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Original scientific paper

Growth Modulation of Human Cells *in vitro* by Mild Oxidative Stress and 1,4-Dihydropyridine Derivative Antioxidants

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A B S T R A C T

Reactive oxygen species and lipid peroxidation products are not only cytotoxic but may also modulate signal transduction in cells. Accordingly, antioxidants may be considered as modifiers of cellular redox signaling. Therefore, the effects of two novel synthetic antioxidants, analogues of 1,4-dihydropyridine derivatives, cerebrocrast and Z41-74 were analysed in vitro *on human osteosarcoma cell line HOS, the growth of which can be modulated by lipid peroxidation. The cells were pretreated with either cerebrocrast or Z41-74 and afterwards exposed to mild, copper induced lipid peroxidation or to 4-hydroxynonenal (HNE), the end product of lipid peroxidation. The results obtained have shown that both antioxidants exert growth modulating effects interfering with the lipid peroxidation. Namely, cells treated with antioxidants showed increased metabolic rate and cell growth, thereby attenuating the effects of lipid peroxidation. Such biomodulating effects of cerebrocrast and Z41-74 resembled growth modulating effects of HNE, suggesting that the antioxidants could eventually promote cellular adaptation to oxidative stress interacting with redox signaling and hydroxynonenal HNE-signal transduction pathways. This may be of particular relevance for better understanding the beneficial role of hydroxynonenal HNE in cell growth control. Therefore, cerebrocrast and Z41-74 could be convenient to study further oxidative homeostasis involving lipid peroxidation.*

Key words: *oxidative homeostasis, growth regulation, hormesis, reactive aldehydes, HNE, bone cells*

Introduction

Oxidative stress is a process where reactive oxygen species (ROS) are formed in excess and the steady-state equilibrium between formation and elimination of ROS is disturbed[1]. Excessive production of ROS, often caused by transition metals (copper and iron) in the vicinity of cellular membranes induces lipid peroxidation, having as a result production of reactive aldehydes, namely malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acrolein[2]. One of the most intensively studied aldehydes is HNE, a major lipid peroxidation product of ω-6 polyunsaturated fatty acids (e.g. linoleic and arachidonic acid), nowadays considered as a major bioactive marker of lipid peroxidation[3].

Depending on its concentration, HNE can be considered as cell growth modulator under physiological concentrations and as inhibitor of cell growth under high, pathological concentrations[4]. Usually, 1 μM HNE is referred as a physiological concentration of the aldehyde, 10 μM as a supraphysiological, while higher values are pathological with 50 μM considered as LD50 for majority of the cell types treated *in vitro*[2]. Exposure to pathological concentrations of HNE causes strong inhibition of several cellular functions; mitochondrial respiration, DNA, RNA and protein synthesis[2,5]. Recently, it has been demonstrated that HNE acts as a signaling molecule in

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pathological but also in physiological conditions[3,6]. The presence of physiological concentrations of HNE (1 μM) could depend on the presence of serum factors, but not serum albumin which is known to couple with HNE thus decreasing the effects of the aldehyde[7–9]. HNE can induce *in vitro* proliferation of malignant cells, such as HeLa, as well as of non-malignant cells, like aortic smooth muscle cells, interfering with the activity of cytokines thereby acting as a growth-regulating factor[5,10]. Hence, HNE modulates also cell proliferation of human osteosarcoma cells (HOS cell line), which often show functional differentiation *in vitro* into osteoblasts and are considered as convenient model to study human bone cells[11].

While these biomedical activities are not commonly well known, oxidative stress is usually considered as pathological background of numerous diseases. Consequently, antioxidants are often considered as substances that counteract harmful effects of free radicals thereby preventing the onset of various diseases. However, recent concepts of oxidative homeostasis suggest also beneficial effects of mild oxidative stress and consider antioxidants as biological modifiers of oxidative homeostasis[12,13]. Recently, many new antioxidants have been synthesized, which possess potent free radical scavenging capacities. However, solubility, permeability, stability, and pharmacokinetic parameters like absorption, distribution, metabolism and excretion are the important properties that affect the dose efficiency of antioxidants[14]. Therefore, synthetic antioxidants should be tested *in vitro* and *in vivo* before being considered for human use, as in case of other potential medical remedies. In the present study we tested *in vitro* effects of two derivatives of 1,4-dihydropyridine using human osteosarcoma cell line (HOS), which is known to be sensitive not only to the cytotoxic but also to the growth modulating effects of HNE acting as mediator of oxidative homeostasis and of lipid peroxidation[15].

Z41-74 is an N,N,N-trimethylammonium analogue of the 1,4-dihydropyridine compound described by Urtti et al.[16], while cerebrocrast (CER) exerts broad spectrum of protective properties (neuroprotective, memory-enhancing, anti-ischemic, cardioprotective, anti-diabetic, anti-inflammatory)[17,18]. This antioxidant prevents cell death, free radical production and decrease in mitochondrial membrane potential in MPP^+ neurotoxicity model (cerebellar granule cells)[19]. Oxygen-glucose deprivation cell damage is also prevented by cerebrocrast[20]. Cerebrocrast has an anti-inflammatory effect[21], and was shown to protect the immune system in streptozocin-induced diabetic rats from streptozocin toxicity, preventing thymus and lymph node mass loss, as well as pancreatic beta cell damage[22].

Aiming to see if CER and/or Z41-74 could be involved in growth modulation under mild oxidative stress and lipid peroxidation, HOS cells were cultured in the presence of CER or Z41-74 and were exposed to copper-induced lipid peroxidation or to the ranging concentrations of HNE.

Materials and Methods

Compounds

Tested compounds denoted cerebrocrast 4-(2-difluoromethoxyphenyl)-2,6-dimethyl-3,5-bis[(2-propyloxy)ethoxycarbonyl]-1,4-dihydropyridine (Mw=511.6) and N,N'-[3,5-bis-methyloxycarbonyl-4-phenyl-1,4-dihydropyridin-2,6-diyl]dimethylene]bis-N,N,N-trimethylammonium dibromide (Z41-74) (Mw=577.37) were synthesized at the Latvian Institute of Organic Synthesis. All of these compounds are studied as potential antioxidants and according to high performance liquid chromatography (HPLC) data are at least of 98% purity.

Cell culture

The human osteosarcoma (HOS) cell line was obtained from the American Type Culture Collection (ATCC). Cells were maintained in DMEM with 10% (v/v) FCS (Sigma, USA) in an incubator (Heraeus, Germany) at 37 °C, with a humid air atmosphere containing 5% CO_2. The cells of semiconfluent cultures were detached with 0.25% w/v trypsin solution and viable cells were counted on Bürker-Türk hemocytometer upon trypan blue exclusion.

4-Hydroxynonenal

HNE was obtained from Karl-Franzens University in Graz, Austria in the form of HNE-DMA (dimethylacetal) 1 mg/mL preserved in chloroform and kept at –20 °C. Before use HNE was activated as described before[3].

^{3}H-thymidine (^{3}H-TdR) incorporation assay

HOS cells were seeded in 96 microwell plates at density of 2×10^4 *per* well, and were allowed 24 h to adhere. After adhering period, cells were treated with CER and Z41-74 at concentrations 0.1, 1 and 10 μM for 1h, when $CuSO_4$ was added at concentrations of 20, 50 and 100 μM. After 48h, ^{3}H-thymidine (1 μCi/well, Amersham, USA) was added to each well and left for additional 24h, as described before[24].

Using the above mentioned concentration range of $CuSO_4$ allowed induction of moderate lipid peroxidation and the insight into the growth modulating activities of the antioxidants under such a mild oxidative stress.

MTT assay

HOS cells were seeded in 96 microwell plates at density of 2×10^4 *per* well. After allowing cells to adhere for 24 h, media were removed and cells were treated with CER and Z41-74 at concentrations 0.1, 1 and 10 μM for 1h, when HNE was added in concentrations of 1, 10 and 100 μM. After 24h incubation period, MTT assay (EZ4U, Biomedica Austria) was done as described before, since it reflects well mitochondrial metabolic activity of the cells thus reflecting their growth[23].

Using the above mentioned concentration range of HNE allowed the insight into the growth modulating activities of the antioxidants under mild oxidative stress.

Statistics

All assays were carried out in quadruplicates. Significance was calculated according to the Student's t-test. The values of $p<0.05$ were considered as statistically significant.

Results

The effects of CER and Z41-74 on the $CuSO_4$-affected cell proliferation

The influence of antioxidant compounds CER and Z41-74 in combination with $CuSO_4$ as oxidant is shown on Figure 1. Oxidant $CuSO_4$ alone significantly decreased cell proliferation in a concentration-dependent manner ($p<0.05$), with maximum inhibition of 30% observed after treatment with the highest concentration of $CuSO_4$ used (100 mM). CER alone only at the concentration of 10 mM caused significant increase in cell proliferation ($p<0.05$). On the other hand, Z41-74 alone significantly increased cell proliferation in concentrations of 0.1 and 1mM respectively ($p<0.05$). The highest concentration of Z41-74 used (10 mM), did not enhance the proliferation of the cells ($p>0.05$).

Negative effects of $CuSO_4$ on cell proliferation were reduced in a concentration-dependent manner by CER at all concentrations used ($p<0.05$). At the lowest $CuSO_4$ concentration used, negative effect of the stressor were completely abolished by CER and even overwhelmed to level of significant stimulation of cell proliferation. Decrease in cell proliferation caused by the highest $CuSO_4$ concentration was suppressed by all concentrations of CER used ($p<0.05$). Accordingly, neither 20 nor 50 μM $CuSO_4$ did significantly affect the stimulation of cell proliferation caused by CER.

Z41-74 was more effective than CER at the lowest dose (0.1 μM), while opposite was observed for the highest concentration of the antioxidants (10 μM), in which case CER was more efficient than Z41-74.

The effects of CER and Z41-74 on the HNE-affected cell growth

The effects of HNE and tested compounds, CER and Z41-74, on the growth of HOS cells are shown on Figure 2. HNE enhanced cell growth at concentrations of 1 and 10 μM, while at concentration of 100 mM HNE significantly ($p<0.05$) inhibited the cell growth. Both compounds, CER and Z41-74, significantly increased cell growth in all used concentrations (0.1, 1 and 10 μM), although CER was shown to be more effective.

CER, in combination with 1 and 10 μM HNE showed modulation of cell growth. These combinations of CER and HNE significantly increased cell growth in comparison to cells treated with the same concentrations of HNE but without CER ($p<0.05$). Still, the stimulating effects of CER and HNE did not reach the level of stimulation by CER alone. At 100 μM HNE, CER was not able to diminish the inhibition of cell growth caused by HNE.

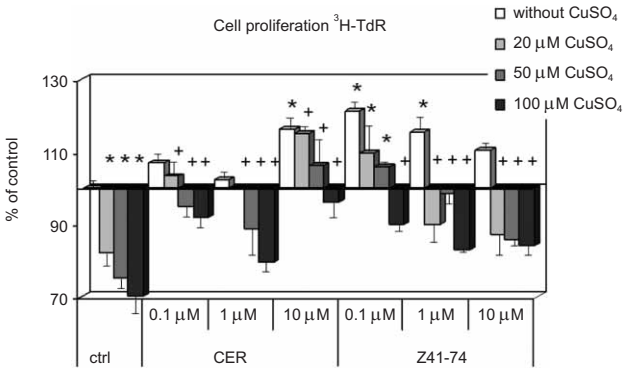

*Fig. 1. Influence of novel compounds CER and Z41-74 on HOS cell proliferation – 3H-TdR incorporation assay. Cells were treated with different concentrations of CuSO4 alone (denoted as ctrl on Figure), or in combination with either cerebrocrast (CER) or Z41-74. Results were expressed as mean±SD in percentage of untreated cells (cells cultured without any stressor or compound tested). *significantly different compared to untreated cells (without CuSO4 and without antioxidant) + significantly different compared to the same CuSO4 concentration but without antioxidant.*

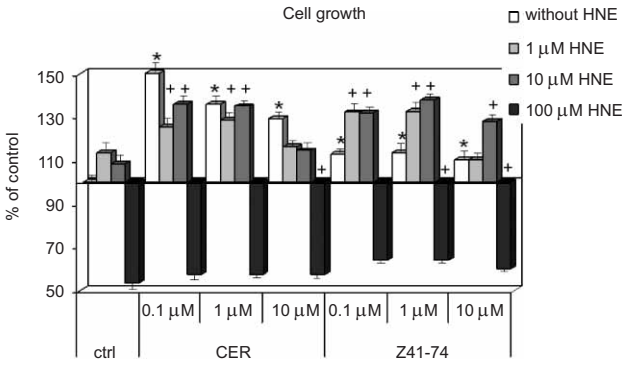

*Fig. 2. Influence of novel compounds CER or Z41-74 in combination with HNE on cell viability determined by MTT assay. Cells were treated with different concentrations of HNE alone (denoted as ctrl on Figure), or in combination with either cerebrocrast (CER) or Z41-74. Results were expressed as mean ± SD in percentage of untreated cells (cells cultured without any stressor or compound tested). * significantly different compared to untreated cells (without HNE and without antioxidant) + significantly different compared to the same HNE concentration but without antioxidant.*

Z41-74 showed different reaction pattern than CER. Namely, Z41-74 alone increased cell growth at the same level regardless of concentration used. In combination with 1 and with 10 mM HNE the cell growth was additionally increased in the presence of 0.1 or 1 μM Z41-74 (in both cases, $p<0.05$). The highest concentration of Z41-74 used (10 μM) only in combination with 10 μM HNE significantly increased cell growth ($p<0.05$). Similar to CER, Z41-74 also did not modify the growth inhibition of HOS cells treated with 100 μM HNE.

Therefore, major difference between the two antioxidants was observed in respect to the interference with HNE because CER showed better stimulatory effect when

added alone whereas Z41-74 showed better growth stimulation when combined with HNE.

Discussion and Conclusion

Oxidative stress and lipid peroxidation are considered usually only as negative factors of cell growth regulation. However, recent findings provide better insight into the role of oxidative stress in physiological processes introducing the term of oxidative homeostasis[12]. Namely, oxidative homeostasis can be considered as fundamental mechanism of hormesis, implying adaptation to stress upon exposure to mild stress, eventually resulting in increased resistance to the more aggressive stress[25]. Thus, the purpose of our study was to evaluate possible interactions between different concentrations of lipid peroxidation agents causing mild oxidative stress with novel 1,4-dihydropyridine derivatives, which might exert antioxidant bioactivities.

We have used two lipid peroxidation mediators, $CuSO_4$, well known oxidizing agent[26,27] and HNE, the end product of lipid peroxidation product, which acts as signaling and cell growth regulating factor[3,5,7,8,15,28]. Even $CuSO_4$ may be considered to be effective due to the activity of copper ions that induce lipid peroxidation resulting in production of reactive aldehydes, such as HNE. In our study we have used ^{3}H-thymidine incorporation assay to monitor effects of $CuSO_4$ on cell proliferation due subtle differences in cell growth caused by mild oxidative stress. Addition of novel antioxidant compounds, CER and Z41-74, under such conditions modulated cell proliferation. The basis of the stimulating effects on cell proliferation could lie in the interactions between copper, serum albumin and antioxidants, while according to our knowledge none of the antioxidant substances tested is able to bind and inactivate directly copper or HNE. It is certain however that higher doses of copper (>50 μM) cause oxidation of serum with a delay, but also to a lesser degree than lower doses due to presence of antioxidants in the serum[29]. These interactions could result in cell adaptation to mild oxidative stress consequentially increasing tolerance to oxidative stress, i.e promoting hormesis. That might be especially important for the cells and tissues which have physiologically present high amounts of HNE and for which HNE seems to play certain physiological, not only pathological roles[11,12,30,31]. Taking into account that lipid peroxidation end products, especially HNE, affect signaling pathways relevant for the bone regeneration, we have tested effects of HNE on growth of HOS cells directly by adding HNE to HOS cells and indirectly by causing lipid peroxidation with $CuSO_4$ that is followed by HNE formation[26]. To enhance HNE effects as a cell growth modulator, we combined it with CER and Z41-74, respectively.

Obtained results indicated that the growth stimulating concentrations of HNE (ranging from 1 to 10 μM) could be enhanced either by CER or by Z41-74, indicating possible role of these compounds in modulation of cell growth through modulation of redox signaling pathways. Namely, HNE is a cell growth regulating factor, which affects expression of several proto-oncogenes and interferes with bioactivities of cytokines, such as TGF beta, PDGF, FGF and EGF. The effects of HNE are therefore very complex, but their concentration dependence *in vitro* usually points to the growth stimulation if used at low concentrations and suppression when supraphysiological or toxic concentrations are used. Accordingly, we may assume that the tested antioxidants interfere with the cellular redox signaling and growth regulating effects of HNE. Furthermore, our findings suggest differential influence of the tested substances in respect to the concentration dependent bioactivities of HNE, which might further open possibilities of the differential usage of the tested substances and HNE for the treatment of different cell types, such as malignant and non-malignant counterpart cells aiming to modulate their growth in desirable way.

This could be of particular importance for better understanding and control of bone cell growth since it was previously shown that HOS cells are convenient model to study not only cytotoxic but also growth modifying effects of lipid peroxidation[11]. Namely, HNE itself modulates HOS cell growth by interaction with several cytokines present in serum, especially EGF[15]. Also, HNE was shown to display dose-dependent effect modulated by binding to serum proteins. HNE-serum protein adducts reduce interactions between HNE and cell membrane thereby diminishing possible cytotoxic effects of HNE[7,15]. Accordingly, CER and Z41-74 might affect growth regulating effects of HNE by interfering with its affinity to bind to humoral as well as to cellular proteins; however, this assumption has to be further verified. Combining the results of two complementary methods used in this study, measuring cell proliferation and cell mitochondrial activity, it can be concluded that both of the tested compounds, CER and Z41-74 acted as biological response modifiers, modulating cell adaptation to mild oxidative stress induced either by $CuSO_4$ or by HNE.

These results might be relevant for human medicine, especially in context of bone and damaged tissue healing and should be further studied considering a possibility of HNE involvement in signaling pathways of tissue regeneration.

Thus, our results showed that both CER and Z41-74 may be efficient in regulation of oxidative homeostasis and might therefore support the hormesis signaling based on HNE, which shall be further studied, especially in respect to the possible differences in regulation of oxidative homeostasis between malignant and non-malignant cells.

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MODULACIJA RASTA LJUDSKIH STANICA *IN VITRO* BLAGIM OKSIDACIJSKIM STRESOM I ANTIOKSIDACIJSKIM DERIVATIMA 1,4-DIHIDROPIRIDINA

SAŽETAK

Reaktivni kisikovi radikali i produkti lipidne peroksidacije osim citotoksičnog učinka mogu utjecati i na prijenos signala u stanici. Antioksidansi pak mogu biti modulatori bioloških aktivnosti koje su posljedica oksidacijskog stresa. Stoga smo u ovom radu istražili ulogu dva antioksidansa, derivata 1,4-dihidropridina, nazvanih cerebrokrast ili Z41-74 na *in vitro* rast humanih osteosarkomskih stanica, model humanih osteoblasta, osjetljivih na oksidacijski stres i lipidnu peroksidaciju. Stanice su bile sat vremena izložene trima različitim koncentracijama sintetskih antioksidansa, cerebrokrasta ili Z41-74, nakon čega su izložene niskim koncentracijama bakrenog sulfata ili 4-hidroksinonenala, krajnjeg produkta lipidne peroksidacije. Oba antioksidansa pokazala su pozitivan učinak na rast stanica. Naime, stanice prethodno izložene antioksidansima bolje su rasle i imale su povećanu metaboličku aktivnost nego stanice koje su bile izložene samo oksidacijskom stresu. Ovaj biomodulacijski učinak cerebrokrasta i Z41-74 podsjeća na biomodulacijske učinke 4-hidroksinonenala, stoga može biti posljedica stanične adaptacije na oksidacijski stres s obzirom da antioksidansi djeluju na redoks procese i signalne putove 4-hidroksinonenala. To bi moglo biti posebno značajno za razumijevanje uloge 4-hidroksinonenala u cijeljenju tkiva. Dakle antioksidansi, cerebrokrast i Z41-74 bi mogli kontrolirati procese oksidacijske homeostaze što bi trebalo potvrditi daljnjim istraživanjima.