Background was determined by the level of radioactivity in a reaction mixture from which iNOS was omitted. This amount was subtracted from the level detected in the experimental samples. Enzyme activity, i.e. the amount of citrulline formed, was deduced from the specific activity of the [^{3}H]arginine.

Nitrite Assay

Nitrite production was measured by the Griess reaction as described in Chan *et al.* [5].

Statistical Analysis

Data were analyzed by Student's *t*-test, and the level of significance was determined at $P < 0.05$.

RESULTS

Effect of Curcumin on Ex Vivo *Cultured Murine Peritoneal Macrophages*

The time course for induction of iNOS production with LPS has been analyzed in many systems. In murine macrophages, for example, Kamijo *et al.* [23] have shown that the mRNA level is nondetectable at 0 hr, increased from 6 to 8 hr, and then declined by 18 hr after stimulation. Therefore, inhibition of iNOS mRNA expression by curcumin was observed at 4 hr after stimulation with LPS and IFNγ, when the mRNA production was increasing. Figure 2A shows a competitive-based RT–PCR analysis. A concentration-dependent trend of inhibition by curcumin was detected. The amount of iNOS cDNA was reduced from 0.045 amol in the sample without curcumin to 0.026,

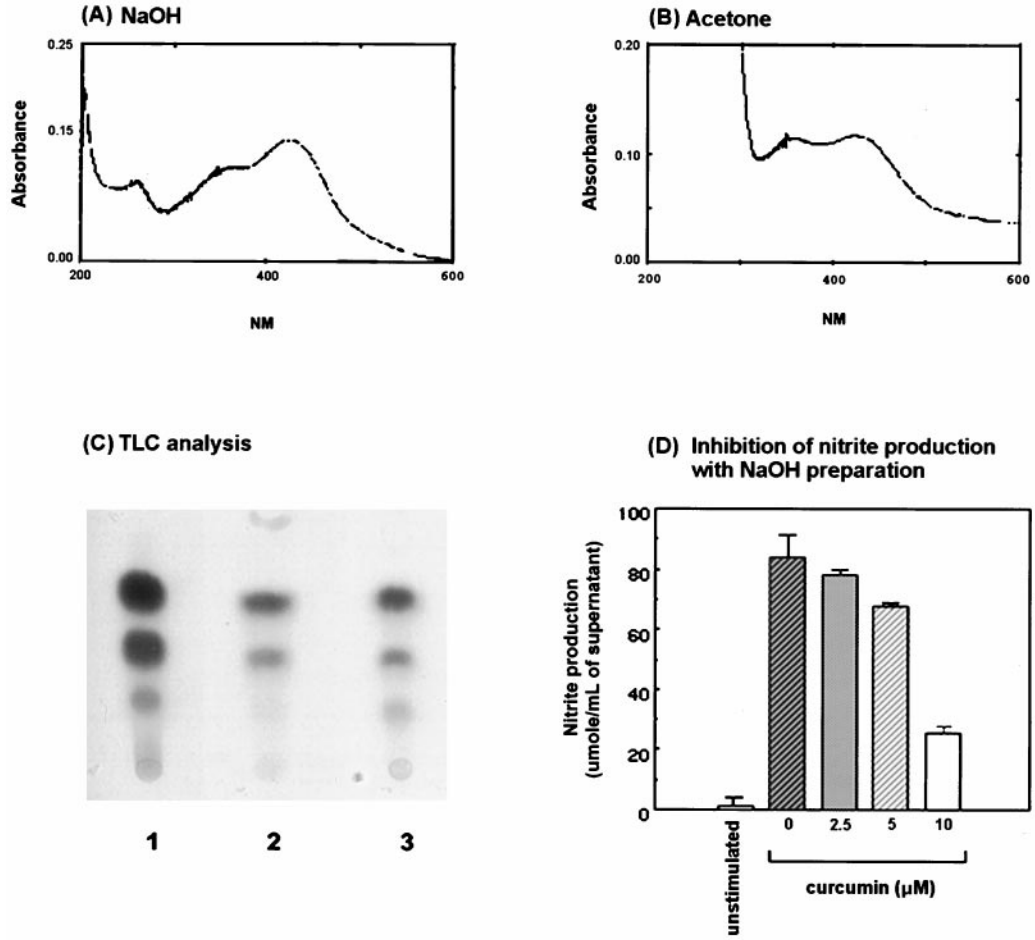

FIG. 3. Comparisons of curcumin from different methods of preparation. UV spectra of curcumin prepared by (A) dissolving in 0.5 N of NaOH and then diluted to 10 μM in PBS and (B) dissolving in acetone and then diluted to 10 μM in PBS containing 0.1% acetone. (C) TLC of (1) a 100 mM solution of curcumin prepared by dissolving in acetone, (2) 100 μM of curcumin prepared by dissolving as 100 mM in acetone and then diluted to 100 μM with PBS containing 0.1% acetone, and (3) a 100 μM of curcumin solution prepared by dissolving in NaOH and then diluted to 100 μM in PBS. The solutions were extracted with ethyl acetate, spotted on a silica gel TLC plate, and separated with chloroform:methanol (96:4) for 15 min. (D) Mouse peritoneal cells were cultured in the presence or absence of LPS (0.1 μg/mL), IFNγ (10 U/mL), or curcumin, which was prepared by dissolving in NaOH. After 24 hr of incubation, the culture supernatants were collected, and the amount of nitrite present was determined by the Griess reaction. The values are means ± SD, N = 3.

0.021, 0.018, and 0.015 amol in the samples to which curcumin was added at 1, 2.5, 5, and 10 μM. The level of GAPDH, a housekeeping gene, was unaffected by treatment of up to 10 μM of curcumin. Correspondingly, a decrease in nitrite production was also observed in the culture supernatants (Fig. 2B). A decrease in iNOS mRNA production was observed in three similar experiments.

Use of NaOH for Preparation of Curcumin Solution for Gavage

For oral feeding, curcumin was dissolved in NaOH. We found that curcumin was freely soluble in 0.5 N of NaOH and remained dissolved upon further dilution in PBS (pH 7.0). The UV spectral analyses of the curcumin solution thus prepared and that of the compound prepared by dissolving in acetone are shown in Fig. 3, panels A and B. The major peaks of both spectra are between 350 and 430 nm, indicating that the compound, in each case, was for the most part not degraded. In Fig. 3B, the peak at 200–300 nm was produced by acetone. TLC confirmed this result (Fig. 3C). Identical migration patterns showed the presence of curcumin, demethoxycurcumin, and bisdemethoxycurcumin in the curcumin sample that was prepared by dissolving 100 mM of the compound in acetone and then immediately diluting to 100 μM in PBS containing 0.1% acetone, and in the sample that was dissolved in NaOH and then immediately diluting to 100 μM in PBS. The UV spectrum profile and TLC spot intensity also showed that the concentration of curcumin was higher in the sample that was prepared with NaOH than in the one that was prepared with acetone. Because the solutions were supposed to be of the same concentration, this difference suggested when dissolved with acetone some of the compound might be inadvertently lost, perhaps due to colloidal precipitation

TABLE 1. Effect of curcumin on iNOS mRNA expression in mouse liver

Experiment	Treatment	iNOS (amol/μg RNA × 10^{-5})	% Inhibition*
1	Curcumin	0.125 ± 0.028	
	LPS	1.040 ± 0.110	
	LPS + curcumin	0.280 ± 0.043	73 ($P < 0.0002$)
2	Curcumin	0.051 ± 0.016	
	LPS	0.955 ± 0.047	
	LPS + curcumin	0.490 ± 0.357	50 ($P < 0.0010$)
3	Curcumin	0.647 ± 0.331	
	LPS	1.511 ± 0.319	
	LPS + curcumin	0.898 ± 0.035	57 ($P < 0.0028$)
4	Curcumin	0.037 ± 0.031	
	LPS†	4.140 ± 0.871	
	LPS + curcumin	1.609 ± 0.402	63 ($P < 0.0051$)

Mice were given two doses of 0.5 mL of 10 μM (5 nmol) of curcumin solution orally by gavage and 0.5 μg/g of body weight of LPS intravenously, and their livers were collected. RNA was extracted, and competitive-based RT-PCR was performed on iNOS and non-competitive PCR was performed for GAPDH in parallel. The reactions for iNOS were carried out for 40 cycles, whereas for GAPDH it was for 25 cycles. For each sample, the RT–PCR reactions were performed at least in triplicate. The PCR products from iNOS cDNA were quantified by comparison to the products from the competitive iNOS cDNA template. These amounts were then adjusted by the ratio of iNOS to GAPDH. The values are means ± SD, N = 3. The differences between the LPS control and the curcumin-treated samples were determined by Student's *t*-test, and the *P* values are given in parentheses.

*The amount of iNOS in the curcumin-treated samples was calculated as a percent of the control that was not treated with curcumin by the following formula: % inhibition = 100 × [1 − (LPS + curcumin)/(LPS)].

†For experiment 4, the mice were killed at 6 instead of 5 hr after injection with 10 instead of 5 μg/g of LPS.

and adherence to the vessel wall, upon further dilution in PBS containing 0.1% acetone. Furthermore, the biological activity of the solution prepared with NaOH was also unaltered, as indicated by the inhibition of nitrite production by LPS-stimulated macrophages (Fig. 3D).

In Vivo *Effect of Curcumin in Murine Liver*

Using aqueous solutions of curcumin dissolved with NaOH, we investigated the effect of curcumin on LPS-induced iNOS mRNA expression *in vivo*. Mice were placed on the restricted feeding regimen (Fig. 1) to avoid intermittent feeding. Behavioral changes that would indicate stress or starvation in the experimental animals were not noted. LPS-injected mice that were fed twice with 0.5 mL of a 10-μM curcumin solution (equivalent to 5 nmol or 92 ng/g body weight) expressed 50–70% less iNOS when compared with the untreated controls (Table 1). Thus, curcumin was effective in inhibiting LPS-induced production of iNOS, a free radical generator, when given twice at 10 nmol (i.e. 184 ng) per g of body weight. The inhibitory action of curcumin was specific for iNOS because the expression of GAPDH mRNA was largely unaffected, as shown in Fig. 4. Nonetheless, in Table 1, we have used the iNOS/GAPDH ratio to determine the degree of inhibition so as to adjust for minor differences in RT efficiency among the samples.

Curcumin, at nanograms per gram of body weight, however, inhibited iNOS expression only in mice that had been put on the restricted diet regimen, as illustrated in Fig. 1 (Fig. 4A). Even though the curcumin solution was prepared and administered identically, no inhibition was observed in the mice that were given food *ad lib*. The amounts of iNOS PCR product in the curcumin-fed and non-curcumin-fed mice were 2.25×10^{-5} and 1.77×10^{-5} amol/g of RNA, respectively (Fig. 4B). Thus, curcumin, at a low dose, appeared to inhibit iNOS expression only in mice that had been fasted.

Effect of Curcumin on iNOS Enzyme Activity

Previously, we have found that curcumin reduces $NO^{\cdot}$ production by LPS-stimulated peritoneal exudate cells [5]. This may result from one or more mechanisms: down-regulation of iNOS gene expression, inhibition of iNOS enzyme activity, and/or scavenging of $NO^{\cdot}$ molecules. Sreejayan and Rao [24] have shown that curcuminoids may scavenge $NO^{\cdot}$. To deduce the mechanism of inhibition further, we have found that curcumin did not inhibit iNOS enzyme activity. Whereas 10 μM of N^{G}-methyl-L-arginine, the positive control, inhibited the activity of iNOS by 97%, addition of 25, 50, and 100 μM of curcumin inhibited citrulline formation to 0, 6, and 14%, respectively, when the reaction occurred in 0.135 μM of arginine and 60 μM of tetrahydrobiopterin with 126 pmol/min of the 100,000 *g* iNOS preparation. At 25–100 μM concentrations, curcumin did not inhibit the enzyme activity of iNOS significantly. At 100 μM, inhibition was 14 ± 11%, $P = 0.33$.

DISCUSSION

In India, curcumin is used routinely as a household anti-inflammatory remedy. In this report we have shown in a concentration-dependent manner that the actions of curcumin include inhibition of LPS-induced iNOS gene ex-

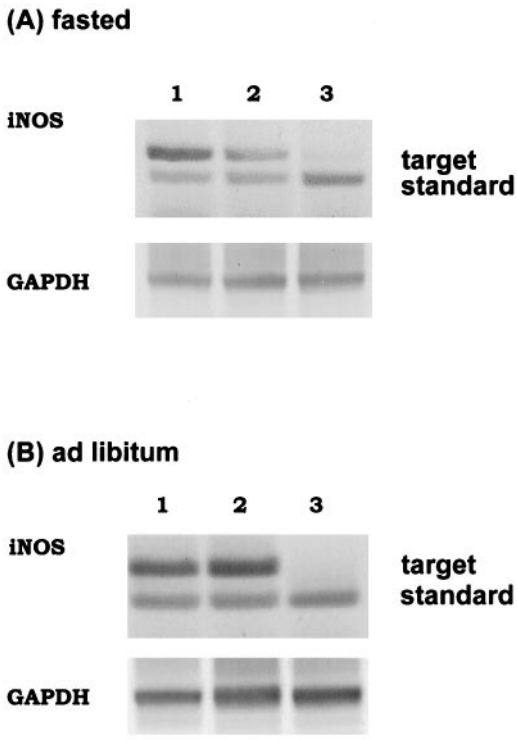

FIG. 4. Influence of food intake on the *in vivo* efficacy of curcumin. Mice were treated with (1) LPS, (2) LPS plus curcumin, or (3) curcumin only and fed according to (A) the regimen illustrated in Fig. 1, or (B) *ad lib*. Their livers were collected, RNA was extracted, and RT–PCR was performed as described in Table 1. The bands shown are the PCR products derived from amplification of the RNA in the livers of mice.

pression, as demonstrated in *ex vivo* cultured peritoneal exudate cells. The effective concentration range was similar to that observed in other components of the inflammatory cascade such as inhibition of the activation of NFκB, production of TNFα and IL-1β, cyclooxygenase and lipoxygenase activities, lipid peroxidation, and synthesis of prostaglandin [reviewed in Ref. 25]. The 5′ flanking sequence of murine iNOS gene contains consensus elements for the transcription factors interferon-γ responsive element (γIRE), NFκB, AP-1, and tumor necrosis factor response element (TNF-RE) [6, 7]. That curcumin inhibits the production of TNFα and the activation of NFκB and AP-1 may have contributed to this effect [4, 26, 27].

We have also shown that curcumin is an inhibitor of iNOS activation *in vivo*. For both the *in vitro* and *in vivo* systems used in this study, the inhibition was specific for iNOS as GAPDH was unaffected, although it has been reported that curcumin may inhibit RNA synthesis in HeLa cells, as indicated by [^{3}H]uridine incorporation, when they are cultured under serum-free conditions [28]. In many cancer prevention studies, curcumin has to be given at a very high concentration for tumor reduction, e.g. at hundreds of milligrams per kilogram or continuous administration for long periods of time at 0.2 to 5% (w/w) of the diet [8, 9]. Curcumin inhibited LPS-induced iNOS gene expression in the liver when given at 10 nmol or 184 ng/g body weight (Table 1). To our knowledge, this is the first report to show that orally administered curcumin has bioactivity *in vivo* in nanomoles per gram body weight. This activity is similar to that obtained *in vitro* or when given topically in acetone (0.1 to 10 μmol in 10 μL) to inhibit 12-*O*-tetracanoylphorbol-13-acetate (TPA)-induced hyperplasia or tumor formation in mouse skin [27, 28]. It is up to 100-fold higher than when given through i.p. injection to inhibit croton oil-induced edema in mouse ear (10 μg/g) [11], to inhibit DNA adduct formation in rat mammary gland [10], ischemia-induced biochemical changes in cat heart (100 μg/g) [12], or glutathione *S*-transferase activity in rat liver (200 μg/g daily) [10]. It is also up to 1000-fold higher than when it is administered orally as a suspension in oil to reduce the levels of lipid peroxidation of microsomal fatty acids [13, 14] and glutathione *S*-transferase in rat liver [15], to inhibit carrageenan-induced thickening in rat paw [13], melanoma metastasis to mouse lung [16], micronucleation in mouse bone marrow [17], benzopyrene-induced tumor in mouse stomach [18], or to reduce glutathione *S*-transferase level in rat lens [19].

In India, the practice is to mix curcumin in lime, $Ca(OH)_2$ [2]. By immediately diluting the curcumin in PBS (pH 7) after it was dissolved in NaOH, we were able to avoid degradation, even though the half-lives of curcumin, demethoxycurcumin, and bisdemethoxycurcumin at pH 10.2 have been reported as 0.4, 1.0, and 5.0 hr, respectively [29]. Nevertheless, we detected significant degradation when curcumin was left in 0.5 N NaOH for 2 hr. Wahlstrom and Blennow [30] have dissolved curcumin in 1 N of NaOH for i.v. injection, and Ravindranath and Chandrasekhara [31] have similarly prepared curcumin for studying the absorption of curcumin through everted intestinal sacs. Degradation, if any, was not reported in these studies. However, curcumin has been reported to be metabolized in the liver and the intestine [32, 33]. Thus, whether it is curcumin or its metabolites that inhibited the iNOS production of mRNA in the liver remains to be investigated.

In addition to dissolution in base, the use of restricted feeding for the mice probably contributed to the increase in *in vivo* efficacy. When food was not available to mice prior to curcumin treatment, the *in vivo* efficacy of curcumin was in the nanomole range. This efficacy was lost when mice were fed *ad lib*. Perhaps interaction with food may reduce the efficacy of orally administered curcumin, and ingestion of curcumin on an empty stomach may be a key to efficacy. In previous studies, when rats were fasted, a moderate level of the compound was detected in the liver of the animals even though the curcumin was not dissolved. For example, Khana [34] fasted rats for 24 hr prior to feeding with curcumin in 2% gum acacia suspension at 10 mg/kg of body weight. She found that, at 0.5 hr after administration, 85% of the compound was in the stomach and small intestine. By 3 h, 11, 9.5, and 21.8% of the curcumin was detected in the liver, kidney, and large intestine/feces, respectively. Similarly, Ravindranath and Chandrasekhara [32, 35] also

conducted two studies in which they fasted rats for 24 hr before feeding 400 mg of nonradioactive or [^{3}H]curcumin suspension in water containing 0.01% Tween 20 per animal. In these experiments, where the rats were not fed afterwards, they found that 60% of the curcumin was absorbed. With the radioactive compound, they found that at 0.5 to 24 hr, 7–14 and 4–5% of the radioactivity was in blood and liver, respectively, although 59% was excreted through the feces when measured after 12 days.

It has been shown that alendronate, an osteoporosis preventive compound whose oral bioavailability is considered limited, is best absorbed when taken in the fasting state. Its efficacy is impaired drastically if food is taken 2 hr before, with, or 2 hr after dosing. The recommended regimen for the administration of alendronate to humans is after an overnight fast and at least 30 min before any food intake [36]. Some polyphenols may have a tendency to bind to proteins and dietary fibers. Rock and Swendseid [37] found that β-carotene can bind to unabsorbed dietary fiber, such as pectin. Concordingly, Micozzi *et al.* [38] have shown that when β-carotene is ingested as a supplement, its plasma levels exceed that obtained when it is ingested with food. Addition of milk to tea also significantly inhibits the *in vivo* antioxidative effect of tea polyphenols, probably due to binding to milk protein [39]. One possible explanation for this phenomenon is that the compound had become bound to some dietary constituents that make it unavailable for systemic absorption. This study underlines the fact that *in vivo* efficacy may depend on how a compound is prepared and when it is administered.

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