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In-vivo effects of nociceptin and its structural analogue [Orn^9] nociceptin on the antioxidant status of rat blood and liver after carrageenan-induced paw inflammation

Research Article

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Abstract: The production of reactive oxygen species (ROS) in cells is well balanced with their elimination by the antioxidant defence system. This balance is essential for maintenance of physiological conditions, and its disturbance (oxidative stress) has been suggested as a potential pathogenic mechanism in a variety of diseases, accompanied by inflammation. In this study, the in-vivo effects of nociceptin (N/OFQ(1-13)NH_2) and its structure analogue [Orn^9]N/OFQ(1-13)NH_2 were studied on markers of oxidative stress in erythrocytes and liver of rats 4 hours after subplantar administration of carrageenan (CG) (1%, 100 μl) in the right hind paw. A considerable inflammatory oedema of the paw was observed. CG did not change blood haemoglobin content, hematocrit value, glutathione level and antioxidant enzyme activities in the erythrocytes, but there was an increase in lipid peroxidation. In liver, CG-induced imbalance was manifested by an increase in lipid peroxidation and a decrease in glutathione level. Both peptides (20 μg, i.p.), when administered alone, had no effect on all parameters tested. When either [Orn^9]N/OFQ(1-13)NH_2 or N/OFQ(1-13)NH_2 was injected simultaneously with CG or 15 minutes before it, they did not affect the CG-induced changes in the antioxidant status of the erythrocytes and liver. Our results suggest that the peptides tested did not play a role in the free radical processes that accompany CG-induced paw inflammation.

Keywords: *Paw inflammation • N/OFQ(1-13)NH_2 • [Orn^9]N/OFQ(1-13)NH_2 • Liver • Erythrocytes • Glutathione level • Lipid peroxidation • Antioxidant enzymes*

1. Introduction

Nociceptin (N/OFQ), a neuropeptide sharing sequence homology with classical opioid peptides but with a distinct pharmacological profile, is the endogenous agonist of the NOP receptor. Although there are differences in the peptide precursor, the sequence of this heptadecapeptide is the same in mouse, rat, and human; for details see the review of Calo et al. [1]. On the basis of pharmacological and physiological characteristics, NOP receptor ligands (agonists and antagonists) could be useful for treatment of pain, anxiety, depression, dementia, parkinsonism, stress-induced anorexia, neurogenic bladder, oedema, or drug dependence [2].

Nociceptin is localized on primary sensory neurons, projecting into the majority of peripheral organs and tissues. N/OFQ plays a role in cardiovascular, gastrointestinal, and immune regulation. mRNA of the N/OFQ precursor prepronociceptin (ppN/OFQ) is expressed in the aorta, pulmonary artery, and renal artery and vein at high levels comparable to the amounts found in the brain, whereas no expression of ppN/OFQ mRNA is shown in other peripheral tissues, including the ventricle, liver, lung, and kidney [3]. N/OFQ is also expressed in many regions of the central nervous system (CNS) [4]. It has been found that intracerebroventricular (i.c.v.) injection of N/OFQ causes water diuresis (aquaresis) that may be useful in the treatment of oedema or hyponatremia, occurring in the end stage of various diseases, including liver cirrhosis [5].

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In blood, the N/OFQ/NOP system has been investigated mainly in immune cells. Following degranulation, freshly isolated peripheral-blood neutrophils (PMNs) were found to express and secrete N/OFQ, which identified these inflammatory cells as a novel source of the neuropeptide [6]. It has been shown that functionally active N/OFQ/NOP is present in human neutrophils. In the study by Williams et al. [7], in which NOP mRNA, but not protein for NOP was detected, the presence of a low-density NOP expression in human peripheral blood mononuclear cells (PBMC) was suggested. It is assumed that N/OFQ, which is produced by these immunocytes, may be involved in the control of immune function and autoregulation. This has been supported by further investigation, indicating that PBMCs transcribe ppN/OFQ [8].

N/OFQ is involved in many physiological and pathological processes, including pain regulation and inflammation. In patients with various pain states, depression, and hepatic disease, altered plasma levels of N/OFQ have been found. Plasma N/OFQ concentrations increase in critically ill patients with sepsis [9]. High plasma levels of N/OFQ have also been measured in patients with Wilson's disease and in those with acute or chronic pain [10,11]. During development of hepatocellular carcinoma (HCC), the plasma N/OFQ level also strongly increases [12,13]. An N/OFQ content that was increased 15-fold was found in HCC tissue, compared to that found in the tumour-free tissue sample of liver taken during autopsy [13]. According to Horvath et al. [13], the elevated N/OFQ level might represent a compensatory mechanism of N/OFQ/NOP system to modulate pain perception in the CNS. This mechanism could explain why some patients with a very high plasma level of N/OFQ do not experience pain, despite the advanced stage of malignant liver tumour. The authors consider the progressive elevation of plasma N/OFQ level as an indicator of HCC during tumour development.

N/OFQ involvement in the maintenance of neuropathic pain is realised by activating neuronal nitric-oxide synthase (nNOS), and the analgesic effect of some NOP-antagonists (such as JTC-801) on neuropathic pain is mediated via inhibition of nitric oxide (NO) production by nNOS [14]. Following fluid percussion brain injury (FPI), it has been demonstrated that N/OFQ at concentrations found in the cerebrospinal fluid increases superoxide anion radical (O_2^-) production in a cyclooxygenase-dependent manner [15,16]. The peptide also increases protein kinase C (PKC)-dependent O_2^- production after insult [17].

It is well known that oxidative stress, provoked by overproduction of ROS, underlies the pathogenesis of various clinical disorders (ischemia, reperfusion injury, atherosclerosis, acute hypertension, diabetes mellitus, and cancer), as well as inflammation. According to Chen and Sommer [18], the nociceptin system may be involved in the modulation of neuropathic and inflammatory pain at the level of the primary afferent neuron.

Data have also shown that ROS induce an expression of two neuropeptide genes, the opioid proenkephalin and pronociceptin that suggests a role for nociceptin in the injury and stress responses of the CNS and peripheral pathophysiological conditions involving reactive oxygen species [19].

The role of N/OFQ and its mechanism of action on neurogenic inflammation have been widely studied. Despite the fact that the inflammation is accompanied by free-oxygen processes, we did not find any data on pro- or antioxidant-capacity of N/OFQ under these conditions. That is why we aimed in this study to investigate the *in-vivo* effects of N/OFQ(1-13)NH_2 and its structural analogue [Orn^{9}]N/OFQ(1-13)NH_2 (a highly potent NOP-receptors agonist [20] on the antioxidant status of rat erythrocytes and liver associated with acute carrageenan (CG)-induced peripheral inflammation. The effects of these peptides on endogenous antioxidant levels in red blood cells and liver from healthy animals is also of interest.

2. Material and Methods

2.1. Materials

N/OFQ(1-13)NH_2 and [ORN^{9}]N/OFQ(1-13)NH_2 were synthesized in the Department of Organic Chemistry, University of Chemical Technology and Metallurgy (Sofia, Bulgaria). The reagents (γ-carrageenan, 2-thiobarbituric acid, $NADP^+$, NADPH, reduced and oxidized glutathione, riboflavine, methionin) were obtained from Sigma-Aldrich (Germany).

Carrageenan (CG), as 1% solution, was prepared in 1% dimethylcellulose; the solutions of N/OFQ(1-13)NH_2 and [Orn^{9}]N/OFQ(1-13)NH_2 were freshly prepared in saline before each experiment; all other solutions were prepared with over-glass re-distilled water.

2.2. Animals

Male Wistar rats, weighing 180-200 g, were housed at 22^{o}-25^{o}C. The animals were allowed to adapt with free access to food and water and a natural day/night light cycle. Prior to the experiments, they were given no food for 24 hours, but with access to water as desired by the animal.

2.3. Treatment and tissue preparations

The rats were divided in eight experimental groups: 1) Controls; 2) N/OFQ(1-13)NH_2-treated; 3) [Orn^9]N/OFQ(1-13)NH_2-treated; 4) CG-treated; 5) simultaneously treated with N/OFQ(1-13)NH_2 and CG; 6) simultaneously treated with [Orn^9]N/OFQ(1-13)NH_2 and CG; 7) N/OFQ(1-13)NH_2-treated, 15 minutes before CG; 8) [Orn^9]N/OFQ(1-13)NH_2-treated, 15 minutes before CG.

CG was given in a volume of 0.1 ml via an intraplantar injection into the right hind paw. N/OFQ(1-13)NH_2 and [Orn^9]N/OFQ(1-13)NH_2 (20 µg/ml) were administered via an intraperitoneal injection in a volume of 0.1 ml/100g body weight [21]. Four hours after the CG treatment, the volume of the injected paw was measured plethysmographically, and the animals were sacrificed under light ether anaesthesia.

Blood was taken by a heparinized syringe through a puncture of the left heart ventricle, and erythrocytes were obtained after centrifugation at 600 g for 10 minutes. The plasma was discarded, and the red blood cells were washed twice with 0.9% sodium chloride and were centrifuged under the same conditions. A 5%-erythrocyte suspension in saline was used to measure lipid peroxidation and glutathione levels. Part of the 5%-erythrocyte suspension was lysed through freezing (-20°C) for 24 hours and used to measure enzyme activities.

The liver was perfused with cooled 0.15 M KCl to remove the blood cells, and a 10%-homogenate was obtained by a Potter-Elvehjem glass homogenizer with Teflon pestle; the homogenate was centrifuged in a refrigerated centrifuge (*Janetzki* K24) for 10 minutes at 3000 rpm, and a postnuclear homogenate was obtained. This preparation was used for quantitative measurement of the levels of glutathione and lipid peroxidation. Part of the postnuclear homogenate was centrifuged for 20 minutes at 12,000 rpm (temperature control, between 0° and +4°C). The resulting postmitochondrial supernatant was used for measuring the enzyme activities.

2.4. Analytical methods

Protein content was measured by the method of Lowry et al. [22].

Haemoglobin (Hb) amount was determined by Merck test cat. No 3317.

Lipid peroxidation (LP) was determined by the amount of thiobarbituric acid reactive substances (TBARs) formed in fresh biological preparations. Erythrocyte suspension (0.5%, according to Hb) in 0.9% NaCl-10 mM potassium phosphate buffer, pH 7.4, was incubated for 60 minutes at 37°C in the presence and in the absence of 10 mM H_2O_2 [23]. Postnuclear liver homogenate (mg protein/ml) in 0.15 M KCl-10 mM potassium phosphate buffer, pH 7.4, was incubated for 60 minutes at 37°C in the presence and in the absence of 0.05 mM $FeCl_3$-0.5 mM ascorbic acid [24]; the 600nm absorbance was considered to be a nonspecific baseline and was subtracted from A_{532}. The values were expressed in nmoles malondialdehyde (MDA) per mg protein, with a molar extinction coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$.

Total glutathione (GSH) level was measured according to Tietze [25] and was expressed in ng/mg protein or haemoglobin, with glutathione oxidized (GSSG) as a reference standard.

Glutathione peroxidase (GSH-Px) activity was measured by the method of Gunzler et al. [26] and was expressed in nmoles NADPH oxidized per minute per mg protein or haemoglobin, with a molar extinction coefficient of $6.22 \times 10^6 M^{-1}cm^{-1}$.

Glutathione reductase (GSSG-Red) activity was measured by the method of Pinto and Bartley [27] and was expressed in nmoles NADPH oxidized per minute per mg protein or haemoglobin, with a molar extinction coefficient of $6.22 \times 10^6 M^{-1}cm^{-1}$.

Glucose-6-phosphate dehydrogenase (Glu-6-P-DH) activity was determined by the method of Cartier et al. [28] and was expressed in nmoles $NADP^+$ reduced per minute per mg protein or haemoglobin, with a molar extinction coefficient of $6.22 \times 10^6 M^{-1}cm^{-1}$.

Cu,Zn-Superoxide dismutase (SOD) activity, determined according to the method of Beauchamp and Fridovich [29], was expressed in U/mg protein or haemoglobin (one unit of SOD activity is the amount of the enzyme, producing a 50% inhibition of nitroblue tetrazolium reduction).

Catalase (CAT) activity was determined according to the method described by Aebi [30]; enzyme activity was expressed as ΔE_{240}/min/mg protein or haemoglobin.

2.5. Statistical analysis

The results were statistically analyzed by one-way ANOVA (Dunnett post-test), with $p<0.05$ accepted as the minimum level of statistical significance of the established differences.

The experiments were performed according to the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985), and the rules of the Ethics Committee of the Institute of Physiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

Table 1. *In vivo* effects of N/OFQ(1-13)NH_2 and [ORN^9]N/OFQ(1-13)NH_2 on haemoglobin and hematocrit levels in control and CG-treated rats: Haemoglobin is expressed in g/100 ml blood and hematocrit in per cent. Values represent the mean ± SEM of 7 animals.

	Haemoglobin	Hematocrit
	I. Control animals	
Controls	13.9 ± 0.58	51.3 ± 2.23
N/OFQ	13.7 ± 0.83	51.0 ± 2.26
[ORN^9]N/OFQ	14.4 ± 0.72	49.7 ± 1.17
	II. Carrageenan-treated animals	
Carrageenan (CG)	15.0 ± 0.44	52.4 ± 1.89
	III. Carrageenan-treated animals (the peptides are applied simultaneously with CG)	
N/OFQ	14.0 ± 0.76	48.5 ± 1.89
[ORN^9]N/OFQ	14.7 ± 0.73	51.8 ± 2.22
	IV. Carrageenan-treated animals (the peptides are applied 15 minutes before CG)	
N/OFQ	13.7 ± 0.28	47.0 ± 2.51
[ORN^9]N/OFQ	13.9 ± 0.27	49.6 ± 0.38

Figure 1. *In vivo* effects of N/OFQ(1-13)NH_2 and [ORN^9]N/OFQ(1-13)NH_2 on GSH level in erythrocytes from control and CG-treated rats: Each data point represents the mean ± SEM for 7 animals.

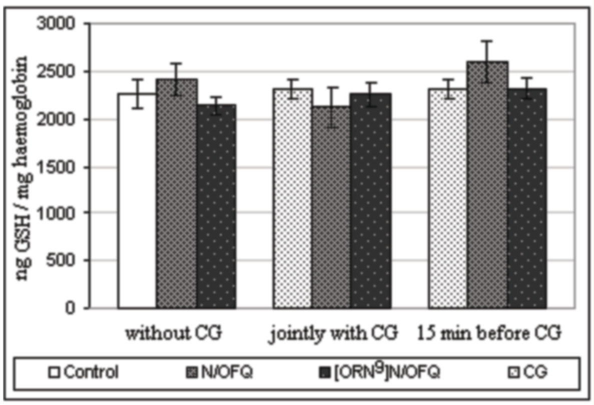
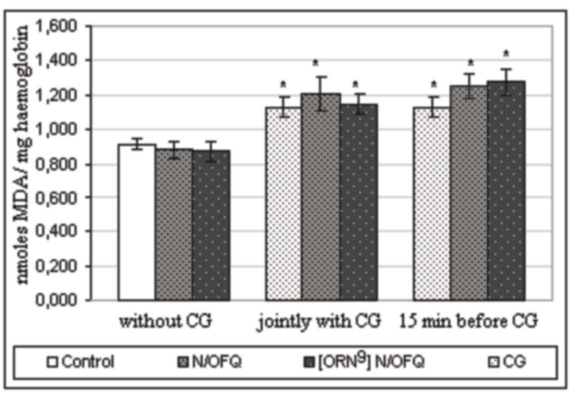


Figure 2. *In vivo* effects of N/OFQ(1-13)NH_2 and [ORN^9]N/OFQ(1-13)NH_2 on lipid peroxidation in erythrocytes from control and CG-treated rats: Each data point represents the mean ± SEM 7 animals. Significant differences versus controls at *P<0.05.



3. Results

Four hours after CG treatment, the volume of the right hind paw of the rats was increased by about 70%, compared to the pre-carrageenan value. Using this CG-model of acute inflammation [31], we studied the effects of N/OFQ(1-13)NH_2 and [Orn^9]N/OFQ(1-13)NH_2 on antioxidant status of erythrocytes and liver of control and GC-treated animals.

Compared to the controls, the blood indexes, hemoglobin and hematocrit, were unchanged in the CG-treated animals (Table 1). At the fourth hour after CG injection, we failed to find any changes in the levels of non-enzyme (total GSH) (Figure 1) and enzyme (SOD, CAT, GSH-Px) antioxidants as well as in Glu-6-P-DH activity (Table 2) in red blood cells; only an increase of the amount of TBARs (an index of lipid peroxidation) was observed (Figure 2). In control animals, all the checked parameters were unchanged by both peptides. Moreover, independent of the administration time (simultaneously or 15 min, before CG), neither N/OFQ(1-13)NH_2 nor [Orn^9]N/OFQ(1-13)NH_2 modified the CG-induced changes.

Acute paw inflammation caused by CG resulted in some changes in liver antioxidant status. At the fourth hour after CG injection, an increase in the spontaneous (Figure 3A) and Fe/ascorbate-induced lipid peroxidation (Figure 3B) and a decrease in GSH level (Figure 4) were observed, but the activity of antioxidant enzymes (SOD, CAT, GSH-Px, GSSG-Red), as well as of Glu-6-P-DH, was unchanged (Table 3). In addition, neither N/OFQ(1-13)NH_2 nor [Orn^9]N/OFQ(1-13)NH_2 affected the parameters tested in the liver of both control and CG-treated animals; only a tendency toward an increased GSH level after the injection of [Orn^9]N/OFQ(1-13)NH_2 was observed in the liver of CG-treated animals (Figure 4).

Table 2. *In vivo* effects of N/OFQ(1-13)NH_2 and [ORN^9]N/OFQ(1-13)NH_2 on antioxidant enzyme activities in erythrocytes from control and CG-treated rats: Antioxidant enzyme activities were measured in erythrocyte lysate. The corresponding enzyme activities are expressed in: nmoles NADP(H)/min/mg Hb (for GSH-Px and Glu-6-P-DH); U/mg Hb (for SOD) and ΔE_{240}/min/mg Hb (for CAT). Values represent the mean ± SEM of 7 animals.

	SOD	CAT	GSH-Px	Glu-6-P-DH
		I. Control animals		
Controls	7.7 ± 0.44	1.39 ± 0.046	110 ± 5.3	11.45 ± 0.601
N/OFQ	7.7 ±0.79	1.35 ± 0.133	111 ± 3.5	12.33 ± 0.138
[ORN^9]N/OFQ	7.3 ± 0.58	1.32 ± 0.061	106 ± 8.7	10.15 ± 0.683
		II. Carrageenan-treated animals		
Carrageenan (CG)	7.7 ± 0.53	1.45 ± 0.071	113 ± 7.2	10.88 ± 0.648
		III. Carrageenan-treated animals (the peptides are applied simultaneously with CG)		
N/OFQ	8.8 ± 0.43	1.35 ± 0.089	122 ± 5.3	10.51 ± 1.131
[ORN^9]N/OFQ	9.0 ± 1.01	1.42 ± 1.000	108 ± 9.7	11.77 ± 0.601
		IV. Carrageenan-treated animals (the peptides are applied 15 minutes before CG)		
N/OFQ	9.2 ± 0.95	1.56 ± 0.078	128 ± 7.6	11.08 ± 0.819
[ORN^9]N/OFQ	8.3 ± 0.51	1.41 ± 0.045	105 ± 3.3	10.96 ± 0.164

Figure 3. *In vivo* effects of N/OFQ(1-13)NH_2 and [ORN^9]N/OFQ(1-13)NH_2 on spontaneous (A) and Fe^{3+}/ascorbic acid-induced lipid peroxidation (B) in the liver from control and CG-treated rats: Each data point represents the mean ± SEM for 9 animals. Significant differences versus controls at *P<0.05.

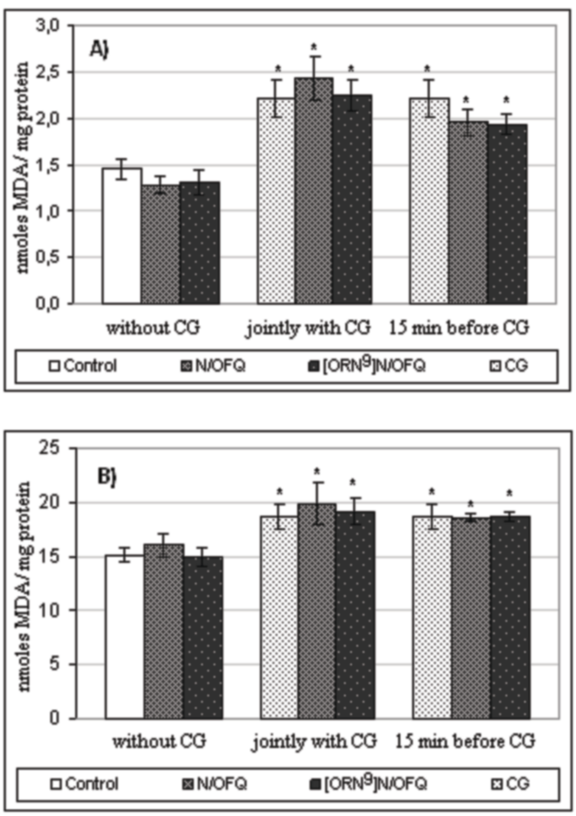
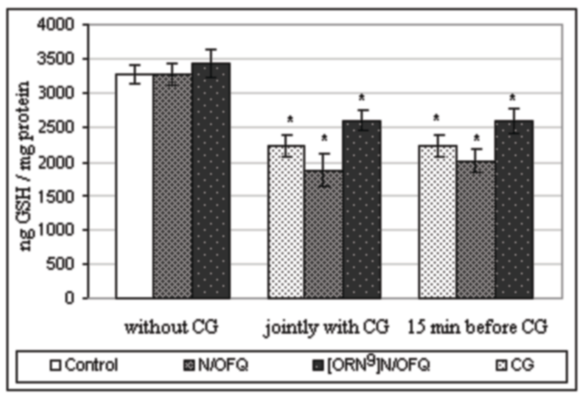


Figure 4. *In vivo* effects of N/OFQ(1-13)NH_2 and [ORN^9]N/OFQ(1-13)NH_2 on GSH level in the liver from control and CG-treated rats: Each data point represents the mean ± SEM for 9 animals. Significant differences versus controls at *P<0.05.



4. Discussion

It is well known that ROS are normally generated in body cells and that the defence systems against them involve both enzyme and nonenzyme cell antioxidants. Under physiological conditions, an equilibrium of prooxidant and antioxidant status in cells exists.

The increase in ROS production and the decrease in cell-defence systems capacity (oxidant/antioxidant imbalance) is termed OS. OS underlies the pathogenesis of many diseases and is accompanied by inflammation. OS could be manifested by direct injurious effects, and it is involved in the molecular mechanisms that control inflammation. The possible cell sources for ROS include NAD(P)H oxidases, mitochondrial electron transport enzymes, xanthine oxidase, cyclooxygenase (COX),

Table 3. In vivo effects of N/OFQ(1-13)NH_2 and [ORN9]N/OFQ(1-13)NH_2 on antioxidant enzyme activities in the liver from control and CG-treated rats: Antioxidant enzyme activities were measured in liver supernatant. The corresponding enzyme activities are expressed in: nmoles NADP(H)/min/mg Hb (for GSH-Px, GSSG-Red and Glu-6-P-DH); U/mg Hb (for SOD) and ΔE_{240}/min/mg Hb (for CAT). Values represent the mean ± SEM of 9 animals.

	SOD	CAT	GSH-Px	GSSG-Red	Glu-6-P-DH
			I. Control animals		
Controls	76 ± 5.4	5.9 ± 0.26	238 ± 26	34.3 ± 1.26	27.2 ± 1.55
N/OFQ	84 ± 4.2	5.6 ± 0.36	226 ± 23	34.7 ± 0.88	30.1 ± 2.35
[ORN9]N/OFQ	85 ± 7.9	6.7 ± 0.47	223 ± 7	35.5 ± 1.39	26.7 ± 1.82
			II. Carrageenan-treated animals		
Carrageenan (CG)	75 ± 5.6	6.5 ± 0.38	285 ± 42	37.5 ± 1.02	27.6 ± 1.72
			III. Carrageenan-treated animals (the peptides are applied simultaneously with CG)		
N/OFQ	81 ± 5.6	5.7 ± 0.33	260 ± 22	35.1 ± 2.84	30.0 ± 2.91
[ORN9]N/OFQ	87 ± 5.7	6.1 ± 0.37	215 ± 27	37.5 ± 1.16	32.4 ± 1.47
			IV. Carrageenan-treated animals (the peptides are applied 15 minutes before CG)		
N/OFQ	81 ± 5.6	5.7 ± 0.33	299 ± 17	34.8 ± 0.91	32.8 ± 2.51
[ORN9]N/OFQ	87 ± 5.7	6.1 ± 0.37	229 ± 25	37.9 ± 1.37	26.6 ± 2.81

lipoxygenase, and uncoupled nitric oxide synthase (NOS). Hence, activation of these sources under pathological conditions might contribute to increased ROS production and, subsequently, to OS.

In experimental practice, the mucopolysaccaride CG is most commonly used for induction of an acute local paw inflammation. In addition to paw oedema, blood-vessel permeability and release of pro-inflammatory mediators CG have led to the activation of NAD(P)H oxidases; iNOS expression; an increase in arachidonic acid metabolism and in prostaglandin E_2 (PGE_2), urate, and caeruloplasmin levels; iron overload; and decreased glutathione content [32-37]. An important factor in the carrageenan-induced inflammatory response is NO [38-40]. The increase in COX activity, NO, and oxygen-derived free radicals can cause lipid peroxidation and cellular damage [38-47], since O_2^- and peroxynitrite ($NOOO^-$) play a critical role in the development of an inflammatory response [33,48-50]. All of these features make CG-induced inflammation an appropriate model for testing the antioxidant capacity of different substances [34,35,51-56].

Using the CG model, we tested the effects of N/OFQ(1-13)NH_2 and its structural analogue [Orn9]N/OFQ(1-13)NH_2 on the antioxidant status of red blood cells and liver. Four hours after CG injection, the volume and the tissue mass of the inflamed paw increased, i.e., a local paw inflammation was achieved. Under these experimental conditions, no changes in the values of blood haemoglobin and hematocrit or in the endogenous enzyme and non-enzyme antioxidant levels in erythrocytes of CG-treated animals were observed; only an increase in the LP was measured. Elevated TBARs values were also found in blood plasma [57]. The slight CG-induced effects on erythrocyte antioxidant status might be explained by the finding that a significant alteration in the morphology of red blood cells has been observed at the 24th hour after carrageenan administration [33].

In our study we have shown that the acute paw inflammation from the CG led to an increase in the spontaneous and Fe/ascorbate-induced lipid peroxidation in liver. This finding agrees with that of Dhuley et al. [58] who showed that CG-induced rat paw oedema is in a direct co-relationship with liver LP. Decreased GSH level and unchanged antioxidant enzyme activity in liver were also observed in our model of CG inflammation. A co-relation between paw inflammation from CG and liver antioxidant status has been shown by Lu et al. [37]. Investigating the antiinflammatory mechanism of aqueous extract of Glycine tomentella (AGT) via detection of the antioxidant enzyme activities in liver and the levels of NO and malondialdehyde (MDA) in paw oedema, these authors suggested that the AGT antiinflammatory mechanism is related to the decrease in MDA and NO levels in the paw oedema via the increase in liver antioxidant enzyme activity.

Our results demonstrate that neither N/OFQ(1-13)NH_2 nor [Orn9]N/OFQ(1-13)NH_2 affect the antioxidant indexes, which were measured on follow-up in the blood and liver of both control and CG-treated animals. As observed previously [31], these peptides did not change the antioxidant status of the control- and CG-inflamed paw tissues. This agrees with the data published by Trombella et al. [59] that N/OFQ stimulates chemotaxis of monocytes (via NOP receptor activation) but not of neutrophils and that this peptide does not affect neutrophil-dependent O_2^- production. In healthy animals, the peptides tested in our study did also not affect the endogenous antioxidant levels in erythrocytes and liver. In conclusion, it might be suggested that N/OFQ(1-13)NH_2 and its structural analogue [Orn^9] N/OFQ(1-13)NH_2 do not exert pro- or antioxidant properties.

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