Original Research Report

UP-REGULATION OF NEP-ACTIVITY OF SK-N-SH CELLS BY GREEN TEA EXTRACT AND ITS NATURAL PRODUCTS ON RISK OF ALZHEIMER

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Abstract

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. AD is characterized pathologically by the accumulation of amyloid ß-peptide as senile plaques in the brain. Neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE) belong to the major Aß-degrading enzymes. NEP and ACE are zinc-endopeptidases enzymes acting as ectoenzymes on the outer surface of different cells. In order to test the influence of green tea extract and some of its natural components (caffeine, theophylline, theanine, epicatechin and epigallocatechin), we used the neuroblastoma cell line SK-N-SH then we studied the changes in the specific cellular NEP and ACE activity after long-term treatment with these substances. Despite the high similarity between both enzymes ACE and NEP green tea extract and the tested substances only influenced selectively the specific NEP activity without affecting the specific ACE activity. We have also shown that dibutyryl-cAMP, forskolin and rolipram increase the level of intracellular cyclic adenosine monophosphate (cAMP) which results in an induction of the specific NEP activity without any effect on the specific ACE activity. Green tea extract was also able to increase the level of intracellular cAMP in vitro. These results demonstrate that the enhancement effect of the specific NEP activity, by green tea extract and its natural products, was selective and correlated with an elevated level of cAMP.

Key Words: Neutral endopeptidase (NEP), Angiotensin-converting enzyme (ACE), Alzheimer's disease (AD), Beta-Amyloid peptides (Aß) and Green tea.

1. INTRODUCTION

Green tea is one of the most commonly consumed beverages in the world. Several effects of green tea on life-style related diseases have been reported, including anti-tumor activities (Fujiki 1999), antioxidative activities (Benzie et al 1999), and anti-inflammatory activities (Haqqi et al 1999). Epidemiological studies associated the consumption of green tea with a lower 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 components on cellular systems. Recent studies were conducted to explain the role of green tea in the regulation of gene expression and enzyme induction (Embola et al 2002; Chen et al 2000).

Alzheimer's disease (AD) is one of the main causes of dementia in the elderly. AD is a progressive neurodegenerative disorder characterized by the presence of extracellular plaques of *ß* amyloid and intracellular neurofibrillary tangles (NFTs) in the brain (Selkoe 2001). An imbalance between the generation and the degradation of *ß*-amyloid (A*ß*) leads to an abnormal accumulation of Aß in the brain (Glabe 2000). Therefore, activation of mechanisms which lower the brain A*ß* levels is considered valuable for the treatment of Alzheimer's disease. Neutral endopeptidase (EC 3.4.24.11, NEP), also known as neprilysin, and

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angiotenin-converting enzyme (EC 3.4.15.1, ACE) are zinc-endopeptidases enzymes acting as ectoenzymes on the outer surface of different cells and are included in the metabolism of regulatory peptides, like substance P, bradykinin, enkephalins, natriuretic peptides and other vasoactive hormonal peptides (Hooper, 1996). NEP was found to be one of the major A*β*-degrading enzymes and a malfunction of this enzyme has been postulated in the etiology of AD (Iwata et al. 2001). Up-regulation of NEP in the brain prevents AD development by increasing A*β* clearance, resulting in a decrease of A*β* levels (Yasojima et al. 2001a, 2001b, Iwata et al. 2005). ACE has also been reported to play a role in A*β* degradation based on in vitro and cell-based assays (Hu et al. 2001). In order to elucidate the beneficial effects and protective activities of the green tea and its constituents by daily intake and to clarify the possible mechanism responsible for the pharmacological efficiency, we used the neuroblastoma cell line SK-N-SH and studied the changes in the specific cellular activities of both similar enzymes NEP and ACE after long term treatment with green tea extract and its constituents.

Materials and Methods Chemicals & Test Compounds

Succinyl-L-Ala-L-Ala-L-Phe-7-amido-3-methylcoumarin (SAAP-AMC), aminopeptidas N (APN) and phosphoramidon were obtained from Sigma. Hip-L-His-L-Leu was purchased from Bachem; lisinopril was a gift of Schering & Plough.

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) in distilled deionized water and stored foil-wrapped at 4°C. The Green tea extract (EFLA®85942) was supplied by Emil Flachsman AG (Switzerland). Epicatechin (EC), epigallocatechin (EGC), epigallocatechingallate (EGCG), caffeine, and theophylline were taken from Sigma; L-theanine was acquired from ChromaDex.

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 pyruvat and non-essential amino acids plus 10% fetal calf serum at 37°C in a humidified atmosphere with 5% CO_2 according to ATCC instruction manual (Rockville, USA). Subcultivation was performed in 70-cm^2 culture flasks unit 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 aforementioned 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, 50µl of SAAP-AMC-solution (50µM) and 400µl of HEPES-buffer (50mM + 154mM 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 50µl phosphoramidon (50µM). 400µl of the incubation mixture of each well were transferred in an Eppendorf tube and 20 µl of an APN-solution (1:235 diluted with water) were added to the combination and the reaction mixture was incubated again for 60 min at 56°C. Finally by adding 800µl acetone terminated the reaction. The fluorescence of the released AMC was measured at exit =367 nm, emiss =440 nm and slit =3nm. 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 to controls without an inhibitor. Enzyme activity was calculated in pmol/min per 50,000 cells.

ACE Activity

The assay was performed according to Bormann & Melzig (2000). Briefly, 20µl of Hip-L-His-L-Leu solution (24 mM in water) and 260µl of phosphate-buffer (83mM K2HPO4 X 3 H2O + 326 mM NaCl, pH 8,3) were added to the intact cell layer after removing the growth medium. To investigate the acute inhibitory activity of a test substance, this compound was simultaneously added. The cells with assay solution were incubated for 20 min at 37°C. 250µl of the incubation mixture of each well were transferred in an Eppendorf tube and the reaction was stopped by addition of 1 ml 0,4 N NaOH plus 100 µl o-phthalaldehyde solution (2 % in methanol, freshly prepared). Under exclusion of the light this mixture was incubated for 10 min at 22°C. Adding 300 µl 2 N HCl terminated the reaction. The fluorescence of the formed product was measured at exit =360 nm, emiss =500 nm and slit =3nm. To calculate the enzyme activity, a calibration curve with L-His-L-Leu was determined. During the enzymatic reaction and considering the absorbance of fluorescence light by test compounds, the inhibition rates were calculated in comparison to controls without an inhibitor. Enzyme activity was calculated in pmol/min per 100,000 cells for ACE and NEP.

DNA Assay

The assay was performed in 24-well plates using Hoechst 33258 reagent after cell lysis by freezing in distilled deionized 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 until reaching room temperature and then the plates were frozen again for 1 h. The plates were thawed until reaching room temperature. Next, 400 µl of DNA-buffer (41mM Na2HPO4 12H2O + 9mM NaH2PO4 H2O + 2M NaCl + 2mM EDTA, pH 7,4) was added to each well. 740 µl of the mixture of each well was transferred in an Eppendorf tube, and 60 µl of Hoechst 33258 (10µl of the stock solution + 1 ml water) was added. Simultaneously, the fluorescence of the formed product was measured (at exit =356 nm, emiss =458 nm, slit 5nm). A calibration curve with DNA was determined to calculate the amount of DNA of each well.

Quantification of intracellular cyclic AMP by EIA

In order to measure the intracellular cAMP concentration, the cells were allowed to grow in order to confluence in 6-well plates. The green tea extract in a concentration of 50.0 µg/ml was added to each of the wells and the cells were cultivated for further 72 h. After that the medium was discarded and 0.1 M HCl was added to each well for 20 min, then the cells were scraped and dissociated until the suspensions were homogenous. After centrifugation at 1,000-x g for 10 min., the supernatant from every well was collected, and then cAMP EIA assay was performed. The technique for the detection of cAMP was performed according to the protocols described in the cyclic AMP EIA assay kit (Code No. 581001 Cayman Chemical Co. USA). The value of cAMP was reported as mean ± standard deviation in picomoles of cAMP per µg DNA.

Cell counting

The cells were dissociated with trypsin/EDTA (0.25% /0.02%) and counted with the cell analyzer system CASY (SCHÄRFE System, Germany). The cell numbers we used represent

the mean of at least three independent experiments with two parallel samples.

Statistical Treatment

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

2. Results

Effects of green tea extract, caffeine, theophylline, theanin, epicatechin (EC) and epigallocatechin (EGC) on the specific NEP and ACE activity.

On the cell surface of SK-N-SH cells, where NEP and ACE act as ecto-enzymes, the effect of green tea extracts and some of its substances were tested in the first series of experiments.

The long-term effect of green tea extract, caffeine, theophylline, theanine, EC and EGC was determined on the specific cellular NEP and ACE activity. We found that the treatment of the cells with these natural products induced significantly only the activity of the specific cellular NEP without affecting the specific ACE activity (Tab. 1 and 2).

With the support of a DNA-assay we have studied the cell proliferation. The results of this assay showed that the induction of specific NEP activity was accompanied with an inhibition of cell proliferation (Tab. 1 and 2).

EC and EGC with caffeine and theophylline on specific NEP and ACE activity

To investigate the mechanism of action of green tea and its components on the cellular NEP activity in SK-N-SH, we tested whether the combination of EC and EGC with caffeine and theophylline at the same concentration (1, 10 µM) will lead to an additional increase in specific enzyme activity or not. We found that the combinations of EC and EGC with caffeine and theophylline led to an additional increase in cellular NEP induction to about 150% without inhibition of cell proliferation (Tab. 2). These results suggest that the enzyme induction effect of these substances was independent on the inhibition of cell proliferation (Tab. 2).

The combination of EC and EGC with caffeine and theophylline did not increase the specific ACE activity demonstrating that the induction effect of these substances was only selective for NEP activity (Tab. 2).

Mode of action on the specific cellular enzyme activity

To elucidate the mechanism of the process in which the green tea extract effect the specific cellular NEP induced, we tested whether its induction effect was associated with an increase of the intracellular cAMP levels or not. We found that green tea extract (50.0µg/ml) was able to increase significantly the concentration of intracellular cAMP in SK-N-SH cells to 2.45±0,08 pmol/µg DNA (200%) in comparison to the control without green tea which was 1.22±0.056 pmol/µg DNA (100%). Also, we tested whether the induction effect on the specific NEP and ACE activity was also found in parallel to the increase of intracellular cAMP level or not. The effect of 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 (Vitolo et al 2002), was tested on the specific NEP and ACE activity. All of these substances are able to increase the intracellular cAMP levels.

We found that dibutylryl-cAMP, forskolin and rolipram increased the specific NEP activity to about 150% without affecting the cell

Table 1. Influence of green tea extract (GT), caffeine (Caf), theophylline (ThP) and theanine (ThN) on specific enzyme activity and cell proliferation

Substances		Specific cellular enzyme activity (%)		Cell proliferation (%)
		ACE	NEP	
GT (in µg/ml)	control	100 ± 8	100 ± 6	100 ± 8
	10	101 ± 9	117 ± 7*	90 ± 9
	25	106 ± 11	132 ± 5*	82 ± 7**
	50	104 ± 10	250 ± 4*	45 ± 5**
Caf (in µM)	control	100 ± 7	100 ± 8	97 ± 10
	10	103 ± 13	109 ± 4	84 ± 11
	100	112 ± 10	145 ± 11*	70 ± 10**
	500	119 ± 8*	178 ± 8*	57 ± 10**
ThP (in µM)	control	100 ± 7	100 ± 8	93 ± 7
	10	111 ± 3	115 ± 8	95 ± 7
	100	119 ± 6*	140 ± 7*	95 ± 5
	500	124 ± 6*	170 ± 6*	60 ± 6**
ThN (in µM)	control	100 ± 7	100 ± 8	97 ± 5
	10	116 ± 4*	111 ± 4	89 ± 9
	100	119 ± 5*	119 ± 5*	87 ± 11**
	500	109 ± 4	137 ± 10*	82 ± 6**

*Significant difference to the specific enzymatic activity of the controls (100 ± 6, p < 0.05).
**Significant difference to cell proliferation of the controls (100 ± 8, p < 0.05).

Table 2. Influence of epicatechin (EC), epigallocatechin (EGC), and combination with caffeine (Caf) and theophylline (ThP) on specific enzyme activity and cell proliferation

Substances		Specific cellular enzyme activity in % (1 µM substances)		Specific cellular enzyme activity in % (10 µM substances)		Cell proliferation in %	
		ACE	NEP	ACE	NEP	1 µM	10 µM
EC	control	101 ± 6	111 ± 3	105 ± 8	121 ± 3*	95 ± 7	88 ± 6**
EC + Caf	10	106 ± 7	110 ± 13	110 ± 6	125 ± 3*	95 ± 5	95 ± 5
EC + ThP	100	110 ± 4	119 ± 6*	148 ± 7*	139 ± 6*	94 ± 4	94 ± 4
	500		124 ± 6*	170 ± 6*		60 ± 6**	
EGC	control	100 ± 8	100 ± 7	101 ± 4	129 ± 2*	92 ± 8	84 ± 9**
EGC + Caf	10	99 ± 10	102 ± 5	103 ± 10	150 ± 7*	97 ± 3	95 ± 3
EGC + ThP	100	100 ± 10	119 ± 9*	119 ± 5*	148 ± 4*	97 ± 4	95 ± 3
	500		109 ± 4	137 ± 10*		82 ± 6**	

*Significant difference to the specific enzymatic activity of the controls (100 ± 6, p < 0.05).
**Significant difference to cell proliferation of the controls (100 ± 8, p < 0.05).

proliferation. Also these substances did not induce the specific cellular ACE activity demonstrating that there is no relation between the induction of the specific ACE activity and the elevation of cAMP level (Tab. 3)

3. Discussion

Green tea extract and its constituents inhibited the proliferation of SK-N-SH cells, a human neuroblastoma cell line, and this inhibition was associated with an increase in cellular enzyme activity of NEP but not ACE. Despite the high similarity between both enzymes ACE and NEP the results of this study show that the treatment of SK-N-SH cells, with green tea extract, caffeine, theophyline, theanine, epicatechin and epigallocatechin influenced only the expression of NEP. One 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 activities do support the assumption that the inhibition of cell proliferation was not due to the cytotoxic effect (Uehara et al 2001).

In our investigations the increase of cellular enzyme activity of NEP was only associated with an inhibition in cellular proliferation, demonstrating a clear enhancement of cellular differentiation. Furthermore, we investigated the effect of green tea polyphenols (EC and EGC) in combinations with caffeine or theophyline to illustrate 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 demonstrating that the induction of cellular NEP activity by these polyphenols was independent from the inhibition of cell proliferation. A presumably explanation for the observed enzyme induction effects might be based on the increase of cAMP level, which is induced by methyl-xanthines like caffeine and theophylline. According to the specification of the manufacturer, the green tea extract contained 47,5-52,5% polyphenols (EGCG app. 61%) 5-10% caffeine and 0,3 -1,2% theobromine analysed by HPLC.

The major tea catechins are EGCG, EGC, ECG, and EC. It can be qualified that methylxanthine contributes to the induction effects of specific NEP activity; however, they are not the main

Table 3: Influence of dibutylryl-cAMP, forskolin and rolipram on the specific enzyme activity and cell proliferation

*Significant difference to the specific enzymatic activity of the controls ($p < 0.05$)

responsible constituents for the enzyme induction. It is known that caffeine and other methylxanthine 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).

It has been reported that low concentration 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 *ß*-amyloid-induced neurotoxicity in both vitro and in vivo via blockade of adenosine A2A receptors (Dall'Igna et al 2003). In addition to its neuroprotective effects, caffeine has established cognitive-enhancing effects in humans, in particular to the elderly (Brice & Smith 2002). The cAMP level is a key factor for protection, growth and myelination of injured CNS axons in vivo and recovery of function (Pearse et al 2004). The elevating of cAMP levels is considered as therapeutic potential for the delay of Alzheimer's disease progression. The Alzheimer's disease related increases in the amyloid precursor protein C-terminal derivative (CTDs) can be reversed by treating it with the agents that increase intracellular cyclic adenosine monophosphate (cAMP), such as dibutyryl-cyclic-AMP, theophylline, and isoproterenol (Wolozin et al 1993).

Possibly, the increase of cellular NEP activity correlates to the increase of cAMP levels that could explain our results. In order 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 (PD4) isoform. Rolipram is able to restore the cAMP/cAMP-dependent protein kinase/cAMP regulatory element-binding protein (cAMP/PKA/CREB) pathway activity in the hippocampal and its long-term potentiation (LTP) (Vitolo et al 2002). We found that the treatment of SK-N-SH cells with dibutylryl-cAMP, forskolin and rolipram significantly induced the cellular NEP activity without affecting both ACE activity and cell 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.

Another explanation for the effect of theanine is reported by investigating the neuroprotective effect of theanine on postischemic neuronal death in field CA1 of the gerbil hippocampus using a transient ischemia model suggesting that one of the mechanisms of theanine responsible for its neuroprotective effect is associated with glutamate receptors as an antagonist (Kakuda 2002). In combination with the effects of green tea catechins as well as with the methylxanthines the total effect of the consumed green tea will be the result of a multiple pharmacological activity influencing a broad spectrum of neuronal mechanisms.

The possible physiological importance of the induction effect of green tea and its constituents on the specific cellular NEP activity can be discussed as neuroprotective effect. That is due to an improved degradation of A*ß* by NEP (Yasojima et al. 2001a, 2001b, Iwata et al. 2005) as well as by ACE (Hu et al. 2001) , which may affect the susceptibility to Alzheimer's disease and prevent the accumulation of amyloid plaques in vivo. In animal experiments was shown that the intake of catechins from green tea partially improves the morphologic and functional alterations that occur naturally in the brains (Unno et al 2004), a result which supports our conclusion resulting from in vitro experiments. Long-term intake of green tea-catechins, even at concentrations as low as those in typical green tea preparation, seems to be important, because cells are

constantly exposed to oxidative stress. Beside the antioxidative defense systems which might also prevent oxidative damage in the brain, the discussed NEP system contributes to the neuroprotective effect of green tea consumption.

4. Conclusions

Finally, based on our studies it seems that the green tea and its constituents induced only the specific activity of NEP without any significant effects on ACE. This induction effect depends not only on the differentiation improvement but also on the direct influence on NEP gene expression as well. Possibly, the daily consumption of green tea can diminish risk of Alzheimer's disease and age-related dementia by increasing the degradation of amyloid peptides.

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