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Induction of neutral endopeptidase and angiotensin-converting enzyme activity of SK-N-SH cells *in vitro* by quercetin and resveratrol

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Quercetin and resveratrol are weak inhibitors of neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE) activity of the neuroblastoma cell line SK-N-SH. The long term incubation of the cells for 4 days with quercetin, resveratrol and a combination of both substances in concentrations lower than necessary for inhibition of NEP and ACE activity induced the cellular enzyme activity of NEP and ACE associated with an inhibition of cellular proliferation. The long term treatment of neuroblastoma cells with quercetin and resveratrol enhanced the differentiation state of the cells. Taking into account the significance of NEP and ACE for the degradation of amyloid beta peptides, the effect of quercetin and resveratrol as constituents of red wine for a neuroprotective activity is discussed.

1. Introduction

Neutral endopeptidase (NEP) and angiotensin-converting enzyme (ACE) belong to the group of zinc-endopetidases acting as ectoenzymes on the outer surface of different cells. Both peptidases play a role in the metabolism of regulatory peptides, like substance P, bradykinin, enkephalins, natriuretic peptides and other vasoactive and hormonal peptides. Many attention has focused on the role of NEP in inactivating the natriuretic peptides *in vivo* [1]. The availability of circulating or tissue ANP may be increased by inhibiting its metabolic clearance by NEP-inhibitors. These agents induce a reduction in blood pressure and diuretic effects in animal models, and may become a new class of drugs for the clinical management of patients with hypertension and congestive heart failure. The effects of NEP-inhibitors are synergistically supported by inhibitors of ACE. By simultaneously inhibiting the renin-angiotensin-aldosterone system and stimulating the natriuretic peptide and kinin system, inhibitors of NEP and ACE reduce vasoconstriction, enhance vasodilatation, improve sodium/water balance, and, in turn, decrease peripheral vascular resistance and blood pressure and improve local blood flow [2]. Reports are published about the myocardial and vascular protection of red wine extract containing a complex mixture of polyphenols, flavonoids and related compounds like resveratrol [3]. The so called French paradox suggests that constituents of red wine possess pharmacological activities that favour protection against cardiovascular disease. A red wine extract inhibits both NEP and ACE activity [4]. Also other flavonoids and related natural phenolic compounds are able to act as inhibitors of both enzymes and by that to induce similar pharmacological effects [5]. Most of these investigations studied acute effects of inhibitors on isolated enzymes in biochemical experiments. To observe the inhibitory activity of natural compounds on enzymes in a more physiological environment we used in the following investigation cultivated living cells with intact ACE and NEP as ectoenzymes localized on the surface of the neuroblastoma cell line SK-N-SH. This cell system opened the possibility to study acute as well as long term effects of inhibitors on enzyme activity. We focused the investigations on the influence of quercetin and resveratrol, two natural compounds with assumed pharmacological activity present in red wine.

2. Investigations, results and discussion

Table 1 shows the results of enzyme inhibition by quercetin and resveratrol in the experiments when the test substances were immediately added to the assay mixture (acute inhibition) for 60 min (NEP) or 20 min (ACE). It was demonstrated that quercetin inhibited NEP activity stronger than ACE activity, whereas resveratrol was only a weak inhibitor of ACE without inhibitory activity against NEP. For comparison with both test compounds Table 1 shows the IC_{50} values of two standard inhibitors, lisinopril for ACE and phosphoramidon for NEP activity, indicating that SK-N-SH cells and the assay conditions are a right system for estimation of both enzyme activities on intact living cells. The high concentrations of quercetin and resveratrol necessary for 50% inhibition of both enzymes (IC_{50} values) characterize these compounds as weak inhibitors compared to phosphoramidon and lisinopril.

Taking into account that quercetin and resveratrol are taken up daily in small amounts with vegetables, fruits or

Table 1: IC_{50} values of test compound and standard inhibitors

	IC_{50} NEP	IC_{50} ACE
Phosphoramidon	8.9 nM	no inhibition
Lisinopril	no inhibition	0.9 nM
Quercetin	148 μM	226 μM
Resveratrol	no inhibition	486 μM

Table 2: Influence of long term treatment of SK-N-SH cells on cellular enzyme activity

Treatment	NEP activity in nmol/min per 100.000 cells	ACE activity in nmol/min per 100.000 cells
Control	28.3 ± 4.1	67.7 ± 11.3
Quercetin 1 μM	29.4 ± 3.1	54.9 ± 3.2
Quercetin 2.5 μM	91.7 ± 12.9*	123.7 ± 8.3*
Resveratrol 1 μM	31.6 ± 5.5	n.d.
Resveratrol 2.5 μM	29.3 ± 2.2	n.d.
Resveratrol 5 μM	32.6 ± 7.4	78.7 ± 8.0
Resveratrol 10 μM	55.5 ± 11.4*	154.9 ± 11.0*
Quercetin 1 μM + Resveratrol 5 μM	42.3 ± 3.7*	90.4 ± 4.6*
Quercetin 2.5 μM + Resveratrol 10 μM	47.6 ± 5.7*	110.4 ± 8.3*

n.d. not determined
* significant difference to control, U-test, $P < 0.05$

red wine the question arose if such small concentrations of both substances influence the gene expression of NEP and ACE detectable by determination of cellular enzyme activity. Therefore we investigated the influence of quercetin, resveratrol and a mixture of both in small concentrations on both enzymes after long term treatment of the cell cultures for 4 days. The results are shown in Table 2. Obviously, there is a threshold concentration for both substances after that the cellular activity of ACE and NEP is induced. These concentrations are much lower than the IC_{50} values determined in the inhibition experiments before (Table 1), and indicating that long term incubation over 4 days with these inhibitory active substances caused an up-regulation of the cellular enzyme activity. Interestingly, the combination of 1 μM quercetin with 5 μM resveratrol induced significantly NEP and ACE cellular activity, although both compounds did not inhibit the enzymatic activity nor induce the enzymes when the cells were incubated with each substance in this low concentration alone. Treatment with 2.5 μM quercetin and 10 μM resveratrol, respectively, for 4 days induced also the cellular enzyme activity of NEP and ACE, by combination of both substances no further increase of the enzyme induction rate was observed. The optimal concentration of a mixture seems to be lower than 2.5 μM quercetin and 10 μM resveratrol for induction of NEP and ACE cellular enzyme activity.

A possible explanation for the observed enzyme induction effects might be based on the relation between cellular differentiation and proliferation. The determination of cellular growth under the influence of quercetin, resveratrol and a combination of both is shown in Figs. 1 and 2. Any inhibition of proliferation is associated with an increase in cellular enzyme activity (Table 2), which is equate with an enhancement of cellular differentiation. In all cell types the processes of differentiation and proliferation are mutually exclusive. A non-toxic inhibition of cell proliferation enhances in most cases the cellular differentiation state [6]. The same correlation was observed investigating the differentiating effect of retinoic acid on matrix metalloproteinase-2 (MMP-2) expression, the proliferative activity was inversely correlated to MMP-2 expression in SK-N-SH cells [7].

Also for NEP and ACE it has been reported that in different cell types the up-regulation of the cellular enzymatic activity was correlated with an enhanced differentiation state and with an inhibition of cellular proliferation [8, 9].

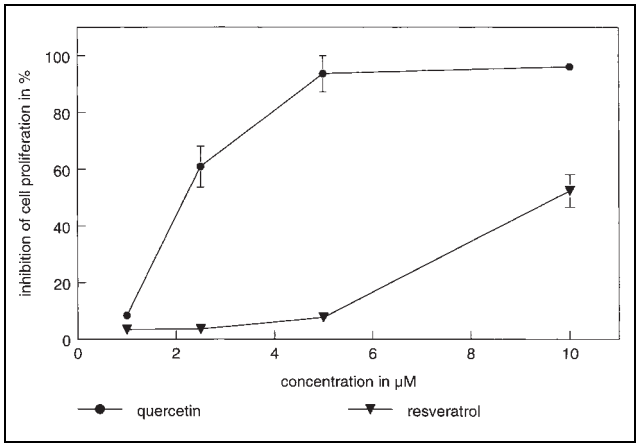

Fig. 1: Influence of quercetin and resveratrol on proliferation of SK-N-SH cells. The cells were incubated with the indicated concentration 24 h after plating for further 4 days.

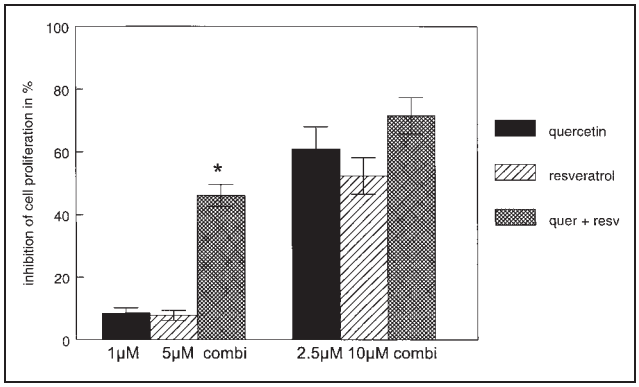

Fig. 2: Influence of selected concentrations of quercetin, resveratrol and a combination (combi) of both substances (quer + resv) on proliferation of SK-N-SH cells. The cells were incubated with the indicated concentration 24 h after plating for further 4 days.

* significant difference to treatment with 1 μM quercetin and 5 μM resveratrol

The enhanced cellular enzyme activities are by that a proof for the assumption that the inhibition of cell proliferation was not the result of a cytotoxic effect.

Our originally thoughts that long term inhibition of NEP and ACE might inhibit the expression of both enzymes or lead to a down-regulation of cellular enzyme activity were not confirmed by the results obtained. The vasoprotective effect of daily red wine consumption seems to be not the result of a down-regulation of both enzymes NEP and ACE. In contrast to the used cellular model with proliferating tumour cells, *in vivo* most of the cells are non-proliferating or arrested in the G_0 cell cycle phase. If quercetin and resveratrol also enhance the cellular enzyme activity under such conditions the differentiation state of the cells has to be clarified in additional experiments with differentiated quiescent cells. On the other hand our results have shown that quercetin, resveratrol and especially the combination of both substances enhance the differentiation state of proliferating cells, an effect which has been described also for other cells *in vitro* [10, 11]. NEP is also known as CALLA (common acute lymphoblastic leukaemia antigen) or CD10 [12] and located on the surface of leukocytes. Mature quiescent lymphocytes show enzyme activity higher than premature proliferating hematopoietic cells [13] indicating a correlation between cellular NEP activity and immune response.

NEP and ACE are also present in the brain. Beside the metabolisms of neuropeptides, both are also included in the degradation of amyloid beta peptides and by that associated with pathogenesis of Alzheimers disease [14, 15]. Under this point of view the up-regulation of NEP and ACE by low concentrations of quercetin and resveratrol can be discussed as neuroprotective effect, because an enhanced degradation of the amyloid beta peptides may affect the susceptibility to Alzheimers disease and preventing the accumulation of amyloid plaques *in vivo* [16].

The occurrence of both quercetin and resveratrol in red wine and the fact that red wine constituents have a neuroprotective ability [17] might support this idea. Beside their established antioxidant properties both substances seem to influence also the expression of neuropeptidases included in the degradation of amyloid peptides and diminish the risk of accumulation of plaque forming peptides.

Summarizing, quercetin and resveratrol induce up-regulation of NEP and ACE by inhibition of cell proliferation in a non-toxic manner and by that they act obviously as cell differentiation enhancing compounds.

3. Experimental

3.1. Materials

Quercetin, resveratrol, Suc-L-Ala-L-Ala-L-Phe-7-amido-3-methylcoumarin (SAAP-AMC), Aminopeptidase N (APN) and phosphoramidon were obtained from Sigma, Hip-L-His-L-Leu was purchased from Bachem. Lisinopril was a gift of Schering & Plough (USA). The cell culture media and fetal calf serum (FCS) were obtained from Biochrom.
Test compounds were dissolved in DMSO and than diluted. The influence of DMSO (lower than 0.1%) was considered in controls.

3.2. Cell culture

SK-N-SH cells, human neuroblastoma cells, were obtained from 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 until confluence and than cells were seeded for the enzymatic experiments in 24-well plates (inoculum 100.000 cells/well).
For investigating the acute effect of test compounds the cells were cultivated for 5 days until confluence in the 24-well plates, the medium was then removed and replaced by NEP- or ACE-assay solution plus the compound to be tested.
For the long term experiments 24 h after plating the cells were incubated with the indicated concentration of the test compound and cultivated for further 4 days. Then the medium was removed and replaced by NEP- or ACE-assay solution.

3.3. Determination of ectoenzyme activities

3.3.1. NEP activity

The assay was performed according to Bormann et al. [5]. Briefly, 50 µl of SAAP-AMC-solution (50 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. To investigate the acute inhibitory activity of a test substance this compound was simultaneously added. The cells with the assay solution were incubated for 60 min at 37 °C. The NEP reaction was stopped by addition of 50 µl phosphoramidon (50 µM). 400 µl 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 and incubated again for 60 min at 56 °C. The reaction was terminated by addition of 800 µl acetone. The fluorescence of the released AMC was measured (excitation 367 nm, emission 440 nm). To calculate the enzyme activity a calibration curve with AMC was determined.

3.3.2. ACE activity

The assay was performed according to Bormann et al. [5]. Briefly, 20 µl substrate solution (24 mM Hip-L-His-L-Leu in water) plus 260 µl phosphate buffer (83 mM $K_2HPO_4 \times 3\,H_2\,O$ + 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 the assay solution were incubated for 20 min at 37 °C. 250 µl 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, fresh prepared). Under exclusion if light this mixture was incubated for 10 min at room temperature and terminated by addition of 300 µl 2 N HCl. The fluorescence of the formed product was measured (excitation 360 nm, emission 500 nm). To calculate the enzyme activity a calibration curve with L-His-L-Leu was determined.
The inhibition rates were calculated in both enzymatic assays in comparison to controls without inhibitor during the enzymatic reaction, considering the absorbance of fluorescence light by test compounds. IC_{50}-values were determined from dose-effect curves by linear regression. Enzyme activities were calculated in nmol/min per 100.000 cells.

3.4. 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 number values represent the mean of at least three independent experiments with two parallel samples.

3.5. Statistics

All values in the tables and figures are expressed as mean ± standard error of the mean of at least 3 independent experiments with 7 parallel samples. Wilcoxon's *U*-test was used to test significance ($P < 0.05$).

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