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Heavy fermentation impacts NO-suppressing activity of tea in LPS-activated RAW 264.7 macrophages

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Abstract

The effect of fermentation on the nitric oxide (NO)-suppressing activity of tea was investigated using an lipopolysaccharide (LPS)-activated RAW 264.7 cell model. Two species of tea, Taiwan tea No. 12 and Chinsin Oolong, commonly cultivated and consumed in Taiwan, were selected and manufactured with three degrees of fermentation. All of the teas including non-fermented green, partially fermented paochong and fully fermented black teas, were prepared from a single batch of fresh tea leaves. Additionally, the freeze-dried solids of tea infusions were used to treat cells and their NO-suppressing activities were compared. The results showed that the two species of black tea inhibited the NO production, inducible nitric oxide synthase (iNOS) catalytic activity and iNOS protein expression in LPS-activated cells, more weakly than did green and paochong teas. Meanwhile, the NO-suppressing activity was highly correlated with the total phenolics content. Clearly, heavy fermentation strongly affects the NO-suppressing activity of tea, and the decline of the NO-suppressing activity is attributed to the elimination of the phenolics.

Keywords: Fermentation; Nitric oxide; Antioxidant; Phenolics; Tea

1. Introduction

Nitric oxide (NO) is a gaseous free radical that can be formed by nitric oxide synthase (NOS) in human cells. A well-balanced level of NO has been shown to be an important regulator of various physiological processes, such as vasodilation, neurotransmission and host defense (Mayer & Hemmens, 1997). Three NOS isoforms have been identified in cells. Both neural NOS and endothelial NOS are constitutive, whereas inducible NOS (iNOS) is inducible in response to various stimuli. Following induction, iNOS can be expressed quantitatively in various cells, such as macrophages, smooth muscle cells and hepatocytes. Once NO is formed in the cell, it can react with superoxide anions to form peroxynitrite, which is a potent oxidizing and nitrating molecule. These reactive nitrogen species damage cellular macromolecules such as proteins, DNA, and lipids, trigger numerous detrimental cellular responses (Radi, Beckman, Bush, & Freeman, 1991a, 1991b; Yermilov et al., 1995). Excess NO production by iNOS has been implicated in the development of several diseases such as cancer, diabetes, renal disease and cardiovascular disease. (Beckman & Koppenol, 1996; Cooke & Dzau, 1997; Yu, Gengaro, Niederberger, Burke, & Schrier, 1994). Based on this information, the suppression of NO, produced by iNOS, may be a promising indicator of the health effects of food.

Tea is one of the most popular beverages in the world. Moreover, tea is one of the main sources of flavonoids in the diet (Arts, Hollman, & Kromhout, 1999). Several protective effects of tea against chronic

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disease have been demonstrated (Dufresne & Farnworth, 2001). Among the beneficial effects of tea, the antioxidant activity of polyphenols is proposed herein to be the most important (Dreosti, 2000). However, the content of polyphenols is greatly influenced not only by factors such as species, leaf-age, climate, and horticultural practices but also by the processing procedure (Lin, Lin, Liang, Lin-Shiau, & Juan, 1998; Lin, Juan, Chen, Liang, & Lin, 1996). People in various countries or regions are used to drinking tea fermented to various degrees. Japanese and northern Chinese prefer non-fermented green tea. Partially fermented teas, such as paochong and oolong, are commonly drunk by people who live in southern China and Taiwan. In northern America and Europe, the most frequently consumed tea is fully fermented black tea. Notably, the degree of fermentation strongly influences the content of polyphenols, particularly the catechins (Lin et al., 1998). During fermentation, polyphenols in the green tea are oxidized and subsequently condensed. Consequently, the total catechins content is reduced and new products, such as theaflavins and thearubigins, are formed in fermented tea. The effect of fermentation on the antioxidant activity of tea has been demonstrated in the study of Lin and Liang (2002). Rather little attention has been paid to the impact of fermentation, particularly light fermentation, on the NO-suppressing activity of tea in a cell system.

In this study, a batch of two species of fresh tea leaves was used to prepare teas with three degrees of fermentation. Their inhibitory effects on the production of NO in LPS-activated RAW 264.7 macrophages were compared. Additionally, the phenolics content and the antioxidant activity were determined to elucidate the relationships among the phenolics content, NO-suppression and antioxidant activity.

2. Materials and methods

2.1. Materials

A RAW 264.7 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL Life Technologies Inc., Grand Island, NY, USA) and maintained at 37 °C in a humidified incubator that contained 5% CO_2. Lipopolysaccharide (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Folin–Ciocalteu reagent, BCIP/NBT liquid substrate system and authentic catichins used in HPLC analysis including (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC) and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical purity.

2.2. Extract preparation

Two species of Taiwanese tea, Taiwan tea No. 12 (TTE12) and Chinsin Oolong, were used to produce teas with three degrees of fermentation, including non-fermented green tea, partially fermented paochong tea and fully fermented black tea. One batch of fresh tea leaves was harvested and processed by tea-making experts at the Yuchih Branch, Tea Research and Extension Station, Council of Agriculture, Executive Yuan (Yuchih, Taiwan), following the procedures described by Lin et al. (1998). Tea extract was prepared by steeping 5 g of the dried tea leaves in 100 ml of 95 °C distilled water for 5 min. The infusion was filtered through No. 1 filter paper. The filtrates were then collected and freeze-dried. The extraction yields of green (TG), paochong (TP) and black (TB) TTE12 teas were 25.0%, 25.2% and 22.0%, respectively. Green (CG), paochong (CP) and black (CB) Chinsin Oolong teas had an extraction yield of 26.8%, 24.0% and 25.5%, respectively. For the cell culture experiment, the weighed extract was dissolved in DMEM and filtered through a membrane with 0.2 μm pores, and then diluted in DMEM to the indicated concentration.

2.3. Determination of NO production

To determine the effect of tea extract on the NO production, the cells were seeded at a density of 6×10^4 cells per well in 96-well culture dishes. Following incubation for 24 h, the adherent cells were washed three times with PBS. The cells were then incubated in the medium with extracts from various teas, with or without 1 μg/ml LPS. After incubation for 24 h, the medium was collected and stored at −70 °C until assay. Moreover, cell viability was evaluated using the MTT method (Mosmann, 1983). Finally, medium nitrite concentration was measured as an indicator of NO production by the Griess reaction (Kim et al., 1995). The rates at which the teas inhibit the production of NO were calculated as follows.

$$\text{Inhibition of rate} = [1 - (NO_{tea+LPS} - NO_{control})/(NO_{LPS} - NO_{control})] \times 100\%.$$

2.4. Evaluation of iNOS enzyme activity

The cells were cultured in a 10 cm culture dish and stimulated with LPS (1 μg/ml) for 12 h. They were then harvested and plated in a 24-well culture dish, and treated with various tea extracts for a further 12 h. The medium was finally collected and assayed for nitrite.

2.5. Evaluation of iNOS protein expression

The cells were seeded at a density of 5×10^6 cells per 6 cm culture dish and incubated for 24 h. After three washes, the adherent cells were incubated for 12 h in the presence or absence of 1 μg/ml LPS and 500 μg/ml of tea extract. The adherent cells were then washed with PBS, collected, suspended in lysis buffer (50 mM Tris, pH 7.6, 0.01% EDTA, 1% Triton X-100, 1 mM PMSF, and 1 μg/ml leupeptin) and centrifuged at 12,000×*g* for 20 min at 4 °C. The protein concentration was determined using a BCA kit (Pierce Co., Rockford, IL, USA). Additionally, iNOS protein levels were determined by Immunoblot analysis. Briefly, samples with equal protein contents were loaded and separated on 8% SDS-polyacrylamide gel and then transferred to PVDF filters. Meanwhile, filters were blocked and probed with antibodies (BD Transduction Laboratories, Josesan, CA, USA). The filters were then incubated with secondary antibody conjugated to alkaline phosphatase and detected using NBT/BCIP solution. Finally, the band intensities were quantified with a software-supported photoimager (ImageMaster VDS; Amersham Pharmacia Biotech Co. Piscataway, NJ, USA).

2.6. Determination of total phenolic compounds

The amount of total phenolics in the tea extract was determined spectrophotometrically using the previously reported, but modified, Folin–Ciocalteu colorimetric method (Singleton, Orthofer, & Lamuela-Raventos, 1999). Briefly, 2 ml of the optimal diluted sample was introduced into the test tube. Folin–Ciocalteu phenol reagent was then added to the sample, which was held for 3 min. Then, 2 ml of 10% (W/V) aqueous sodium carbonate was added and allowed to stand at room temperature for 1 h. The absorbance of the developed color was measured using a spectrophotometer at 765 nm. The total phenolics content in each tea extract was then calculated by a standard curve prepared with gallic acid and expressed in terms of milligrams of gallic acid equivalents per gram of solid extract.

2.7. Evaluation of antioxidant activity

The total antioxidant capacity of the tea extract was measured using a commercial kit (Randox Laboratories Ltd., Crumlin, UK) and expressed in millimoles of Trolox equivalents per gram of extract. This assay was based on 2,2′-azinobis(3-ethylbenzothiazoline sulfonate) (ABTS) incubated with metmyoglobin and hydrogen peroxide to generate the radical cation $ABTS^+$. $ABTS^+$ has a stable blue-green color, and the color can be measured at 600 nm. Antioxidants in the sample suppress the development of color to an extent that is proportionally to their concentration.

2.8. Determination of catechins by HPLC

The catechins content was determined by an HPLC system equipped with a hypersil C_{18} column (5 μm, 25 × 0.46 cm i.d.) and an UV–Vis detector. The mobile phase contained 1% acetic acid (solvent A) and acetonitrile (solvent B), with a linear gradient start with an 92/8 of A/B ratio and change to 73/27 over a period of 40 min and control the flow rate at 1 ml/min. Identification of the individual catechin and gallic acid was based on the retention time of sample peaks to those of reference authentic standards. The amount of each constituent in the tea extracts was estimated by the integrated datum.

2.9. Statistical analysis

All data are presented as mean ± SD for 3 independent tests. The significance of the differences at each sample was analyzed by ANOVA and Duncan's multiple range test using SPSS software. The paired *t* test was used to evaluate the differences between the treatments and the control, with the significant difference set at $p < 0.05$ (SPSS for Windows 10.0; SPSS Inc. Chicago, IL, USA). The correlation between 2 variants was analyzed by the Pearson test.

3. Results

3.1. NO production in unactivated and LPS-activated macrophages

The cells showed an obviously reduced survival rate (<80%) when incubated with tea extract at a concentration of over 1000 μg/ml. Therefore, in this study, a concentration of under 1000 μg/ml is used to treat cells, to prevent the teas from having a cytotoxic effect on NO production. Analysis of NO production by measuring the nitrite with the Griess reaction revealed that placing unstimulated RAW 264.7 cells in culture medium for 24 h produced a basal amount of nitrite (Fig. 1). When the cells were incubated with extracts from these teas in the absence of LPS, medium nitrite concentration was maintained at a background level similar to that of the unstimulated control (data not shown). After treatment with LPS for 24 h, the medium concentration of nitrite increased markedly compared with the control group (Fig. 1). Significant concentration-dependent inhibition of NO production was observed when cells were co-treated with LPS and various concentrations of the tea extracts (Fig. 1). At a treatment concentration of 500 μg/ml, TG, TP, and TB led to 81.3%, 80.2% and 59.8% less production of NO, respectively, than in the LPS-treated control. Similarly, CG, CP, and CB were responsible for 83.3%, 85.0% and 66.4% inhibition of the production of NO, respectively. These results

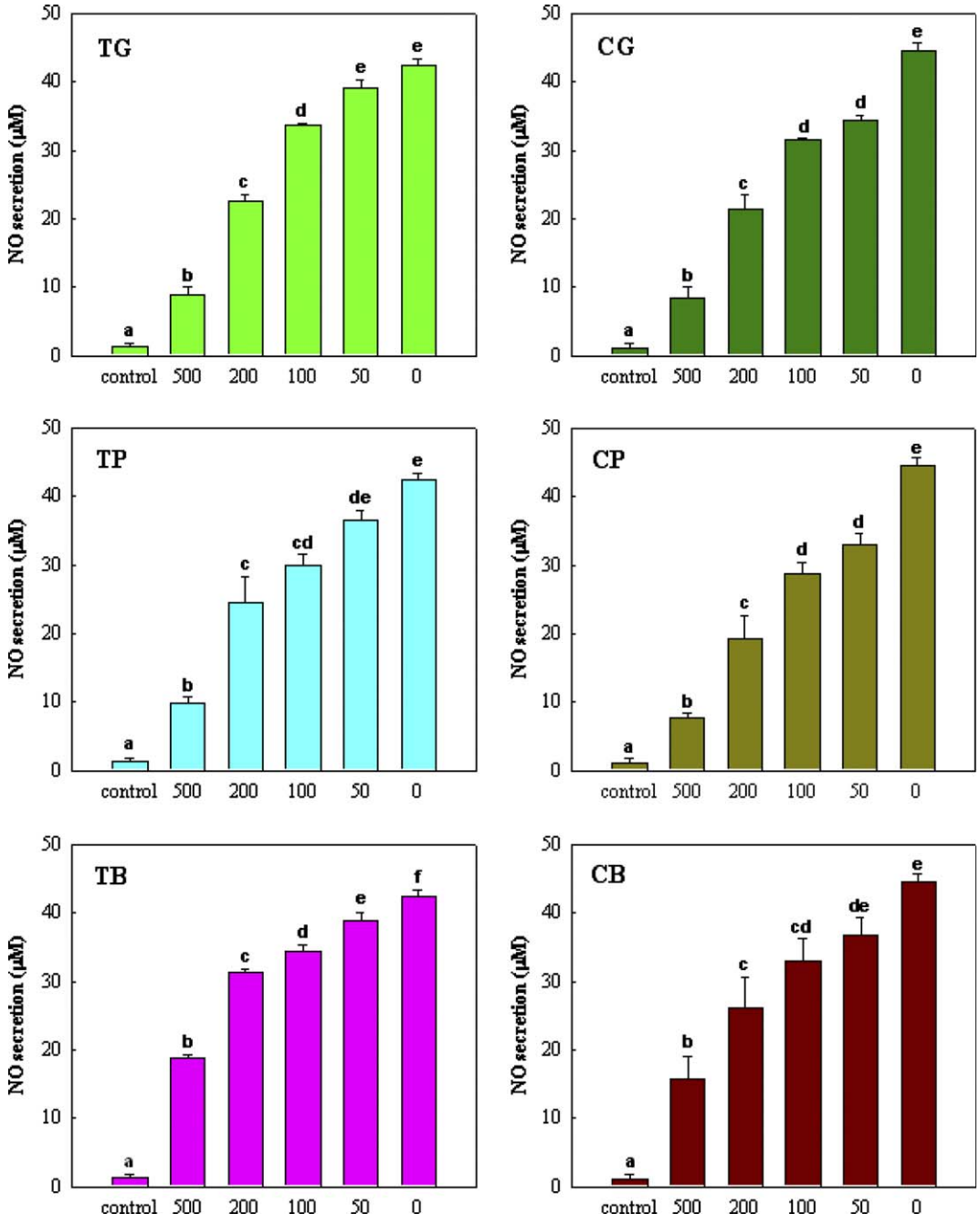

Fig. 1. Effect of teas with various fermented degrees on the NO production in LPS-activated RAW264.7 cells. The values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

demonstrated that light fermentation did not affect the NO-suppressing activity. However, heavy fermentation significantly reduced the NO-suppressing activity of tea in LPS-activated RAW 264.7 cells.

3.2. iNOS catalytic activity

This work explored whether the inhibitory effects on inducible nitrite production resulted from a direct effect of these teas on intrinsic enzyme activity of iNOS. A dose of 500 μg/ml, at which the teas most strongly inhibited the production of NO, was selected to examine the effect of the teas on iNOS enzyme activity. All tea extracts had a marked inhibitory effect. Both green and paochong teas appeared more strongly to inhibit iNOS enzyme activity than did black tea (Table 1). This phenomenon was observed in two species of tea used in this study.

Table 1
Rate of inhibition of teas with various fermented degrees on the iNOS catalytic activity in RAW 264.7 cells

Treatment	NO secretion[B] (μM)	Rate of inhibition[A,C](%)
Control	$0.14 \pm 0.06^{*}$	
+LPS	$35.63 \pm 2.01^{¶}$	
TG	$22.43 \pm 2.29^{*,¶}$	37.28 ± 2.87^{a}
TP	$21.91 \pm 1.66^{*,¶}$	38.69 ± 1.68^{a}
TB	$26.12 \pm 0.41^{*,¶}$	26.68 ± 3.10^{b}
CG	$20.85 \pm 3.31^{*,¶}$	41.87 ± 6.02^{a}
CP	$22.31 \pm 3.94^{*,¶}$	37.80 ± 7.63^{a}
CB	$425.98 \pm 2.55^{*,¶}$	27.29 ± 2.25^{b}

[A] The rate of inhibition was calculated as: percentage inhibition $= [1 - (NO_{tea+LPS} - NO_{control})/(NO_{LPS} - NO_{control}) \times 100\%$.
[B] * and ¶ Represent significant difference when compared with control and LPS-treated control group, respectively, by paired *t*-test ($p < 0.05$).
[C] Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

3.3. iNOS protein levels

Whether these teas affect iNOS protein levels was investigated. Fig. 2 reveals that RAW 264.7 cells did not express a detectable amount of iNOS protein when incubated in the medium without LPS for 12 h, whereas LPS markedly increased the iNOS protein content. At a treatment concentration of 500 μg/ml, all teas exhibited a significant inhibitory effect. TG, TP and TB reduced iNOS protein levels by 87, 81, and 70%, respectively, below those of the LPS-activated group. Similarly, CG, CP and CB reduced LPS-induced iNOS expression to 86%, 87% and 65%, respectively (Fig. 2).

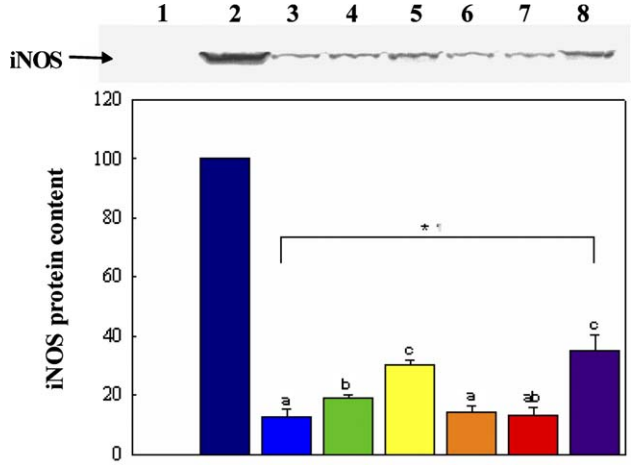

Fig. 2. Suppression of teas with various fermented degrees on the LPS-induced iNOS protein expression. The lanes represent cells treated without (2) or with 500 μg/ml of the tea extract, extracted from TG (3), TP (4), TB (5), CG (6), CP (7) and CB (8), respectively, in the presence of LPS (1 μg /ml) for 12 h. Lane 1 presents the results of the cells without any treatment. The values are expressed as means ± SEM of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

3.4. Total phenolics content and antioxidant activity

Table 2 presents the total phenolics content and antioxidant capacity of the tea extracts. Extracts from green and paochong teas appeared to contain more phenolic compounds than black tea. Similarly, the antioxidant activities of green and paochong teas significantly exceeded those of the black tea. The phenolics content was highly correlated with the antioxidant capacity ($r = 0.983$, $p < 0.01$). Meanwhile, the phenolics content was markedly correlated with the rate of teas' inhibition of 24-h NO production by LPS-activated cells, as determined at a treatment concentration of 500 μg/ml ($r = 0.951$, $p < 0.01$).

3.5. Catechins content

Table 3 shows the catechins content of teas with different fermented degree. It is obvious that heavy fermentation makes the total catechins to be depleted completely, whereas the level of gallic acid is elevated. In contrast to black tea, the lightly fermented tea, paochong tea, still exhibits 85% of total catechins content of that of the green tea.

4. Discussion

Although the NO-suppressing effect of various teas or their components has been extensively investigated, to authors' knowledge, the effect of fermentation on the NO-suppressing activity remains obscure. This work uses a batch of tea leaves to manufacture teas with three degrees of fermentation, and to elucidate the impact of fermentation on the NO-suppression. Clearly, paochong tea, which is a lightly fermented tea, only weakly affects the NO-suppressing and antioxidant activities. In contrast, black tea, which is a

Table 2
Phenolics content and antioxidant activity of teas with various degrees of fermentation

Tea	Phenolics content (mg GAE/g extract)	Antioxidant activity (mmol TE/g extract)
TG	114.6 ± 5.4^{ab}	3.96 ± 0.22^{a}
TP	103.6 ± 3.9^{b}	3.58 ± 0.64^{ab}
TB	67.4 ± 3.9^{c}	1.65 ± 0.15^{c}
CG	117.6 ± 4.0^{a}	4.02 ± 0.65^{a}
CP	108.6 ± 1.2^{ab}	3.22 ± 0.55^{b}
CB	65.5 ± 3.3^{c}	1.70 ± 0.18^{c}

The values are expressed as means ± S.D. of triplicate tests. Means not sharing a common letter were significantly different ($p < 0.05$) when analyzed by ANOVA and Duncan's multiple range test.

Table 3
Catechins content of teas with various degrees of fermentation

Tea	EGCG (mg/g extract)	EGC (mg/g extract)	EC (mg/g extract)	ECG (mg/g extract)	Total catechins (mg/g extract)	Gallic acid (mg/g extract)
TG	154.2	128.7	56.2	15.1	354.2	8.48
TP	173.8	100.0	21.2	13.8	308.8	8.29
TB	ND	ND	ND	ND	0	19.66
CG	141.2	152.4	19.5	0.52	313.6	4.45
CP	137.6	130.9	10.0	0.43	268.9	4.99
CB	ND	ND	ND	ND	0	8.44

ND, not detectable.

heavily fermented tea, exhibited substantially weaker NO-suppressing and antioxidant activities than green and paochong teas of the same species. Moreover, the total phenolics content was strongly correlated with both the activities of NO-suppression and antioxidation. These results demonstrate that the falls of NO-suppressing and antioxidant activities were attributable to the elimination of phenolics during fermentation. Notably, the NO-suppressing activity of black tea was 50–75% of that of the green tea. However, previous reports have demonstrated that the total catechins content, including (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin, (−)-epicatechin gallate, gallocatechin gallate and (−)-epicatechin, of the black tea declines to about 5% of that of the green tea (Lin & Liang, 2002; Lin et al., 1998). In our study, the black tea even had no any detectable catechins. Apparently, the NO-suppressing activity of tea is due to the combined activity of diverse phenolics, rather than being attributable to catechins. This claim is consistent with the report of Paquay et al. (2000).

Foods and phytochemicals can exert NO-suppressing effect via three routes: (1) directly scavenging of NO radicals; (2) inhibition of NOS catalytic activity; (3) suppression of iNOS expression (Sheu & Yen, 2001). Although direct NO-scavenging test was not undertaken in this study, teas were shown to inhibit both the iNOS enzyme catalytic activity and gene expression. Furthermore, the suppression by teas of 24-h NO production is caused mainly by the inhibitory action on iNOS protein expression.

Green and black teas have been reported effectively to scavenge NO and peroxynitrite (Sarkar & Bhaduri, 2001). Catechins, the major phenolics, which account for 30–50% of the solids in a green tea infusion, have been shown to dominate the NO and peroxynitrite scavenging activities. Additionally, the structure of flavan-3-ol linked to gallic acid is an important determinant of the NO, superoxide and peroxynitrite scavenging activities of catechins (Chung et al., 1998; Nakagawa & Yokozawa, 2002). Furthermore, EGCG can completely block the expression of iNOS through down-regulation of the NF-κB transcription factor. It is therefore regarded as a dominant phytochemical in green tea that acts against NO production by LPS-activated cells. In a black tea infusion, in contrast to green tea, polymeric catechins such as theaflavins and thearubigins are formed during fermentation, accounting for 3–6% and 10–30% of the solids, respectively, as the bioactive components (Dreosti, 2000). Indeed, Sarkar and Bhaduri (2001) reconstituted a black tea infusion by stepwise adding various catechins into a catechins-free solution, to identify the NO-suppressing component. They found that theaflavins are the most powerful in abrogating NO production. The theaflavin-3,3′-digallate even exhibited stronger IKK suppressing activity than did EGCG, and hence down-regulating iNOS gene expression (Pan, Lin-Shiau, Ho, Lin, & Lin, 2000). Recently, thearubigin was also found to be a potent inhibitor of iNOS protein expression in the colonic mucosa of rats with colitis (Maity et al., 2003). Besides the three well-known theaflavins, the polymeric catechins in the black tea are diverse and complex, and require further identification. Sang et al. (2004) recently established a horseradish/H_2O_2 enzymatic coupling system to synthesize various theaflavin derivatives, and compared their inhibition of NO production by LPS-stimulated RAW 264.7 cells. Of the 26 tested theaflavin derivatives, only theaflavin-3,3′-digallate, theaflavate and purpurogallin exhibited an NO-inhibitory rate of over 50% of the control value. Although theaflavins and thearubigins have greater NO-suppressing activity, even than EGCG, their activities are compared with reference to the same molar concentration. The dimerized and polymerized reactions during fermentation quantitatively reduce the amount of catechins, but moderately increase the numbers of theaflavin and thearubigin molecules in black tea. In this study, heavily fermented black tea had a lower NO-suppressing activity than both non-fermented green and partially fermented paochung teas. Therefore, we suggest that the moderate rise in the amount of polymeric catechins cannot compensate for the loss of NO-suppressing activity caused by the decline in the amount of catechins in black tea.

Many compounds other than monomeric and polymeric catechins, such as gallic acid, quercetin, rutin and kaempferol have been identified as potent NO-scavenging agents, iNOS enzyme inhibitors and iNOS

expression suppressors (Chen et al., 2001; Lin & Lin, 1997). Heavy fermentation elevates the content of gallic acid, therefore, may help to maintain the NO-suppressing activities of black tea.

The findings of this study indicate that heavy, but not light, fermentation reduces the polyphenol content, affecting the NO-suppressing and antioxidant activities of tea.

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