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Black Tea Is a Powerful Chemopreventor of Reactive Oxygen and Nitrogen Species: Comparison with Its Individual Catechin Constituents and Green Tea

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Production of black tea [BT] results in biotransformation of catechins of green tea [GT] to theaflavins and thearubigins. BT was found to be more efficient than GT and its individual catechin constituents in proportionate amounts in abrogating production of NO and O_2^- in activated murine peritoneal macrophages. In a reconstitution system of BT that is free of all catechins, stepwise addition of catechins showed that though all the constituents contributed to the overall effect of BT, theaflavin was the most powerful in abrogating NO production. RT-PCR analysis also showed theaflavin to be the most important constituent in down-regulating synthesis of iNOS. Clearly, BT containing theaflavin is an excellent chemopreventor against reactive oxygen and nitrogen species. © 2001 Academic Press

Key Words: **black tea; theaflavin; nitric oxide; chemopreventor.**

Black tea (BT) is the most widely consumed beverage worldwide. About 80% of the dried tea manufactured annually is consumed as black tea (1, 2). The 'fermentative' process is crucial for the production of BT. In this process, cut and partially dried green tea leaves are subjected to controlled enzymatic biotransformations at slightly elevated temperature to give the characteristic color, 'briskness' and flavour of BT. Catechins are the main polyphenolic flavanols of tea that undergo major biotransformations during this operation. Oxidation, ring expansion to tropolones and dimerisation or more extensive polymerisation of catechins and their gallates are the most important biochemical conversions that take place at this stage to lead to the formation of theaflavins and thearubigins that are the characteristic constituents present exclusively in BT. The chemistry and biochemistry of BT production has been a subject of continuing research interest for a long time (3–7).

In recent years considerable interest has been generated on green tea (GT) as a health beverage. Based on extensive animal experiments and some epidemiological data GT has been suggested to act as a chemopreventor against various forms of cancer. Antimutagenic (8), anti-oxidative (9) and anti-proliferative (10, 11) properties of green tea have been documented. The protective activity of GT has generally been assumed to be due to the powerful scavenging and anti-oxidative capacity of high concentrations of unpolymerised catechins and their gallates in GT. These works have been reviewed recently by Yang and Wang (12) and by Katiyar and Mukhtar (2). Amongst the various catechin constituents of GT, epigallocatechingallate (EGCG) has been found to be the most effective as a scavenger for reactive oxygen radicals (2). Other sites of action for EGCG have also been suggested. It acts as an inhibitor for urokinase, an enzyme critically involved in tumor growth (13). Recently, reports have appeared to show that EGCG can also prevent induction of nitric oxide synthase (iNOS) in macrophages by down-regulating NF-κB (14, 15).

In contrast to the situation with GT, hardly any work has been done with BT till recently. Black tea was assumed to be much less beneficial than GT because of its lower content of unpolymerised polyphenols, particularly EGCG (2, 12). Only recently work has been initiated with black tea or its characteristic constituents, theaflavins and thearubigins. We have recently shown that black tea can totally protect human red blood cells against oxidative damage brought about by various inducing agents (16). Parallely, Lin *et al.* have reported that theaflavin-3-3′-digallate can very efficiently down regulate induction of nitric oxide synthase in stimulated macrophages (17). In view of this growing literature, it is desirable to assess the importance and capability of BT in abrogating the generation of reactive oxygen and nitrogen species (ROS and RNS)

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and compare the effectiveness of BT with the individual catechin constituents of BT and with that of GT.

For phagocytic macrophages, reactive oxygen species [ROS] such as superoxide anion [O_2^-], H_2O_2 and hydroxyl radical [OH] are crucially involved in its defense function. A large amount of NO, synthesized by inducible nitric oxide synthase [iNOS], also participates in host defense mechanism. NO can combine with superoxide anion to produce highly reactive peroxynitrite radical that is also involved in the defense mechanism. Prolonged and excess production of NO may, however, lead to deleterious effects including death by apoptosis (18). The complex role of NO in the physiology and pathophysiology of many systems including macrophages is still a subject of active investigation (19, 20). Activated murine peritoneal macrophage, thus, provides an ideal, *in vitro* model system to monitor and assess the usefulness of BT or its individual constituents in scavenging superoxide anion and in abrogating production of NO. In this paper, using a stimulated murine macrophage system and a reconstitution system for BT that is devoid of all catechins, we unambiguously demonstrate that the powerful and potentially beneficiary effect of BT is more profound than its individual constituents and is fully comparable with that of GT. In short, polymerised products of BT such as theaflavins obtained during 'fermentation' not only do not adversely affect the chemopreventive properties of black tea but in fact potentiates it further to a significant extent.

MATERIALS AND METHODS

Reagents. Epicatechin (EC), epigallocatechin (EGC), epicatechingallate (ECG), epigallocatechingallate (EGCG), theaflavin (marketed as 80% black tea extract), lipopolysaccharide (LPS) (*E. coli* 0127:B8), sulphanilamide, naphthylethylenediamine dihydrochloride, superoxide dismutase (SOD), nitrate reductase, NADPH, α-ketoglutarate, Phorbol myristate acetate (PMA) were purchased from Sigma. RPMI 1640 medium and fetal calf serum (FCS) were from Gibco. The primers for iNOS and HPRT were the kind gift of Dr. P. K. Das of the institute. All other reagents used were of analytical grade.

Macrophage cell culture. Mice of Balb/C strain were used as a source of normal, nonelicited peritoneal exudate macrophages. Cells were obtained by sterile lavage with ice-cold phosphate-buffered saline (PBS), washed and resuspended in culture consisting of RPMI 1640 (without phenol red) supplemented with 10% heat-inactivated FCS (Gibco), penicillin (100 units/ml), streptomycin (100 μg/ml) and plated in tissue culture plates (10^6 cells/ml). When the cells became a confluent layer, they were activated by incubating with medium containing lipopolysaccharide or heat killed *E. coli*. Various concentrations of test compounds were added as per requirement of the experiment.

Superoxide anion and nitrite assay. Superoxide induced reduction of ferricytochrome *c* to ferrocytochrome *c* was monitored spectrophotometrically at 550 nm (21). Briefly, cells were stimulated with heat-killed *E. coli* or 10 μg/ml LPS or 1 μM PMA in the presence of 100 μM cytochrome *c*. After around 3 h incubation, different test compounds including SOD was added. The rate of superoxide production is given by the difference in the rate of cytochrome *c* reduction in presence and absence of test compounds. Medium with cytochrome *c* served as a control.

The nitrite concentration in the culture medium was measured as an indicator of NO production by the Greiss Reaction as described by Ding *et al.* (22). Briefly, 500 μl of each supernatant was mixed with the same volume of Greiss Reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water); and absorbance of the mixture at 550 nm was determined. The concentrations of nitrite were determined by comparison to a standard curve, constructed using sodium nitrite ranging from 3 to 100 μM.

Extraction of iNOS and its assay. Murine iNOS was extracted from murine macrophage cells lavaged as mentioned earlier. Cells were stimulated with 10 μg/ml LPS or 1 μM/ml PMA or heat killed *E. coli*, incubated for 4 h and then harvested. The washed cells were lysed and enzyme extracted following Chan *et al.* (14) with slight modifications (23).

Enzyme assay studies of iNOS were conducted by standard assay system (24). Briefly, sample containing NO_2^- and NO_3^- was first incubated for 30 min at room temperature with 60 mU nitrate reductase and 25 μM NADPH to reduce NO_3^- to NO_2^-, followed by addition of 200 mU L-glutamate dehydrogenase, 100 mM NH_4Cl and 4 mM freshly prepared α-ketoglutarate. Further incubation was allowed for 10 to 15 min at room temperature to consume any residual NADPH that interferes in the Greiss reaction. Finally equal volume of Greiss reagent was added, incubated at 37°C for 5–10 min and absorbance measured at 543 nm versus a blank containing buffer and Greiss reagent.

Reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR was performed to determine the level of iNOS gene expression (25). Briefly, total RNA was isolated by acid guanidinium thiocyanate–phenol–chloroform extraction. Total RNA (700 ng to 1 μg) was converted to cDNA, from which 50 ng of cDNA was amplified by incubating in 100 mM Tris–HCl buffer, pH 8.3, containing 500 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs and 50 U/ml of Super Taq DNA polymerase with oligonucleotide primers of iNOS and HPRT (used as control). A thermal cycle of 45 s at 95°C, 2 min at 50°C and 1 min at 72°C was used for 30 cycles. PCR products were analysed on 1% agarose gels.

Preparation of black tea (BT) and BT free of all its individual polyphenol constituents (BT-F). For preparation of black tea extract, 10 g of commercially available black tea was soaked in 100 ml of boiling water. After 2 min, it was filtered and the filtrate was designated as black tea extract (BT). For preparation of BT, free of all polyphenols (designated as BT-F) that was used as the starting material for the reconstitution experiment, black tea extract was further treated with polyvinyl-polypyrolidone batchwise 2 to 3 times until filtrate turned colorless (6). Aliquots of this filtrate, free of all coloring materials were further passed through Sephadex G-50 spin column at 2000 rpm for 2 min at least twice to remove the remaining small molecular weight components in the colorless filtrate. Spectral analysis confirmed insignificant presence (<3%) of any 280 nm absorbing or coloring material.

Quantitative estimation of catechin constituents in BT. Aliquots of the three different samples of BT were analyzed for quantification of polyphenol content by HPLC column chromatography using Waters' Reverse Phase-HPLC C-18 column (μ-Bondapak) against standards of individual polyphenols from Sigma. The samples after injection were eluted using an acetonitrile-aquous acetate buffer (2% v/v), mobile phase programmed linearly from 0–30% (0–10 min), then 30–60% (10–40 min) and finally 60–80% (40–60 min) at a flow rate of 0.7 ml min^{-1} and absorption followed at 280 nm. It was estimated that 100 μl of black tea was equivalent to 50 μg of total catechins and the black tea passed through Sephadex G50 spin column was devoid of all individual polyphenols [data not shown]. The HPLC quantification of individual polyphenols broadly matched

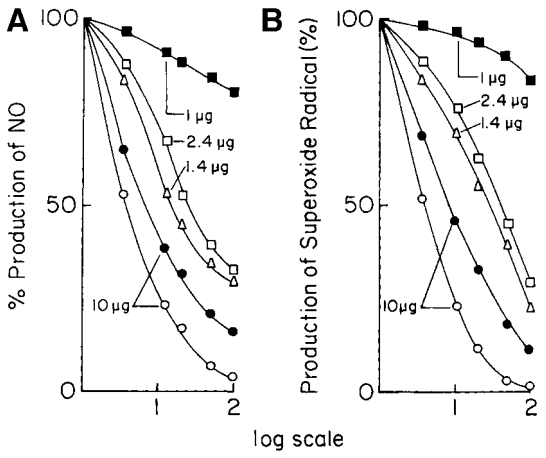

FIG. 1. Abrogation of superoxide anion [O_2^-] and NO production by black tea and its constituents. Macrophage (10^6 cells/well) were incubated with heat killed *E. coli* (10 μg LPS) in the presence of BT (open circle), GT (closed circle), EGCG (open square), theaflavin (open triangle), and epicatechin (closed square). [A] The amount of NO_2^- released in the medium, which serves as a measure of NO, was determined using Greiss reaction. [B] O_2^- production was measured by ferrocytochrome *c* reduction.

with the results of other standard quantifications published recently (26, 27).

RESULTS

Effect of BT and Its Individual Catechin Constituents on Production of NO and O_2^-

Stimulation of nonspecific host defense mechanism in the mononuclear phagocytic systems was carried out with diverse types of agents that include LPS, PMA and dead or live bacteria. Figure 1 shows that when purified murine peritoneal macrophages were stimulated with extracts of heat-killed *E. coli* containing approximately 10 μg of lipopolysaccharide (Materials and Methods), profuse amounts of both NO (24 nmoles h^{-1} 10^6 $cells^{-1}$) and O_2^- (7.8 nmoles h^{-1} 10^6 $cells^{-1}$) were released at the end of 24 h. Simultaneous preincubation with increasing amounts of BT resulted in the decrease of NO and O_2^- production in a dose-dependent manner. GT was also found to be quite but not as effective as BT. In contrast, individual catechin constituents when supplemented for BT in the relative proportions in which they occur in BT were found to be significantly less effective individually in abrogating NO (Fig. 1A) or superoxide anion (Fig. 1B) production. Typically, 10 μg of total catechins in BT (20 μl of BT) contains approximately 1.4 μg of theaflavins, 2.4 μg of EGCG and 1.0 μg of epicatechin. These were the values obtained by us with our locally available sample of BT (see Materials and Methods) and agrees fairly well with other recently published values (26, 27). BT at 10 μg concentration of total catechin (Fig. 1A) abrogates NO production by 70%, whereas 1.0 μg of epicatechin present in this amount of BT can inhibit production of NO by only 10%. Individually, theaflavin (1.4 μg) and EGCG (2.4 μg) play much more significant roles than epicatechin in inhibiting NO production but these are still much less effective than BT. Clearly, the powerful abrogating effect of BT towards NO production is not due to any particular individual catechin component but is most likely the result of the combined effect of all these components. Most importantly, theaflavin, the characteristic and exclusive component of BT is a highly potent inhibitor of NO production and is definitely more effective than EGCG, on molar basis. An essentially similar picture emerged when the superoxide anion scavenging efficiency of BT was assessed in the background of GT and relative amounts of individual catechin constituents present in BT. BT is definitely more effective than GT in inhibiting O_2^- production. Further, the powerful scavenging property of BT appears to be the result of the combined effect of individual catechin components including theaflavin (Fig. 1B). In fact, theaflavin is found to be more potent in scavenging O_2^- than EGCG that was earlier identified as the most powerful anti-oxidant in GT (2, 12, 13). SOD, in this experiment, was used as a control [not shown]. All these experiments were repeated at least thrice and the results presented here are from one such experiment. Further, stimulation of peritoneal macrophage for a longer period (48 h) or with live bacteria or purified LPS reproduced the same basic features of this experiment (data not shown).

Assessment of Relative Contributions of Individual Catechin Constituents by Employing a Reconstitution System

In order to demonstrate more convincingly that the inhibitory effect of BT on NO and O_2^- production is indeed a combined effect of all the catechin constituents present in BT, we carried out a reconstitution experiment with BT that was devoid of all small organic molecules (BT-F, see Materials and Methods). Table I shows that while 20 μl of BT (10 μg of total catechins) can prevent production of NO by more than 75%, BT-F has no inhibitory effect ($<$3%) on NO production. Stepwise addition of free catechins (catechin and epicatechin, 0.8 and 1.0 μg respectively); EGCG (2.4 μg) and theaflavins (1.4 μg) in their relative proportions of occurrence in black tea extract to BT-F resulted in a stepwise increase in inhibitory capacity of BT-F and a final abrogation of NO production by 63% was achieved. It is likely that addition of other catechin constituents of BT, e.g., EGC, ECG and thearubigin would have resulted in 76% inhibition of NO production by this reconstituted system as was obtained with BT. More importantly, this experiment reconfirms the earlier observation that theaflavin, the exclusive constituent of

TABLE I

Relative Contributions of Individual Catechin Constituents in Abrogating NO and O_2^- Production

	Nitric oxide		Superoxide anion	
	Production (nmoles μg protein^{-1} min^{-1})	Abrogation (%)	Production (nmoles μg protein^{-1} min^{-1})	Abrogation (%)
1 Complete	0.38	0	0.141	0
2 Complete + BT [10 μg]	0.09	76.4	0.037	73.0
3 Complete + (BT-F) [I]	0.37	2.8	0.134	3.5
4 [I] + catechin (0.8 μg) + epicatechin (1.0 μg) [II]	0.33	11.9	0.011	18.8
5 [II] + EGCG (2.4 μg) [III]	0.27	30.5	0.084	40.0
6 [III] + theaflavin (1.4 μg) [IV]	0.13	63.7	0.054	61.2

Note. The complete system consists of macrophages (10^6 cells/well) activated with 10 μg LPS. Time of incubation was 24 h and temperature was 37°C. Further additions are presented in the table. Estimation of NO and O_2^- were carried out as described under Materials and Methods.

BT, is probably more efficient than EGCG in abrogating NO production. Similarly, all the catechin constituents contributed individually and significantly in scavenging superoxide anion (Table I).

Reconstitution of Catechin-Free BT (BT-F) Prevents Induction of iNOS

Decreased presence of NO in stimulated macrophages in presence of BT (Fig. 1A) or its reconstituted catechin constituents (Table I) may be due to the free radical scavenging property of polyphenolic catechins. It may also be due to the down-regulation of induction of iNOS that is known to be induced in activated macrophages. Both EGCG and theaflavin-di-gallate have been recently shown to block iNOS induction by down-regulating the activation of its cognate transcription factor NF-κB in a macrophage cell-line (15, 17). Direct assay of iNOS (Materials and Methods) in activated macrophages in presence of varying concentration of BT showed that the expression of the enzyme is strongly down regulated (>80%) in presence of BT containing 25 μg of total catechin. To ascertain relative contributions of EGCG and theaflavins on transcriptional control of iNOS, an RT-PCR analysis for total mRNA samples extracted from activated macrophages was carried out. Figure 2 shows that high level of expression of mRNA in activated macrophages (lane 1) is drastically down-regulated in presence of BT (lane 3) whereas incubation with BT-F that is free of all catechins had no demonstrable inhibitory effect (lane 2) on mRNA expression. When reconstitution of BT-F was carried out with appropriate amounts of EGCG (6 μg) and theaflavins (3.5 μg), full inhibitory activity of BT was restored (lane 6). More importantly, theaflavin (lane 5) was found to be much more effective than EGCG (lane 4) in down-regulating the mRNA expression of iNOS.

DISCUSSION

Employing an *in vitro* activated macrophage system; we produce convincing evidence to show that on equal

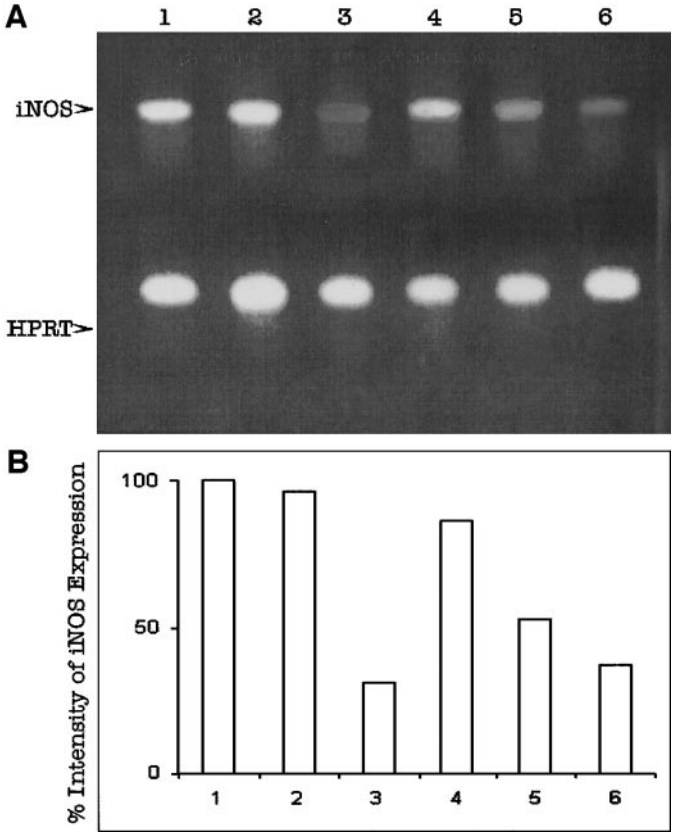

FIG. 2. RT-PCR analysis of mRNA expression of inducible NO synthase. [A] Macrophages (10^6 cells/well) were incubated with heat killed *E. coli* (10 μg LPS) along with BT, BT-F, EGCG, theaflavin, and BT reconstituted. Lane 1, LPS activated macrophage; lane 2, LPS+ BT-F (black tea free of all polyphenols); lane 3, LPS+ BT (25 μg total catechin); lane 4, LPS+ EGCG (6 μg); lane 5, theaflavin (3.5 μg); lane 6, Reconstituted BT (25 μg total catechin). [B] Band intensities were quantified by densitometry.

catechin weight basis BT is probably more effective than GT in scavenging superoxide anion and in abrogating production of NO (Fig. 1). The reconstitution experiment shows that the powerful chemopreventive property of BT is not due to any single catechin constituent but is an additive effect of all these components in BT (Table I). Most significantly, theaflavin is extremely efficient in scavenging O_2^- (Fig. 1B) and also in down-regulating the synthesis of iNOS (Fig. 2) that leads to decreased generation of NO (Fig. 1A). Clearly, the enzymatic biotransformation of monomeric catechins and their gallates to theaflavins during the processing of dried green tea leaves for the production of BT does not in any way adversely affect the chemopreventive properties of BT. In short, BT is as good as GT as a potential health beverage. This is, of course, based on the assumption that elimination of excess reactive oxygen and nitrogen specii is generally beneficial for health (28–30).

Macrophages and endothelial cells seem to be particularly sensitive to the toxic effects of NO and continuous intracellular generation of NO may often lead to apoptosis (20, 31–33). Interaction of NO with O_2^- led to the formation of peroxynitrite, a highly toxic nitrating and oxidizing agent (33). The overall oxidative stress and global damage to DNA and proteins stalled the cell cycle and led to apoptosis (34). Catechin polyphenols were earlier shown by Pannala *et al.* to be very efficient scavenger of peroxynitrite (35). We now show and confirm earlier works by Lin and co-workers (15, 17) that any possible production of peroxynitrite can be eliminated by BT or its characteristic constituent, theaflavin by simply preventing the induction of iNOS synthesis. It is likely that the highly efficient scavenging property of theaflavin and other polyphenolic constituents of BT coupled with their capacity to abrogate enzymatic NO production will prevent apoptosis and other pathological manifestations in various susceptible cellular systems. Our present work is progressing in that direction.

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