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Induction of neutral endopeptidase (NEP) activity of SK-N-SH cells by natural compounds from green tea

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Abstract

Deposition of amyloid β-peptide as senile plaques in the brain is one of the neuropathological hallmarks of Alzheimer's disease, which is the most prevalent progressive neurodegenerative disease leading to dementia. Neutral endopeptidase is one of the major β-amyloid-degrading enzymes in the brain. To examine the influence of different polyphenols and other natural products from green tea extract (from *Camellia sinensis*, Theaceae), we used the neuroblastoma cell line SK-N-SH and studied the changes in the specific cellular neutral endopeptidase activity after long-term treatment with these substances. We have shown that caffeine leads to an increase in specific cellular neutral endopeptidase activity more than theophylline, theobromine or theanine. We have also shown that the combination of epicatechin, epigallocatechin and epigallocatechingallate with caffeine, theobromine or theophylline induced cellular neutral endopeptidase activity. It is suggested that the enhancement of cellular neutral endopeptidase activity by green tea extract and its natural products might be correlated with an elevated level of intracellular cyclic adenosine monophosphate.

Introduction

Alzheimer's disease, the most common cause of dementia in the elderly, is characterized pathologically by the accumulation of β-amyloid peptides (Aβ40 and Aβ42) in the brain (Selkoe 2001). The abnormal accumulation of β-amyloid (Aβ) in the brain and amyloid plaque formation may be caused either by increased generation or by decreased degradation of Aβ. Therefore, activation of mechanisms that lower brain Aβ levels is considered valuable for the treatment of Alzheimer's disease. Neprilysin (NEP; EC 3.4.24.11), also known as neutral endopeptidase, is a type II membrane protein on the outer surface of different cells and is involved in the metabolism of regulatory peptides like substance P, bradykinin, enkephalins and other vasoactive hormonal peptides (Turner & Tanzawa 1997). Neprilysin was found to be one of the major Aβ-degrading enzymes and a malfunction of this enzyme has been postulated in the aetiology of Alzheimer's disease (Iwata et al 2001). Over the past decade, intense focus has been given to investigating the process of Aβ metabolism as a possible target for Alzheimer's disease therapy. Up-regulation of NEP in the brain prevents development of Alzheimer's disease by increasing Aβ clearance, resulting in a decrease of Aβ levels (Yasojima et al 2001a, b; Iwata et al 2005). Various synthetic and naturally occurring compounds have been analysed for their efficiency in the modulation of these pathological events. One such naturally occurring compound achieving worldwide popularity for its therapeutic application is green tea. Several effects of green tea on lifestyle-related diseases have been reported, including anti-tumour (Fujiki 1999), antioxidative (Benzie et al 1999) and anti-inflammatory activity (Haqqi et al 1999). The consumption of green tea is associated with a low risk of several types of cancers (Weisburger & Chung 2002). Moreover, green tea has a neuroprotective effect (Hong et al 2000).

A lot of studies were performed to investigate direct and acute effects of green tea and its constituents on cellular systems. Recent studies were conducted to explain the role of green tea in the regulation of gene expression and enzyme induction (Chen et al 2000; Embola et al 2002). Also, green tea extract seems to enhance the activity of the neuropeptidase NEP independently from inhibition of cell proliferation (Melzig & Janka

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2003). Recently, caffeine consumption was proposed to be a protective factor in the development of Alzheimer's disease (Maia & De Mendonca 2002). Consequently, that proposal led us to test whether caffeine and other constituents of green tea could influence the activity of the specific cellular NEP after long-term treatment using the neuroblastoma cell line SK-N-SH, to elucidate the beneficial effects and protective activity of green tea and its constituents by daily intake and to clarify the possible mechanism responsible for the pharmacological efficiency.

Materials and Methods

Chemicals and test compounds

Succinyl-L-Ala-L-Ala-L-Phe-7-amido-3-methylcoumarin (SAAP-AMC), aminopeptidase N (APN) and phosphoramidon were obtained from Sigma. The cell culture media and fetal calf serum (FCS) were obtained from Biochrom. Hoechst 33258 was obtained from Sigma. A stock solution of Hoechst 33258 was made ($1\,mg\,mL^{-1}$) in distilled de-ionized water and stored foil-wrapped at 4°C.

Epicatechin (EC), epigallocatechin (EGC), epigallocatechingallate (EGCG), caffeine, theobromine and theophylline were obtained from Sigma; L-theanine was acquired from ChromaDex. Rolipram was obtained from MB Biomedicals and forskolin and dibutyryl-cAMP were acquired from Sigma.

Cell culture

SK-N-SH cells, human neuroblastoma cells, were obtained from American Type Culture Collection (ATCC) (No. HTB-11) and cultivated in Minimal Essential Medium (MEM with Earls salts) with sodium pyruvate and non-essential amino acids plus 10% fetal calf serum at 37°C in a humidified atmosphere with 5% CO_2 according to the ATCC instruction manual (Rockville, USA). Subcultivation was performed in 70-cm^2 culture flasks to confluence and then cells were seeded for the enzymatic experiments in 24-well plates. For the long-term experiments the cells were incubated with the indicated concentration of the test compound 24 h after plating and cultivated for further 4 days. Then the medium was removed and replaced by NEP-assay solution.

NEP activity

Determination of the specific NEP activity was performed according to Bormann & Melzig (2000). Briefly, 50 μL of SAAP-AMC-solution (400 μM) and 400 μL of HEPES buffer (50 mM + 154 mM NaCl, pH 7.4) were added to the intact cell layer after removing the growth medium. Cells with assay solution were incubated for 60 min at 37°C. The NEP reaction was stopped by adding 50 μL phosphoramidon (50 μM). Four-hundred microlitres of the incubation mixture from each well were transferred into an Eppendorf tube and 20 μL of an APN solution (1:235 diluted with water) were added and the reaction mixture was incubated again for 60 min at 56°C. Adding 800 μL acetone terminated the reaction. The fluorescence of the released AMC was measured at $\lambda_{excit} = 367$ nm, $\lambda_{emiss} = 440$ nm and slit = 3 nm. To calculate the enzyme activity, a calibration curve with AMC was determined. During the enzymatic reaction, and considering the absorbance of fluorescence light by test compounds, the inhibition rates were calculated in comparison with controls without an inhibitor. Enzyme activity was calculated in pmol/min/50 000 cells. In all investigations the effect of dimethyl sulfoxide (DMSO) was considered in separate control experiments.

DNA assay

The assay was performed in 24-well plates using Hoechst 33258 reagent after cell lysis by freezing in distilled de-ionized water (Labarca & Paigen 1980; Rago et al 1990). After determination of NEP activity, the 24-well plates were washed with saline solution (0.9% NaCl). After removing the saline solution, 400 μL of distilled water was added. The plates were frozen at −20°C. On the day of assay the plates were thawed to room temperature and then the plates were frozen again for 1 h. The plates were thawed to room temperature. Next, 400 μL of DNA-buffer (41 mM Na_2HPO_4 $12H_2O$, 9 mM NaH_2PO_4 H_2O, 2 M NaCl, 2 mM EDTA, pH 7.4) was added to each well. Seven hundred and forty microlitres of the mixture in each well was transferred into an Eppendorf tube, and 60 μL of Hoechst 33258 (10 μL of the stock solution + 1 mL water) was added. The fluorescence of the formed product was measured (at $\lambda_{ecxit} = 356$ nm, $\lambda_{emiss} = 458$ nm, slit = 5 nm). A calibration curve with DNA was determined to calculate the amount of DNA of each well.

Cell counting

The cells were dissociated with trypsin–EDTA (0.25%:0.02%) and counted with the cell analyser system CASY (Scharfe System, Germany). The values of the cell numbers represent the mean of at least three independent experiments with two parallel samples.

Statistics

The assays were performed in at least three independent experiments with four parallel samples. All values in the tables and figures are expressed as mean ± standard error. During enzyme reaction, the inhibition rates were calculated as percentage of controls without inhibitors. Wilcoxon's *U*-test was used to test significance ($P < 0.05$).

Results

Effects of caffeine, theophylline, theobromine, theanine, epicatechin (EC) epigallocatechin (EGC) and epigallocatechingallate (EGCG) on specific NEP activity

In the first series of experiments we tested the effect of some substances that are present in the green tea extract.

The long-term effects of caffeine, theophylline, theobromine and theanine were determined, at a range of concentrations (1–1000 μM), on specific cellular NEP activity. We found that the treatment of the cells with caffeine, theophylline, theobromine and theanine induced the activity of the specific cellular NEP (Figure 1).

The simultaneous adding of these substances (short-term effect) had no effect on the specific cellular NEP activity (data not shown).

In Table 1 the cell proliferation was shown with the assistance of DNA assay. The enzyme induction effect was accompanied by an inhibition of cell proliferation (at a concentration > 100 μM). The long-term treatment of the SK-N-SH cells with low concentration of EC and EGC (1, 10 μM) induced significantly the specific cellular NEP activity (Figures 2, 3) with a slight inhibition of cellular

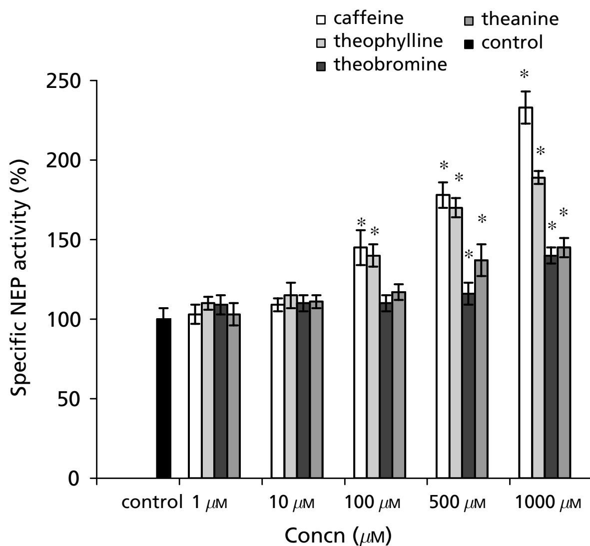

Figure 1 Influence of caffeine, theophylline, theobromine and theanine on specific NEP activity of neuroblastoma cell line SK-N-SH. The cells were incubated with the indicated concentration 24 h after plating and cultivated for further 3–4 days. *$P < 0.05$ compared with the specific enzymatic activity of the control without any treatment.

Table 1 Influence of caffeine, theophylline, theanine and theobromine on cell proliferation

Substance	Cell proliferation (% of control)				
	1 μM	10 μM	100 μM	500 μM	1000 μM
Caffeine	97 ± 9	95 ± 10	83 ± 9	64 ± 11*	45 ± 9*
Theophylline	93 ± 8	88 ± 9	88 ± 9	66 ± 11*	56 ± 9*
(−)-Theanine	99 ± 4	96 ± 4	91 ± 4	83 ± 11	82 ± 5*
Theobromine	99 ± 4	99 ± 6	99 ± 5	90 ± 5	75 ± 8*

Cell proliferation of control: 100 ± 7%. *$P < 0.05$ compared with cell proliferation in the controls.

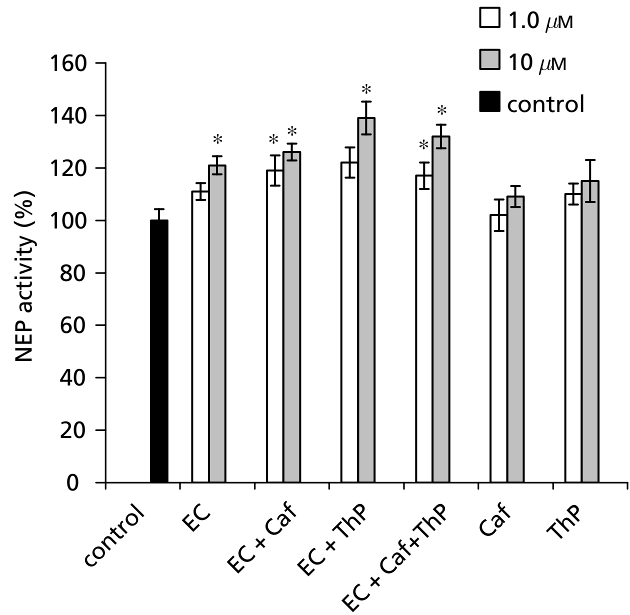

Figure 2 Influence of epicatechin (EC) and the combination with caffeine (Caf) or theophylline (ThP) (or both) on specific NEP activity of neuroblastoma cell line SK-N-SH. The cells were incubated with the indicated concentration 24 h after plating and cultivated for further 3–4 days. *$P < 0.05$ compared with the specific enzymatic activity of the control without any treatment.

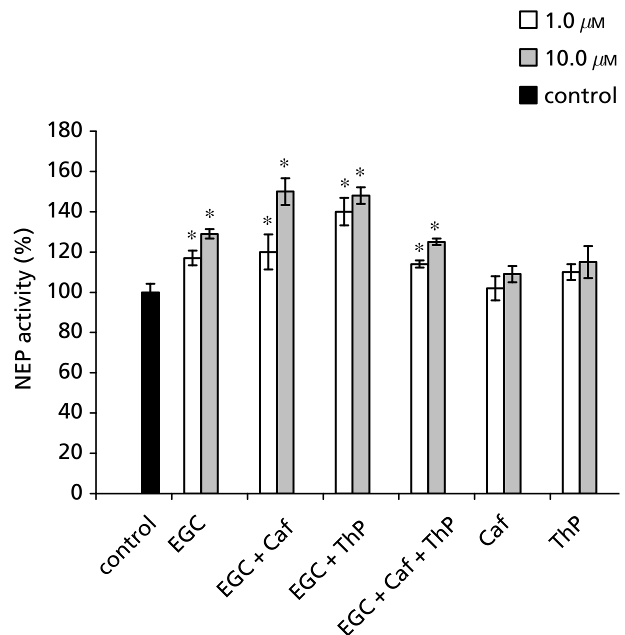

Figure 3 Influence of epigallocatechin (EGC) and combination with caffeine (Caf) or theophylline (ThP) (or both) on specific NEP activity of neuroblastoma cell line SK-N-SH. The cells were incubated with the indicated concentration 24 h after plating and cultivated for further 3–4 days. *$P < 0.05$ compared with the specific enzymatic activity of the control without any treatment.

Table 2 Influence of epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG) and combinations with caffeine (Caf), theophylline (ThP) and theobromine (ThB) on cell proliferation

Substances	Cell proliferation (% of control)		
	1 μM	10 μM	Control (%)
EC	95 ± 7	88 ± 6*	100 ± 6
EC + Caf	95 ± 5	95 ± 5	100 ± 4
EC + ThP	94 ± 4	94 ± 4	100 ± 4
EC + ThB	98 ± 3	98 ± 4	100 ± 6
EC + Caf + ThP	98 ± 1	94 ± 2	100 ± 4
EGC	89 ± 9	84 ± 9*	100 ± 6
EGC + Caf	97 ± 3	95 ± 3	100 ± 3
EGC + ThP	97 ± 4	95 ± 3	100 ± 3
EGC + ThB	100 ± 3	99 ± 3	100 ± 6
EGC + Caf + ThP	99 ± 2	96 ± 4	100 ± 3
EGCG	99 ± 7	99 ± 5	100 ± 8
EGCG + Caf	100 ± 7	103 ± 6	100 ± 8
EGCG + ThP	100 ± 4	97 ± 6	100 ± 8
EGCG + ThB	104 ± 5	100 ± 6	100 ± 8
EGCG + Caf + ThP	97 ± 6	97 ± 5	100 ± 8

*$P < 0.05$ compared with cell proliferation in the controls.

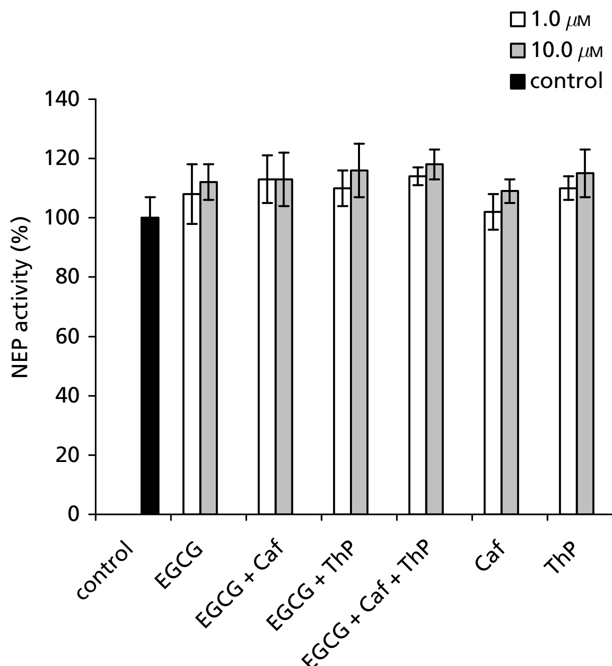

Figure 4 Influence of epigallocatechingallate (EGCG) and combination with caffeine (Caf) or theophylline (ThP) (or both) on specific NEP activity of neuroblastoma cell line SK-N-SH. The cells were incubated with the indicated concentration 24 h after plating and cultivated for further 3–4 days.

proliferation (Table 2). On the other hand, EGCG led to a slight insignificant induction of the specific cellular NEP activity (Figure 4) without inhibition of cellular proliferation (Table 2).

Effect of the combination of EC, EGC and EGCG with caffeine, theophylline and theobromine on specific NEP activity

To investigate the mechanism of action of green tea on cellular NEP activity in SK-N-SH, we tested whether the combination of EC, EGC and EGCG with caffeine, theophylline and theobromine at the same concentration (1, 10 μM) leads to an additional increase in specific NEP activity (Figures 2, 3, 4). We found that the combination of EC and EGC with caffeine and theophylline led to an additional increase in cellular NEP induction to about 150% (Figures 2, 3). But EC and EGC combined with caffeine and theophylline, all in one combination, did not cause an additional increase in cellular NEP induction. Treatment of SK-N-SH cells with the combination of EGCG and caffeine did not lead to an additional increase in cellular NEP induction. Nevertheless, with the treatment of SK-N-SH cells with EGCG together with caffeine and theophylline there was a slight additional increase in cellular NEP activity (Figure 4). The combination of EC, EGC and EGCG with theobromine had no effect on the cellular NEP activity (data not shown).

With the support of DNA assay we have shown cell proliferation. Table 2 shows that long-term treatment of the SK-N-SH cells with EC and EGC in combination with caffeine, theophylline and theobromine led to a significant decrease in the inhibition of cell proliferation and an increase in cellular NEP induction, whereas with EGCG there was no inhibition of the cell proliferation with a slight insignificant induction of the cellular NEP activity. It seem to be that the enzyme induction effect of EC, EGC and EGCG was independent from the inhibition of cell proliferation

Additional investigation to refine the knowledge about the mode of action of green tea components on specific NEP activity

In further experiments, we tested whether the induction effect on the specific NEP activity was also found in parallel to the increase of intracellular cAMP level. We tested the effect of dibutyryl-cAMP (as a protein kinase A activator (Graf et al 1995)), forskolin (an adenylate cyclase activator (Wan Kim et al 2004)) and rolipram (a specific inhibitor of the phosphodiesterase type 4 (PDE4) isoform (Vitolo et al 2002)); all of these substances increase the intracellular cAMP levels.

We found that dibutyryl-cAMP increased the specific NEP activity to about 126% without any effect on the proliferation of cells. Forskolin also led to an induction of specific cellular of NEP activity to about 135%, though there was no difference in cellular proliferation. Rolipram induced significant specific NEP activity to about 150% without affecting the cell proliferation (Figure 5)

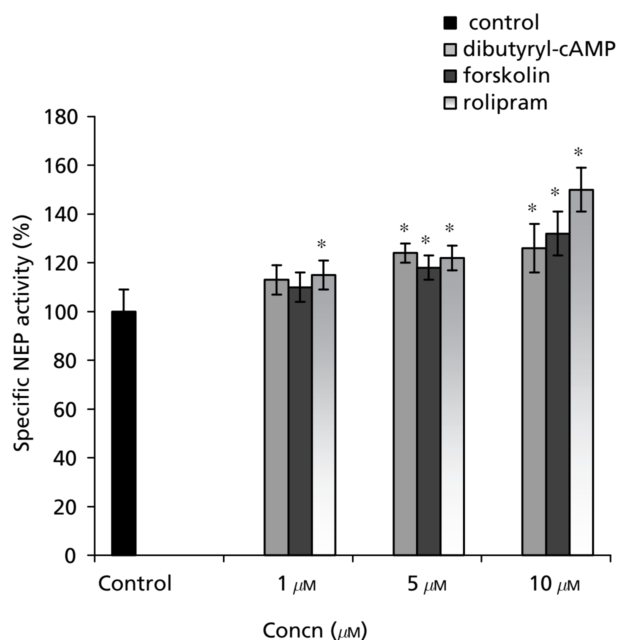

Figure 5 Influence of dibutyryladenosine-3′,5′-cyclic monophosphate, forskolin and rolipram on specific NEP activity of neuroblastoma cell line SK-N-SH. The cells were incubated with the indicated concentration 24 h after plating and cultivated for further 3–4 days. The control for rolipram and forskolin was cells treated with 5 μg mL^{-1} DMSO 0.5% as solvent. *$P < 0.05$ compared with the specific enzymatic activity of the control without any treatment.

Discussion

The data presented here show that the treatment of SK-N-SH cells, a human neuroblastoma cell line, with caffeine, theophylline, theobromine, theanine, epicatechin, epigallocatechin and epigallocatechingallate leads to an increase in cellular NEP activity and this induction depends on the concentration of these substances. A possible explanation for the observed enzyme induction effects might be based on the relationship between cellular differentiation and cellular proliferation. In the case of NEP, it has been reported that in different cell types the up-regulation of the cellular enzymatic activity is correlated to an enhanced cellular differentiation state as well as to the inhibition of cellular proliferation. At the same time, the enhanced cellular enzyme activity supports the assumption that the inhibition of cell proliferation was not the result of a cytotoxic effect (Uehara et al 2001).

In our investigations the increase in cellular enzyme activity of NEP was only associated with a slight inhibition in cellular proliferation, demonstrating a clear enhancement of cellular differentiation. Furthermore, we investigated the effect of green tea polyphenols (EC, EGC and EGCG) in combination with caffeine, theophylline or theobromine to exemplify the synergistic effect of these substances that present in green tea extracts. We have found that these combinations lead to additional increases in the cellular NEP induction without decrease in cell proliferation or with an increase in the cell proliferation, demonstrating that the induction of cellular NEP activity by these polyphenols was independent from the inhibition of cell proliferation.

A possible explanation for the observed enzyme induction effects might be based on the increase in cAMP level, which is induced by methylxanthines like caffeine, theophylline and theobromine. According to the specification of the manufacturer, the green tea extract contained 47.5–52.5% polyphenols (EGCG approximately 61%), 5–10% caffeine and 0.3–1.2% theobromine analysed by HPLC. This green tea is able to induce the cellular NEP activity (Melzig & Janka 2003). The major tea catechins are EGCG, EGC, ECG and EC. It can be qualified that methylxanthines contribute to the induction effects of specific NEP activity; however, they are not the main constituents responsible for the enzyme induction. It is known that caffeine and other methylxanthines, like theophylline and theobromine, are able to enhance the differentiation of neuroblastoma cells via cAMP-dependent histone H1 phosphorylation induced by inhibition of phosphodiesterase activity (Ajiro et al 1990). The clinically relevant concentration of caffeine (10–50 μM) attenuated cAMP accumulation by adenosine, implicating an action apart from the inhibition of phosphodiesterase enzyme (PDE) (Belibi et al 2002). Caffeine ingestion at a dose of 5 mg per kg body weight elicits a plasma concentration of $\sim$ 45 μmol L^{-1} (Graham & Spriet 1995) and resulted in significantly higher concentrations of cAMP (Thong et al 2002). We have found that caffeine and theophylline at 100 μM induce the activity of specific cellular NEP activity to about 145% without significant inhibition of cell proliferation. It has been reported that low concentrations of circulating methylxanthines, less than 100 μM, can suppress the pharmacological effects of adenosine in nerve tissue (Daly et al 1981). Recent studies have shown that caffeine blocks β-amyloid-induced neurotoxicity both in-vitro and in-vivo via blockade of adenosine A_{2A} receptors (Dall'Igna et al 2003). In addition to its neuroprotective effects, caffeine has established cognitive-enhancing effects in man, particularly in the elderly (Brice & Smith 2002). Caffeine has a volume of distribution similar to that of body water and rapidly crosses the blood–brain barrier and penetrates into the brain (Axelrod & Reisenthal 1953). Lethal intoxication is observed with a blood concentration higher than 500 μM (Rall 1980). In our studies we found that the significant induction of the cellular NEP activity with 500 μM caffeine was accompanied by inhibition of cell proliferation to about 64%, which means that the induction at this concentration was accompanied by cellular toxicity.

After administration of a single oral dose of green tea (20 mg tea solids/kg, equivalent to $\sim$ 2 cups of tea) the maximum plasma concentrations of EGCG, EGC and EC in the three repeated experiments were 77.9 ± 22.2, 223.4 ± 35.2 and 124.03 ± 7.86 ng mL^{-1}, respectively, which is equivalent to 0.17, 0.73 and 0.43 μM, respectively, and the time needed to reach the peak concentrations was in the range of 1.3–1.6 h.

The aforementioned values could serve as a reference for designing in-vivo experiments to explain the mechanisms of action of these catechins. Because of the fact that the in-vitro experiments are not so highly sensitive compared to the in-vivo models, we used in our study in cell culture system 1 and 10 μM of EC, EGC and EGCG, and we found that there was only a slight induction of cellular NEP activity. In the plasma, EGCG was mostly present in the free form, whereas EGC and EC were mostly in the conjugated form (Lee et al 2002). The EGC level detected in plasma corresponded to 0.2–2.0% of the ingested amount (Nakagawa et al 1997).

The cAMP level is key for protection, growth and myelination of injured CNS axons in-vivo and recovery of function (Pearse et al 2004). The elevation of cyclic adenosine monophosphate (cAMP) levels is considered to have therapeutic potential for the delay of Alzheimer's disease progression. The Alzheimer's-disease-related increases in the amyloid precursor protein C-terminal derivatives (CTDs) can be reversed by treatment with agents that increase intracellular cAMP, such as dibutyryl-cyclic-AMP, theophylline and isoproterenol (Wolozin et al 1993).

Possibly, the increase in cellular NEP activity correlates to the increase of cAMP levels, which could explain our results. To see if the increase of intracellular cAMP levels influences the expression of endopeptidase NEP, we studied the changes in the specific cellular enzyme activity of NEP and cell proliferation after long-term treatment of neuroblastoma cell line SK-N-SH with dibutyryl-cAMP, as protein kinase A activator (Graf et al 1995), forskolin, as adenylate cyclase activator (Wan Kim et al 2004) and rolipram, a specific inhibitor of the phosphodiesterase type 4 (PDE4) isoform. Rolipram is able to restore the cAMP/cAMP-dependent protein kinase/cAMP regulatory element-binding protein (cAMP/PKA/CREB) pathway activity in the hippocampus and its long-term potentiation (LTP) (Vitolo et al 2002). We found that the treatment of SK-N-SH cells with dibutyryl-cAMP, forskolin and rolipram significantly induced cellular NEP activity and that they had no effect on cellular proliferation. Based on the previous discussion, we can see strong evidence that these substances enhance the NEP gene expression via increasing the intracellular cAMP level.

The possible physiological importance of the induction effect of green tea and its constituents on the specific cellular NEP activity in the neuroblastoma cell line SK-N-SH can be discussed as a neuroprotective effect. The increase in cellular NEP activity might be associated with an increased neuropeptide metabolism, which is necessary to maintain the functional integrity of the brain. Besides the metabolism of regulatory peptides, NEP is also included in the degradation of amyloid beta peptides and by that linked with the pathogenesis of Alzheimer's disease (Shirotani et al 2001). It was shown that unilateral intracellular injection of lentiviral vector expressing human neprilysin (Lenti-Nep) in transgenic mouse models of amyloidosis, which produced an activity of 617 nmol min^{-1} mg^{-1}, reduced amyloid-β deposits by half relative to the untreated side (Marr et al 2003). The up-regulation of NEP activity by green tea and its constituents can be considered as neuroprotection. That is due to an enhanced degradation of β-amyloid peptides (Aβ), which may affect the susceptibility to Alzheimer's disease and prevent the accumulation of amyloid plaques in-vivo.

Conclusions

Finally, judging from our studies, it seems that the increase in the cellular activity of NEP by green tea and its constituents not only depends on the differentiation improvement but also on the direct influence on NEP gene expression. This induction might be correlated with the degradation of amyloid peptides; it also helps in diminishing the risk of accumulation of plaque-forming peptides. Possibly, the daily consumption of green tea can diminish risk of Alzheimer's disease and age-related dementia.

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