Bulgarian Chemical Communications, Volume 48, Number 1 (pp. 33 – 37) 2016

Comparative study of the antioxidant activity of some nociceptin analogues

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Received October 21, 2014, Revised October 16, 2015

Nociceptin (N/OFQ(1-13)NH_2) suppresses the neurogenic inflammation during which enhanced reactive oxygen species (ROS) production is detected. So the question arises about a possible antioxidant mechanism of this suppression. The aim of this study was to investigate and compare the antioxidant effects of nociceptin and its new synthesized structural analogues, in which the lysine (Lys) at position 9 was substituted with ornithine (Orn), diaminobutanoic acid (Dab), diaminopropanoic acid (Dap) or canavanine (Cav). The peptides were tested in concentrations between 1 μM and 100 μM against hydroxyl radicals ($^{\bullet}OH$) and superoxide anion radicals ($^{\bullet}O_2^-$).

The $^{\bullet}OH$ and $^{\bullet}O_2^-$ were generated *in vitro*. Deoxyribose (DR) was used as a detector of $^{\bullet}OH$ radicals. The DR degradation was measured in terms of the formation of thiobarbituric acid reactive substances, which were quantified spectrophotometrically. Superoxide anion radicals were generated photochemically and O_2^--produced nitro-blue tetrazolium (NBT) reduction was measured.

The results showed that in concentrations up to 10 μM neither nociceptin nor its analogues inhibited the $^{\bullet}OH$ - provoked DR degradation; in concentration of 10 μM only [Cav^9]N/OFQ(1–13)NH_2 suppressed the $^{\bullet}O_2^-$-provoked NBT-reduction. However, the higher concentration (100 μM) exerted inhibitory effects in both ROS generating systems. These effects were weakest in presence of [Dap^9]N/OFQ(1–13)NH_2 and strongest in presence of [Cav^9]N/OFQ(1–13)NH_2.

In conclusion, only [Cav^9]N/OFQ(1-13)NH_2 possesses certain antioxidant activity, whereas the antioxidant capacity of the other tested neuropeptides was relatively poor, which makes unlikely an antioxidant mechanism for suppression of inflammation.

Key words: antioxidant properties; nociceptin; nociceptin analogues.

INTRODUCTION

Nociceptin, also known as orphanin FQ (N/OFQ), is a neuropeptide, structurally related to opioid peptides. However, N/OFQ does not bind to classical opioid receptors [1]. It interacts with its own receptor - N/OFQ peptide (NOP) receptor (previously known as opioid receptor like-1, ORL-1). The NOP receptors are coupled to a G-protein. They are located on primary sensory neurons projecting to most peripheral organs and tissues, and act as regulators of neurogenic inflammation. As a lot of diseases are accompanied by inflammation, the N/OFQ effects are intensively investigated. In addition diverse analogues of N/OFQ are synthesized. The impact of N/OFQ and its analogues on inflammation *in vivo* is hard to be predicted, because of its controversial effects on different systems involved in the inflammation reaction. Helyes et al. [2] have found that N/OFQ suppresses the release of the pro-inflammatory mediators: substance P and calcitonine gene-related peptide from the primary sensory neurons. The investigations of Zamfirova et al. [3] have ascertained that N/OFQ, applied intraperitoneally also suppressed the carrageenan-induced inflammation of rat paw. Since it is well known that the inflammatory process is accompanied by increased production of reactive oxygen species (ROS), the question arises whether the N/OFQ is able to inhibit the neurogenic inflammation not only through activation of peripheral receptors (and subsequent NOP reduced release of pro-inflammatory mediators), but also by influencing the production of ROS. So the aim of our study was to test and compare the antioxidant capacity of the nociceptine tridecapeptide template, N/OFQ(1–13)NH_2, and some of its analogues against superoxide anion radicals ($^{\bullet}O_2^-$) and hydroxyl radicals ($^{\bullet}OH$), both generated *in vitro*, and to attempt to specify whether the antioxidant activity is due to scavenging or chelating nature of the substances. The peptide analogues tested in the

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present study differed from N/OFQ(1–13)NH_2 in their structure by only one amino acid, in particular the Lys at position 9 was substituted with Orn, Dab, Dap or Cav. These analogues have been synthesized because Lys[9,13], as well as Arg[8,12] are crucial for receptor occupation. Probably, they interact with the amino acids present in the second extracellular loop of the NOP-receptor [4]. Thus the substitute amino acids (Orn, Dab, Dap) were chosen in order to diminish the distance of the side-chain amino group from the peptide backbone (Fig.1). The canavanine (Cav) ((2S)-2-amino-4-{[(diaminomethylidene)amino]oxy}butanoic acid) analogue was included in this study, because it possesses a guanidinium group (Fig 1) which could be responsible for a direct antioxidant effect. In addition, the nociceptine (a heptadecapeptide) has been synthesized as tridecapeptide, since it is sufficient for exercising its full biological activities [5] in *in vivo* experiments.

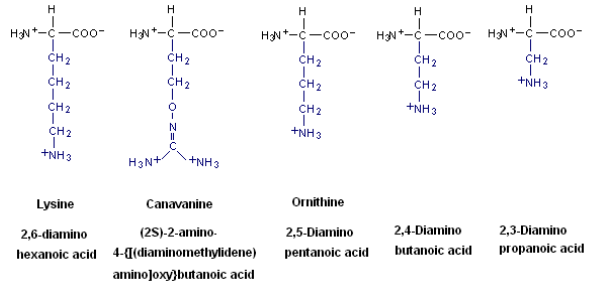

Fig. 1. Amino acids chosen for substitution of Lys at position 9 in the N/OFQ(1–13)NH_2 template.

EXPERIMENTAL

Materials

N/OFQ(1-13)NH_2, [Dab[9]]N/OFQ(1-13)NH_2, [Dap[9]]N/OFQ(1-13)NH_2 and [Orn[9]]N/OFQ(1-13)NH_2 were synthesised at the Department of Organic Chemistry, University of Chemical Technology and Metallurgy (Sofia, Bulgaria); [Cav[9]]N/OFQ(1-13)NH_2 was synthesised at the Department of Molecular Design and Biochemical Pharmacology, Institute of Molecular Biology, Bulgarian Academy of Sciences (Sofia, Bulgaria). The synthesis procedure is described in [6]. Diethylene triamine pentaacetic acid (DTPA), 2-thiobarbituric acid, riboflavin, methionine, nitro-blue tetrazolium and deoxyribose were obtained from Sigma-Aldrich (Germany). All other reagents were of analytical grade; all solutions were prepared with de-ionized water.

Methods

Superoxide anion radicals ($^{\bullet}O_2^-$) were generated photochemically in a medium containing: 50mM potassium phosphate buffer, pH 7.8; 1.17×10^{-6}M riboflavin; 0.2mM methionine; 2×10^{-5}M KCN and 5.6×10^{-5}M nitro-blue tetrazolium (NBT). NBT was reduced by $^{\bullet}O_2^-$ to a blue formazan product, which was measured at 560 nm [7] in the presence of increasing peptide concentrations.

The degradation of DR (a detector of $^{\bullet}OH$ radicals) was measured in terms of the formation of thiobarbituric acid reactive substances (TBARs), according to the method of [8]. The TBARs were quantified spectrophotometrically. Hydroxyl radicals ($^{\bullet}OH$) were generated in a system containing either (a) 10 mM potassium phosphate buffer, pH 7.4; 0.1 mM $FeSO_4$, 0.5 mM H_2O_2 and 2 mM deoxyribose (DR) or (b) 10 mM potassium phosphate buffer, pH 7.4; 0.1 mM DTPA-Fe^{2+} (the DTPA-Fe^{2+} complex was prepared according to [9]), 0.5 mM H_2O_2 and 2 mM deoxyribose (DR). After 30-min incubation at 37°C in the presence of increasing concentrations of the tested drugs, the reactions were stopped by the addition of catalase (20 μg/ml). After addition of 0.2 ml 2.8% trichloroacetic acid, 0.1 ml 5 N HCl and 0,2 ml thiobarbituric acid (2% w/v in 50 mM NaOH), the samples were heated at 100°C for 15 min to develop the color. After cooling, the absorbance was read at 532 nm against blank sample (without drug); A_{600} was considered to be a non-specific base-line drift and was subtracted from A_{532}.

Statistics

Data were reported as mean (SD). Testing for significant differences between mean values was analyzed by using the Student's t-test, $P < 0.01$ being accepted as the minimum level of statistical significance for the differences in population mean values.

RESULTS

In the present study two distinct $^{\bullet}OH$ generating systems with DR as a detector molecule were used. In the first $^{\bullet}OH$-generating system containing only metal ions without chelator (DTPA) and H_2O_2, we did not detect any changes in the rate of DR degradation (Figure 2A) independently of the peptide concentration.

The effects of increasing concentrations (0.001-1 mM) of N/OFQ(1-13)NH_2 and its structural analogues in the $^{\bullet}OH$-generating system containing metal chelator (DTPA), are presented on Figure 2B. In concentrations up to 100 μM the tested peptides

did not exert any protective effect, except [Cav^9]N/OFQ(1-13)NH_2, which at a concentration of 50 μM diminished the DR degradation by about 10%. The presence of 100 μM of peptide in the reaction mixture decreased the formation of TBARs. A considerable and more clearly expressed inhibitory effect was observed in the presence of 1 mM peptide. The augmentation of the peptide's concentration led to a strong decrease in the degradation of DR about 70% for [Cav^9]N/OFQ(1-13)NH_2 and about 40 -50% for the rest.

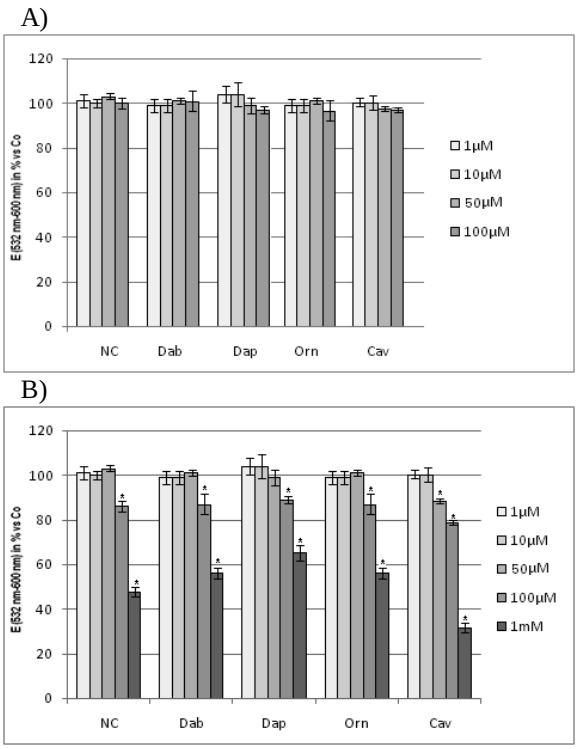

Fig. 2. Effects of N/OFQ(1-13)NH_2, [Dab^9]N/OFQ(1-13)NH_2, [Dap^9]N/OFQ(1-13)NH_2, [Orn^9]N/OFQ(1-13)NH_2 and [Cav^9]N/OFQ(1-13)NH_2 in hydroxyl radical generating system A) without DTPA and H_2O_2, and B) in presence of DTPA and H_2O_2: ·OH-dependent DR degradation was measured at 532 nm. Values represent the mean ± SEM of 7 separate samples. The results are expressed in relative activities (as percentage vs. control). Statistically significant differences vs. controls at *P<0.05.

The effects of the tested peptides in the $^{\bullet}O_2^-$-generating system are presented on Figure 3. All peptides in concentration of 1 μM did not inhibit $^{\bullet}O_2^-$- provoked NBT-reduction. At concentration of 10 μM an inhibitory effect was demonstrated only by [Cav^9]N/OFQ(1-13)NH_2, which decreased the $^{\bullet}O_2^-$-provoked NBT-reduction by about 20%. At concentration of 50 μM again only [Cav^9]N/OFQ(1-13)NH_2 inhibited the process. The presence of 100 μM [Orn^9]N/OFQ(1-13)NH_2 or [Dab^9]N/OFQ(1-13)NH_2 or [Dap^9]N/OFQ(1-13)NH_2 in the reaction mixture led to a decrease in formazan production by about 40%; the inhibitory effect of [Cav^9]N/OFQ(1–13)NH_2 was stronger – about 80%.

DISCUSSION

In this study two deoxyribose tests were used in order to specify the chelating or scavenging potential of the tested peptides. The first test was based on the possibility of DR to bind iron ions. Bound on this detector molecule, the latter in presence of H_2O_2 auto generated in the system, catalyze the site-specific generation of $^{\bullet}OH$ radicals [10].

$$DR + Fe^{2+} \rightarrow DR\text{-}Fe^{2+}$$
$$DR\text{-}Fe^{2+} + O_2 \rightarrow DR\text{-}Fe^{3+} + {}^{\bullet}O_2^-$$
$$2\ {}^{\bullet}O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$
$$DR\text{-}Fe^{2+} + H_2O_2 \rightarrow DR\text{-}Fe^{3+} + OH^- + {}^{\bullet}OH$$

If the tested molecule has a higher binding affinity for iron than the detector, then it can protect the detector molecule, transferring the damage to itself. The protection depends on the concentration of the substance with respect to the detector molecule. In the second case, DTPA chelated the Fe^{2+}, preventing in this manner the metal from association with DR. Thus any $^{\bullet}OH$ generated from the interaction between Fe^{2+}-DTPA and H_2O_2 will have equal access to all components of the reaction medium including DR. Using this method for generation of $^{\bullet}OH$ we found that neither N/OFQ(1-13)NH_2 nor its structural analogues in low concentrations (up to 10 μM) exerted a protective effect (Fig. 2B) . However, the presence of 100 μM peptide in the reaction mixture significantly decreased the formation of TBARs. The highest concentration tested led to a strong decrease in the degradation of DR, especially by [Cav^9]N/OFQ(1-13)NH_2 (about 70%). It seems that the tested peptides act preferentially as radical scavengers, because they were unable to chelate the iron ions in the first chemical medium and thus did not protect the DR molecule from binding to Fe^{2+} and subsequent oxidative fragmentation.

In the NBT test, which provides a simple assay for $^{\bullet}O_2^-$ production and for detection of $^{\bullet}O_2^-$ scavenger effect, again the inhibitory effect of [Cav^9]N/OFQ(1–13)NH_2 was stronger. It decreased the $^{\bullet}O_2^-$-provoked NBT-reduction by about 20% in concentration of 10 μM and by 80% in concentration of 100 μM (Fig. 3). We suggested that this effect could be due to the guanidine group in the molecule of canavanine.

Canavanine is structurally related to L-arginine, the sole difference being the replacement of a methylene group in arginine with an oxa group (i.e. an oxygen atom) in canavanine. But the most

important part in regard to the antioxidant properties is the presence of the guanidine moiety in both molecules. There is evidence that supplementation with L-arginine reduces the production of superoxide from the vessel wall in experimental animals [11]. Also it has been shown that L-arginine reduces Cu^{2+}-provoked oxidation of LDL *in vitro* and this effect is greater than that of vitamin E or ascorbate [12]. Some investigators have hypothesized that this effect may be due to direct antioxidant properties of L-arginine, possibly related to its guanidinium group [12, 13], who comparing the antioxidant activities of aminoguanidine, methylguanidine and guanidine indicated, that guanidine itself, at high concentrations (>0.1 mM), scavenges H_2O_2, HOCl and peroxynitrite, but not the hydroxyl radical. Other guanidine derivatives: aminoguanidine and methylguanidine (at high concentrations, too) have direct scavenging activities against H_2O_2, HOCl, hydroxyl radical and peroxynitrite. Likely, in our system, the guanidinium moiety reacted mostly with H_2O_2 and thus interrupted the possibility of $^{\bullet}OH$ generation.

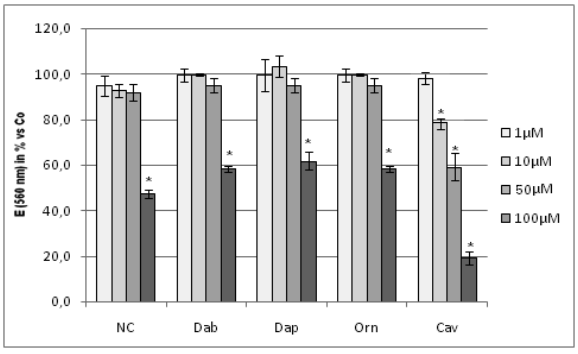

Fig. 3. Effects of N/OFQ(1-13)NH_2, [Dab^9]N/OFQ(1-13)NH_2, [Dap^9]N/OFQ(1-13)NH_2, [Orn^9]N/OFQ(1-13)NH_2 and [Cav^9]N/OFQ(1-13)NH_2 in superoxide radical generating system: O_2^--depending NBT reduction was measured at 560 nm. Values represent the mean ± SEM of 7 separate samples. The results are expressed in relative activities (as percentage vs. control). Statistically significant differences vs. controls at *P<0.05.

CONCLUSIONS

In conclusion, comparing the effects of N/OFQ(1–13)NH_2 with those of its Orn-, Dap- and Dab-analogues we established that at concentration of 100 μM they inhibited both the $^{\bullet}O_2^-$-provoked NBT-reduction and the $^{\bullet}OH$ -provoked DR degradation. Therefore, substitution of Lys in N/OFQ(1-13)NH_2 molecule with other amino acids did not contribute to fundamental changes in its antioxidant properties; the latter depend mainly on the applied concentration. The tested peptides act as radical scavengers. Only [Cav^9]N/OFQ(1–13)NH_2 suppressed the $^{\bullet}O_2^-$-provoked NBT-reduction at a concentration of 10 μM. Therefore, in these experiments only this analogue demonstrated a good antioxidant activity, likely due to the presence of guanidine group in Cav, although it is only one member of the tridecapeptide chain.

The fact that N/OFQ and N/OFQ(1-13)NH_2 display values of potency at the NOP receptor in a low nanomolar range [14] while the antioxidant activities reported here are evident only in the high micromolar range makes unlikely an antioxidant mechanism for suppression of inflammation.

Abbreviations: Cav – canavanine; Dab - diaminobutanoic acid; Dap - diaminopropanoic acid; DR – deoxyribose; DTPA - diethylene triamine pentaacetic acid; NBT - nitro-blue tetrazolium, N/OFQ – nociceptin; N/OFQ(1–13)NH_2 - tridecapeptide template of the nociceptine; $^{\bullet}O_2^-$ - superoxide anion radicals; $^{\bullet}OH$ - hydroxyl radicals; Orn – ornithine; ROS - reactive oxygen species; TBARs - thiobarbituric acid reactive substance.

REFERENCES

1. R. Reinscheid, H. Nothacker, A. Bourson, *Science*, **270** (5237), 792 (1995).
2. Z. Helyes, J. Németh, E. Pintér, J. Szolcsányi, *Br. J. Pharmacol*., **121** (4), 613 (1997).
3. R. Zamfirova, E. Tzvetanova, A. Alexandrova, L. Petrov, P. Mateeva, A. Pavlova, M. Kirkova, S. Todorov, *Central Europ. J. Med*., **4** (2), 170 (2009).
4. G. Calo', R. Guerrini, A. Rizzi, S. Salvadori, D. Regoli, *Br. J. Pharmacol*., 129, 1261 (2000).
5. R. Guerrini, D. Rizzi, M. Zucchini, R. Tomatis, D. Regoli, G. Calo, S. Salvadori, *Bioorg. Med. Chem. Lett*., **13**, 365 (2003).
6. E. Naydenova, V. Zhivkova, R. Zamfirova, L. Vezenkov, Y. Dobrinova, P. Mateeva, *Bioorg. Med. Chem. Lett*., **16**, 4071 (2006).
7. J.M.C. Gutteridge, G.J. Quinlan, In: Experimental protocols for reactive oxygen and nitrogen species. N. Taniguchi, J.M.C. Gutteridge (eds.), University Press, Oxford, 2000, p. 4.
8. B. Halliwell, M. Grootveld, J. Gutteridge, *Methods Biochem. Anal*., **33**, 59 (1988).
9. G. Cohen, In: Handbook of methods for oxygen radical research, R.A. Greenwald (ed.), Florida: CRC Press, Boca Raton, 1985, p.55.
10. A. Miles, M.B. Grisham, *Meth. Enzimol*., **234**, 555 (1994).
11. R.H. Boger, S.M. Bode-Boger, A. Mugge, *Atherosclerosis*, **117**, 273 (1995).
12. A. Philis-Tsimikas, J.L. Witztum, *Circulation*, **92**, 422 (1995).
13. G. Yildiz, A.T. Demiryurek, I. Sahin-Erdemli, I. Kanzik, *British J. Pharmacol*., **124**, 905 (1998).
14. A. Fawzi, H. Zhang, B. Weig, B. Hawes, M. Graziano, *Eur. J. Pharmacol*., **336** (2-3), 233 (1997).

СРАВНИТЕЛНО ИЗСЛЕДВАНЕ НА АНТИОКСИДАНТНАТА АКТИВНОСТ НА НЯКОИ АНАЛОЗИ НА НОЦИЦЕПТИНА

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Постъпила на 21 октомври, 2014 г., коригирана на 16 октомври, 2015 г.

(Резюме)

Ноцицептинът (N/OFQ(1-13)NH_2) потиска неврогенното възпаление, което от своя страна е придружено с увеличена продукция на активни форми на кислорода (АФК). Така възниква въпросът в каква степен този противовъзпалителен ефект се дължи на антиоксидантен механизъм. Целта на изследването беше да се изследват и сравнят антиоксидантните ефекти на ноцицептина и негови новосинтезирани структурни аналози, при които лизинът (Lys) на позиция 9 в структурната верига беше заменен с орнитин (Orn), диаминобутанова киселина (Dab), диаминопропанова киселина (Dap) или канаванин (Cav). Пептидите бяха изследвани в концентрации между 1 и 100 µM в системи, генериращи хидроксилни радикали ($^{\bullet}OH$) и супероксидни анион радикали ($^{\bullet}O_2^-$)

Дезоксирибозата (DR) беше използвана като детектор за $^{\bullet}OH$ радикали. Деградацията на DR беше измервана по формирането на тиобарбитурова киселина реагиращи субстанции, които бяха определяни спектрофотометрично. Супероксид анион радикалите бяха генерирани фотохимично и беше измервана $^{\bullet}O_2$ – предизвиканата редукция на нитроблутетразолиум (NBT).

Резултатите показаха, че в концентрации до 10 µM нито ноцицептинът, нито неговите аналози инхибират $^{\bullet}OH$ – предизвикана деградация на DR; в концентрация 10 µM само [Cav^9]N/OFQ(1–13)NH_2 потискаше $^{\bullet}O_2^-$ – предизвиканата редукция на NBT. Най-високите изследвани концентрации (100 µM) предизвикваха инхибиторен ефект и в двете АФК генериращи системи. Тези ефекти бяха сравнително слаби в присъствието на [Dap^9]N/OFQ(1–13)NH_2 и най-силно изразени в присъствието на [Cav^9]N/OFQ(1–13)NH_2.

В заключение, само [Cav^9]N/OFQ(1–13)NH_2 притежава антиоксидантна активност, докато антиоксидантният капацитет на другите изследвани невропептиди е сравнително слаб, което прави малко вероятно потискането на възпалението да се дължи на антиоксидантен механизъм.