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RAPID COMMUNICATION

Inhibition of Inducible Nitric Oxide Synthase Gene Expression and Enzyme Activity by Epigallocatechin Gallate, a Natural Product from Green Tea

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ABSTRACT. Chronic inflammation has been implicated as the underlying factor in the pathogenesis of many disorders. In the past decade, inflammation-related endogenous production of reactive nitrogen species, similar to oxygen free radicals, has also been suggested as a risk factor for cancer, in addition to the well-studied exogenous nitroso compounds. Epidemiological, *in vitro*, and animal model studies have implicated green tea to be protective against nitroso compound-induced and inflammation-related cancer. Therefore, we investigated the effect of epigallocatechin-3-gallate (EGCG), one of the known biologically active catechins contained in green tea, on the production of nitric oxide ($NO^{\cdot}$). We have shown previously that EGCG reduces $NO^{\cdot}$ production as measured by nitrite accumulation in the culture medium. Expanding on this finding, in this report we show that EGCG may do so by two mechanisms: reduction of inducible nitric oxide synthase (iNOS) gene expression and inhibition of enzyme activity. Addition of 1–10 μM EGCG to lipopolysaccharide- and interferon-γ-activated mouse peritoneal cells reduced iNOS mRNA expression concentration dependently, to 82–14%, as measured by relative reverse transcription–polymerase chain reaction. Addition of 50–750 μM EGCG, in a concentration-dependent manner, inhibited the enzyme activity of iNOS, to 85–14%, and neuronal nitric oxide synthase (nNOS), to 93–56%, as measured by citrulline formation. EGCG competitively inhibited binding of arginine and tetrahydrobiopterin, and the gallate structure is important for this action. BIOCHEM PHARMACOL **54**;12:1281–1286, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. nitric oxide synthase; gene expression; enzyme activity; competitive inhibitor; inflammation; carcinogenesis; green tea; catechin; dietary phytochemical

Nitrite from preserved food and smoke has long been recognized as a potent carcinogen that will cause nitrosylation of amines. However, Tannenbaum and co-workers, studying carcinogenesis, produced the first conclusive evidence that mammals also produce nitrogen oxides endogenously [1]. The simple gas radical, $NO^{\cdot}$,¶ is produced by the enzyme nitric oxide synthase, which has three isoforms. The constitutively expressed isoforms I and III produce $NO^{\cdot}$ at a low level, and they are activated through calcium/calmodulin binding to generate $NO^{\cdot}$ as a signal for physiological functions. Isoform II, iNOS, is regulated by gene expression. During inflammation, it produces $NO^{\cdot}$ at a high level to exert defense against pathogens. Nitrogen oxides, such as $NO^{\cdot}$ and its metabolite, peroxynitrite, are considered mutagenic because they can also cause deamination of DNA and inactivation of DNA repair enzymes [2, 3]. In addition, $NO^{\cdot}$ is also tissue destructive, and it has been implicated to play significant roles in the pathology of many inflammatory conditions, including sepsis, adult respiratory distress syndrome, arthritis, asthma, inflammatory bowel disease, myocarditis, diabetes, gingivitis, and tissue rejection [4]. Therefore, inhibitors of iNOS have been greatly sought.

Tea has been ascribed the properties of being anti-inflammatory, antioxidative, and anti-carcinogenic. In rodent carcinogenesis models, infusion of green tea has been shown to decrease nitrosyl compound-induced tumors in several organs such as esophagus, lung, stomach, colon, mammary gland, and skin [reviewed in Refs. 5–7]. Epidemiological studies in Shanghai, China, and Kyushu, Japan, suggested that frequent consumption of green tea may be associated with a lower incidence of esophageal and gastric cancers [8, 9].

A number of polyphenolic phytochemicals have been

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¶ *Abbreviations:* EC, epicatechin; ECG; epicatechin-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IFNγ, interferon-γ; LPS, lipopolysaccharide; $NO^{\cdot}$, nitric oxide; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; and RT-PCR, reverse transcription–polymerase chain reaction.

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found to inhibit NO· production and iNOS gene expression [10]. With respect to those in green tea, EGCG is the most potent in terms of antioxidative capacity and has been ascribed to have the predominant role in cancer chemoprevention [11–14]. It has been shown to inhibit the proliferation of prostate and breast cancer cells and inhibit 12-*O*-tetradecanoylphorbol-13-acetate-induced skin thickening, activation of protein kinase C, activation of ornithine decarboxylase, and activation of interleukin-1α mRNA and protein expression [15–17]. In addition, it has been reported that topical application of tumor promoter reduces the production of constitutive NOS by epidermis and that pretreatment with green tea extract can reverse this inhibition [18].

The foregoing observations prompted us to investigate the effect of EGCG on NO· production. We have reported previously that EGCG reduces NO· production by LPS- and IFNγ-induced mouse peritoneal cells, as measured by nitrite accumulation in the culture medium [19]. Expanding from this study, we determined here that EGCG inhibits both iNOS gene expression and enzyme activity. To our knowledge, EGCG is the first compound reported to inhibit at both levels.

MATERIALS AND METHODS

Reagents

Purification of EGCG (over 95% pure) and other catechins was performed as described [20]. Recombinant murine IFNγ was provided by Dr. Sidney Pestka of the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School. Murine iNOS, 100,000 *g* fraction, was purchased from Cayman.

Gene Expression

Non-elicited peritoneal exudate cells were obtained from BALB/c mice (Jackson Laboratories) and were cultured at 10^6/mL as described previously [19]. They were stimulated by the addition of 0.01 μg/mL LPS from *Salmonella typhosa* (Difco Laboratories) and 10 units/mL of IFNγ. At 4 hr after stimulation, total RNA was isolated with a Purescript RNA isolation kit (Gentra). RT–PCR was performed to determine the level of iNOS gene expression. From each sample, 300 ng of RNA was reverse-transcribed using 100 units MMLV reverse transcriptase, 20 units RNase inhibitor, 0.6 mM dNTP, and 0.4 mM Oligo(dT_{16}). 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, for 23 cycles, with 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 2.0 units of *Taq* DNA polymerase, and 50 pmol of 5′ and 3′ primers. The reaction condition for competitive RT-PCR was similar to the above, except that an iNOS competitive template, designed to amplify at similar efficiency as the target iNOS cDNA, was added at a range of concentrations from 0.15 to 10 amol. Murine iNOS and GAPDH 5′ and 3′ primers and competitive fragment (MIMIC) were purchased from Clontech. The products were then separated on 1.6% agarose gel and stained with ethidium bromide, and the intensity of the bands was determined by scanning [21].

Enzyme Extraction

Murine iNOS was extracted from the RAW 264.7 murine macrophages. The cells were stimulated with 20 units/mL of IFNγ and 0.5 μg/mL LPS, incubated for 4 hr, and then harvested. The washed cells were lysed in 50 mM MOPS buffer (pH 7.0) which contained 1 mM EGTA, 100 mM NaCl, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPSO), 0.2 mM phenylmethylsulfonyl fluoride, 50 mM E-64, and 1 mM leupeptin [22, 23]. For nNOS, fresh brains from BALB/c mice were homogenized in a 5-fold volume of 50 mM Tris–HCl (pH 7.5) that contained 1 mM EDTA, 5 mM 2-mercaptoethanol and then were centrifuged at 100,000 *g* for 1 hr. After passing through Sephadex G-100 (Pharmacia) to remove arginine, NADPH, and unbound tetrahydrobiopterin, the supernatant was concentrated by ultrafiltration using Centricon 30 microconcentrators (Amicon) [23].

Citrulline Assay

Enzyme inhibition studies for iNOS were conducted in 50 mM HEPES buffer (pH 7.4) containing 1 mM calcium chloride, 1 mM magnesium acetate and 100 mM NADPH. [^{3}H]arginine (New England Nuclear), tetrahydrobiopterin (Biomol) and EGCG were added at the indicated concentrations. For nNOS, 5 μM flavin mononucleotide, 5 μM flavine adenine dinucleotide, 10 μg/mL calmodulin (Calbiochem), and 1.25 mM dithiothreitol were incorporated in addition. The 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 [24]. Reactions were allowed to occur at 37° for 10–20 min since time–course analyses, for both isoforms, 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.

RESULTS AND DISCUSSION

When activated murine peritoneal macrophages were incubated with EGCG, the gene expression of iNOS was inhibited. This effect was verified by two RT–PCR techniques. (1) Relative RT–PCR showed that at 1, 3, 5, and 10 μM, EGCG reduced iNOS mRNA expression in a concentration-dependent manner to 82, 74, 41, and 14%

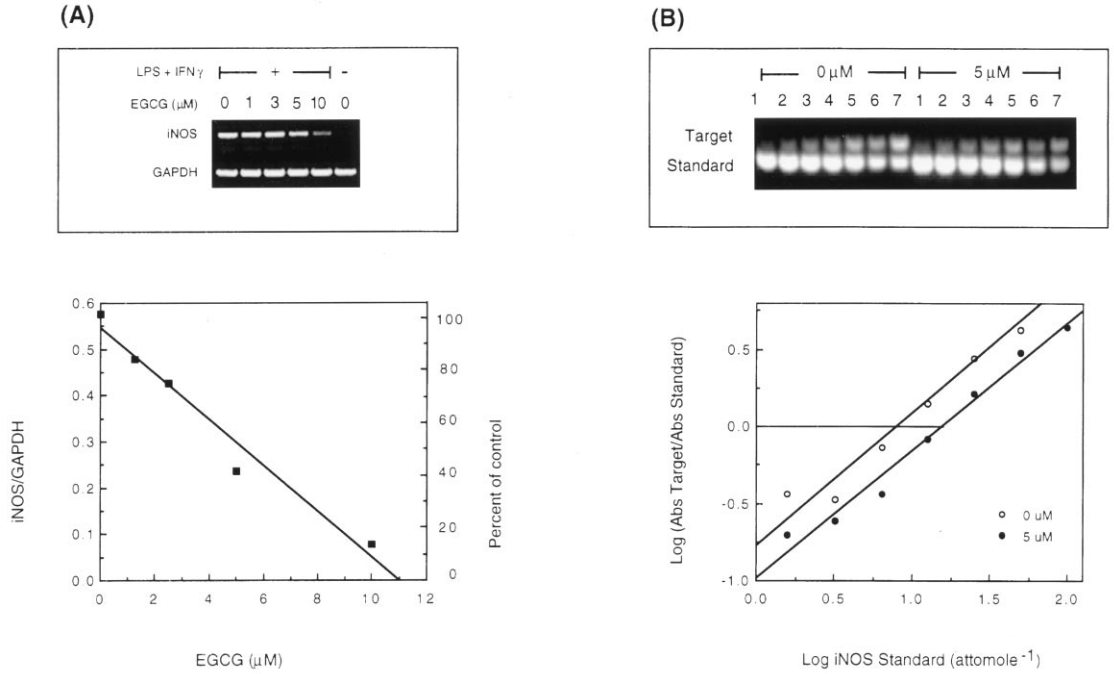

FIG. 1. Effect of EGCG on iNOS gene expression. (A) Relative RT–PCR for iNOS and GAPDH was performed, as described in the text, on RNA that was extracted from mouse peritoneal exudate cells. The cells were stimulated with LPS and IFNγ, and EGCG was added at the indicated concentrations. The inset panel shows the RT–PCR product of iNOS and GAPDH from the experimental samples, treated as indicated. The ratio of iNOS to GAPDH products was calculated, and then the percent of inhibition was determined. (B) Competitive RT–PCR for iNOS was performed on the RNA from the untreated (0 μM) and 5 μM EGCG-treated samples. Competitive DNA fragment for iNOS was added at 10, 5, 2.5, 1.25, 0.6, 0.3, and 0.15 amol (lanes 1–7) to aliquots of the cDNA samples. The inset panel shows the increase in iNOS PCR products from the peritoneal cell RNA extract (target) as the amount of competitive iNOS fragment (standard) that was added decreased. The ratio of the bands was calculated, and linear regression lines were plotted. Then the amounts of iNOS cDNA in the samples and the percent of inhibition were determined.

(Fig. 1A). This inhibition was specific because reduction in mRNA expression of GAPDH, a housekeeping gene, was not observed. Nonetheless, 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. (2) Furthermore, competitive based RT–PCR was performed to semi-quantitatively measure the effect of EGCG at 5 μM (Fig. 1B). The amount of iNOS (target) product increased as the concentration of competitive fragments (standard) added was decreased. Deducing from the slope of the lines, at 5 μM EGCG, the amount of iNOS mRNA was reduced from 0.156 to 0.065 amol/μL, a 42% reduction, which is comparable to that obtained by relative RT–PCR.

The structure of NOS resembles a fusion of a cytochrome P450-like oxygenase at the amino terminal and cytochrome P450-like reductase at the carboxyl end [25]. Since it has been shown that EGCG and other green tea catechins bind to various hepatic cytochrome P450s and inhibit P450-dependent functions, we investigated whether EGCG may inhibit NOS activity by inhibiting enzyme activity [26]. In a concentration-dependent manner, EGCG inhibited the enzyme activity of iNOS, either in the form of crude extract from stimulated RAW 264.7 cells or as 100,000 *g* fraction that was arginine- and NADPH-free. Addition of 50, 250, 500, and 750 μM EGCG inhibited citrulline formation to 85, 40, 27, and 14%, respectively, when the reaction occurred in 0.135 μM arginine and 60 μM tetrahydrobiopterin with 126 pmol/min of the 100,000 *g* iNOS preparation (Fig. 2). The half-maximal effective concentration was 150 μM.

To elucidate the molecular mechanism of inhibition, the kinetic properties of the inhibition were explored. With respect to substrate, iNOS (100,000 *g* fraction) was incubated with 100, 200, 400, and 600 μM EGCG and 0.068, 0.090, 0.135, and 0.270 μM arginine. The potency of the inhibition decreased with increasing concentrations of arginine (Fig. 3A). A Dixon plot of the rate of citrulline formation as a function of EGCG concentration revealed a competitive pattern of inhibition. A series of lines intersected at a common point above the abscissal axis, indicating an apparent K_i of 130 pmol/min.

NOS molecules have to be associated into dimers in order to have enzymatic activity [27]. After association, the oxygenase domains at the amino terminal form the catalytic center that binds arginine, tetrahydrobiopterin, and heme. Interdependently, arginine and tetrahydrobiopterin enhance each other's affinity for binding [28]. Therefore, we proceeded to examine whether EGCG interferes with tetrahydrobiopterin binding. Similarly, iNOS (100,000 *g* fraction) was incubated with 100, 200, 400, or 600 μM EGCG and 12, 60, 90, or 125 μM tetrahydrobiopterin

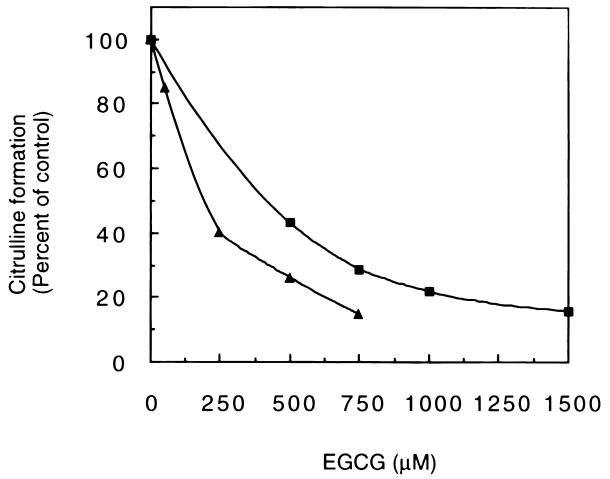

FIG. 2. Effect of EGCG in iNOS enzyme activity. To crude lysate containing iNOS (■) and 100,000 *g* fraction of iNOS with activity of 126 pmol/min (▲), both from RAW 264.7 cells, EGCG was added at the indicated concentrations. Reactions were conducted, as indicated in the text, in 60 μM tetrahydrobiopterin and 0.135 μM [^{3}H]arginine for 10 min. Percent of inhibition was deduced by dividing the amount of citrulline produced in the EGCG-treated sample by that in the untreated sample. The absolute values for citrulline formation were 1.97 pmol/hr for crude lysate and 0.58 pmol/hr for 10,000 *g* fraction.

(Fig. 3B). Although 100,000 *g* fractions contained inherently bound tetrahydrobiopterin, a Dixon plot indicated that EGCG decreased the maximal velocity of iNOS in a competitive manner. It is likely that EGCG may inhibit by binding to the catalytic domain, and its action on iNOS resembles that of nitroindazole on nNOS. Nitroindazole is an arginine and tetrahydrobiopterin competitive inhibitor of nNOS enzyme activity that has been used in many studies to deduce the biochemical structure of the enzyme [29].

Green tea contains several epicatechins, EGCG, ECG, EGC, and EC, that are similar in chemical structure. Their relative efficacy for inhibiting iNOS activity was evaluated so as to determine the structure–function relationship. Figure 4 is representative of three experiments that were performed with 0.270 μM arginine, 125 μM tetrahydrobiopterin, and 126 pmol/min of iNOS. EGCG and ECG, at 800 μM, reduced citrulline formation to 62 and 68%, respectively, whereas EGC was ineffective, with 96% citrulline formation. This suggests that the gallate structure may play a critical role in the inhibition. Moreover, since all three of the compounds have antioxidative capacity and their order of potency is EGCG > ECG > EGC, the fact that EGCG and ECG were equally potent suggested that it is unlikely for the antioxidative action to determine the efficacy of iNOS inhibition, although the possibility of it having a role is not ruled out [11].

Lastly, we explored the effect of EGCG on nNOS. nNOS was prepared as 100,000 *g* fraction from mouse brain extract. Addition of 100, 200, 400, and 600 μM EGCG to nNOS inhibited citrulline formation by 93, 79, 51, and 56%, respectively, when compared with the control in which EGCG was omitted (Fig. 5). Although the result seems to indicate that EGCG was more effective against iNOS than nNOS, its differential selectivity and K_i for the two isoforms remain to be determined. This is because here the enzymes used were partially purified and the conditions that were required for the two isoforms were not equivalent. For measuring nNOS activity, 0.135 μM arginine and 125 μM tetrahydrobiopterin were used. It has been debated that the differential regulation of the action of NOS isoforms may be under indirect control, through the intracellular availability of arginine, tetrahydrobiopterin, and calcium, e.g. nNOS requires calcium/cadmodulin binding. In addition, *in vivo*, the difference in enzyme life-span, shorter for

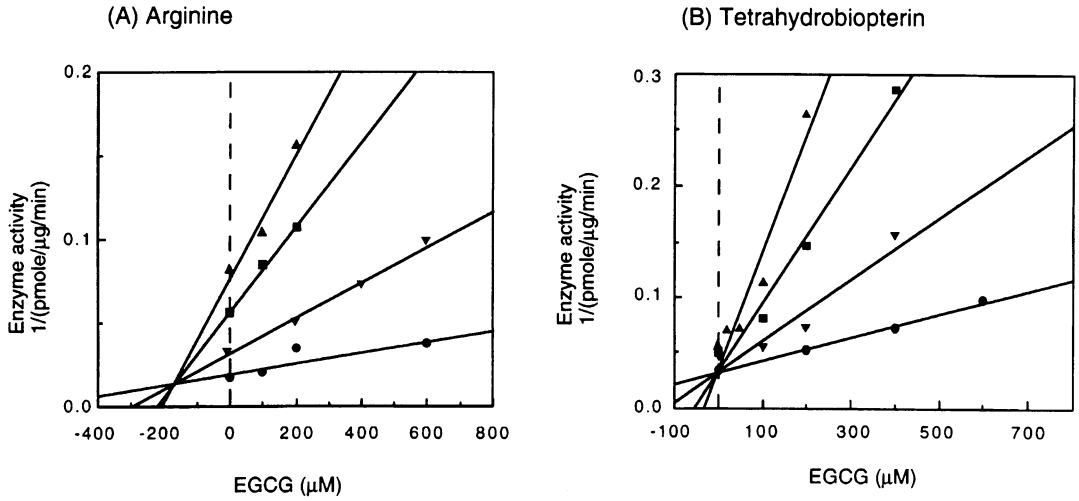

FIG. 3. Kinetic analysis of the action of EGCG on iNOS. The experiments were conducted with 100,000 *g* fraction of iNOS under the conditions described in the legend to Fig. 2, except that (A) arginine was added at 0.068 (▲), 0.090 (■), 0.135 (▼), and 0.270 (●) μM, and (B) tetrahydrobiopterin was added at 12 (▲), 60 (■), 90 (▼), and 125 (●) μM, and EGCG was added at the indicated concentrations. The reciprocal of the velocity of the reaction was plotted against the concentration of EGCG in a Dixon format. The linear regression lines were computer drawn by the "Cricket Graph" program.

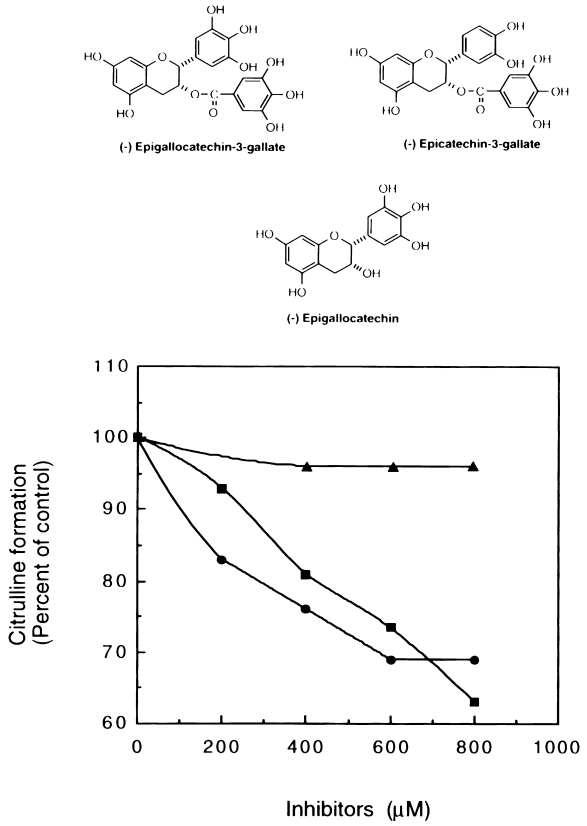

FIG. 4. Structure–function analysis of inhibition. Enzyme assays were conducted as described in the legend to Fig. 2, except that 0.270 μM arginine and 125 μM tetrahydrobiopterin were used in the reaction. EGCG (■), ECG (●), and EGC (▲) were added at the indicated concentrations. The absolute value for citrulline formation was 2.16 pmol/hr.

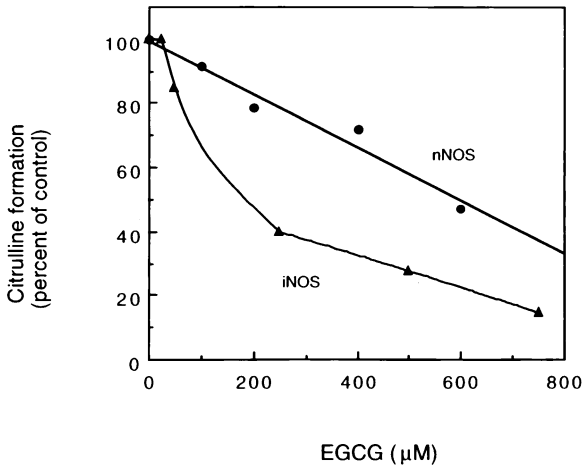

FIG. 5. EGCG action on nNOS. EGCG was added at the indicated concentrations to arginine-free, NADPH-free, 100,000 *g* fraction of mouse brain extract that contained nNOS. Reactions were conducted as indicated in the text, with 125 μM tetrahydrobiopterin and 0.135 μM [^{3}H]arginine added to the reaction buffer. The reaction was allowed to occur at 37° for 20 min, and linearity of activity was checked. Percent of inhibition was deduced by dividing the amount of citrulline produced in the EGCG-treated sample by that in the untreated sample. The absolute value for citrulline formation by nNOS was 0.66 pmol/hr.

nNOS and longer for iNOS, may also be a contributing factor. Nonetheless, the present result suggests that EGCG is capable of inhibiting both nNOS and iNOS. Inhibitors of nNOS have been shown to be antinociceptive, to reduce neuronal damage associated with acute cerebral stroke, AIDS, dementia, and Parkinson's disease, to increase the depth of general anesthesia in the CNS, and to inhibit the distressing behavior effects that follow the withdrawal of morphine-like drugs in dependent animals [30]. EGCG may potentially be applicable in these aspects.

The principal novelty and significance of this study, in our opinion, lie in the discovery that it is possible to have a small molecule that inhibits iNOS at both the level of gene expression and enzyme activity. Presently, most investigators look for iNOS inhibitors that block at one of these two potential therapeutic targets. iNOS is mainly regulated by gene expression; however, once produced, iNOS remains chronically activated and continually produces NO$^\cdot$ for the lifetime of the enzyme. Being able to reduce NO$^\cdot$ production at both iNOS mRNA accumulation and enzyme activity, EGCG will provide a distinctive advantage over inhibitors that may work at only one level, especially in conditions where immediate reduction of NO$^\cdot$ is necessary. The therapeutic potential of EGCG is even greater in light of the report that it may possibly inhibit peroxynitrite, a product from reaction between NO$^\cdot$ and O_2^- that mediates protein damage by the formation of nitrotyrosine and DNA damage by the formation of oxodeoxyguanosine [31]. The structural gene of murine iNOS has 80% nucleic acid sequence homology with human hepatic iNOS [32]. Moreover, both murine and human hepatic iNOS are regulated by similar transcription factors and cytokines [33]. Thus, although the mechanisms that regulate iNOS expression in human macrophages have not been fully determined, it is likely that EGCG may also modulate NO$^\cdot$ production in humans. We are presently in the process of studying the effect of EGCG in human systems.

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