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THE NEURONAL ANTIINFLAMMATORY EFFECTS OF APELIN-13

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ABSTRACT

Objective: Apelin, a peptide with growth factor characteristic, is expressed in many cells and acts by binding to the APJ receptor. Apelin mRNA was detected in the central nervous system (CNS), macrophages and many peripheral tissues. According to recent studies, apelin protects neurons from hypoxia and glutamate-mediated excitotoxicity and can protect cells from oxidative stres (stress) (OS) damage by stimulating catalase activity. Pituitary and neurodegenerative diseases are associated with progressive neuronal loss in the CNS. Neuroinflammation and OS-induced cell damage, caused by microglia and astrocytes activation, have an important role in the pathogenesis and prognosis of these diseases. The aim of this study is to investigate the effects of apelin-13 molecule on the development of inflammation and OS caused by bacterial endotoxin in an invitro astrocyte cell culture medium.
Methods: The effect of apelin -13 on cell viability changes in lipopolysaccharide (LPS) induced astrocyte inflammation was analyzed by enzymatic MTT test. The anti-inflammatory effects were determined by qRT-PCR and ELISA analysis at the level of TNF-α, IL-1β, IL-6 and mRNA transcription and protein. The effects of apelin on endotoxin mediated OS and possible DNA damage, lipid peroxidation, and nitric oxide (NO) production were evaluated by ELISA method.
Results: In our study, LPS application to astrocytes caused inflammation and a significant decrease in inflammation was found with apelin treatment.
Conclusion: The results obtained from this study reveal the antiinflammatory, antiapoptotic and antioxidant effects of apelin, that have widely expressed receptors and role in the development of immune response on the development of neuroinflammation and oxidative cell damage.

Key Words: Apelin, lipopolysaccharide, neuroinflammation, oxidative stres

1. INTRODUCTION

Apelin is a peptide secreted by adipose tissue. The receptor was first detected in 1993, then the apelin molecule was isolated as the endogenous ligand of this receptor (1). Apelin originates from a preproapelin consisting of 77 amino acids and is disintegrated from different parts to produce different numbers of amino acids (apelin-10, apelin-11, apelin-12, apelin-13, apelin-15, apelin-17, apelin-19 and apelin-36 (2). The effects of apelin vary according to these forms. It has been reported that the apelin consisting of 13 and 17 amino acids have stronger biological activity than the apelin containing 36 amino acids. Since apelin-13 has N-terminal pyroglutamate residues, its biological activity is higher than the others. APJ, composed of 380 amino acids and consisting of seven transmembrane regions, is a family of G protein-coupled receptors (3).

Neurodegenerative diseases are a group of diseases with slow and progressive neuronal loss, accompanied by cognitive and cognitive disorders. This group includes Alzheimer's, Parkinson's, Huntington's Diseases, Amyotrophic Lateral Sclerosis, and Multiple Sclerosis. These diseases affect millions of people around the world and it is important because of the expenses made for treatment in the period following diagnosis (4). The underlying causes of the progression of these diseases have not been fully elucidated yet. Recent scientific researches have shown that neuroinflammation and OS in CNS have an important role in the pathogenesis and prognosis of these disorders (5,6). The main cells responsible for the development of neuroinflammation in the brain are microglia and astrocytes (7,8).

The aim of this study is to evaluate the possible anti -inflammatory and antioxidant effects of apelin on astrocytes in in-vitro conditions by using immunological, biochemical and molecular biology methods. By determining the effective concentration and duration of action of apelin on inflammation induced by LPS in astrocytes, its effects on viability changes in cells were analyzed by enzymatic MTT test, and its effects on inflammatory and anti-inflammatory system were determined by TNF-α, IL -1β, IL-6 and IL-10 mRNA measurements determined by qRT-PCR and ELISA analysis at transcription and protein level. The effects of apelin application on OS developed in astrocytes were evaluated with NO measurement, iNOS, nNOS and catalase enzyme level changes were investigated by Western blot method. In addition, phosphorylation changes in the NF-kB transcription factor, which have a central role on the signal transduction pathway of the apelin, have also been demonstrated by Western blot method.

2. MATERIALS AND METHODS

2.1.1. Cell Culture:

The mouse astrocyte cell line C8D1-A was obtained from ATCC, and was passaged every 2-3 days to yield new C8D1-A cells in a complete DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. In a carbon dioxide incubator (5% CO2 + 95% O2; Nuaire Co., Plymouth , MN, USA), the media of the cells kept at 37 °C in a humid environment were changed twice a week. When the cells were confluent, they were removed from flasks using trypsin-EDTA solution and transferred to 96-well plates FOR using in MTT (Thiazolyl Blue Tetrazolium Bromide) assay (9).

2.1.2. MTT Assay:

Using MTT (Thiazolyl Blue Tetrazolium Bromide), the ratio of living cells in a cell population can be determined quantitatively by the spectrophotometric method. This method is based on the principle that MTT can break the tetrazolium ring by mitochondrial reductases in healthy cells. These analyses aimed to determine the effect of apelin on the changes in cellular viability that would develop with the cytotoxic and/or inflammatory effect of LPS. For this purpose, we used 10 ng, 100 ng, 500 ng and 1000 ng doses of LPS in accordance with the literature (10,11) for 3, 24, 48 and 72 hours, and MTT analyses were performed in a microplate reader at 570 nm wavelength (Multiscan GO, Thermo Scientific) (Figure 1). In order to determine the appropriate dose to be used for the detection of apelin activity in LPS-stimulated cells 1, 2.5, 5, 10, 25, 50 and 100 nM of apelin doses were added to the cell treated with 1 µg/ml LPS and incubated for 24 hours. At the end of the period, the viability analysis of the cells was evaluated by MTT test (Figure 2).

2.1.3. ELISA Analysis:

Our study groups are control, LPS, Apelin, LPS+ Apelin. 1000 ng/ml LPS and 10 nM/ml apelin were used for each study group. After 24 hours of incubation, following drug administration, for each group media were collected and centrifuged at 1000 RPMI for 1 minute, and the supernatant was stored at -20 °C until the analysis.

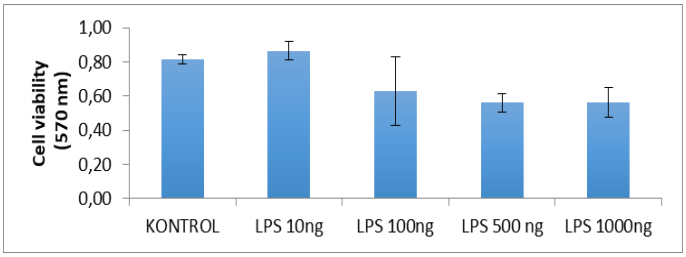

Figure 1. Viability values (MTT test) in cells exposed to different LPS concentrations for 24 hours. No statistical difference was found between the groups (p> 0.05).

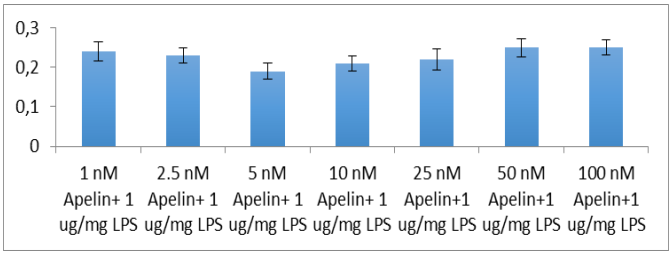

Figure 2. MTT results obtained after induction of 1mcg/ ml LPS and apelin applications at various doses in C8D1-A cells. No statistical difference was found between the groups (p> 0.05).

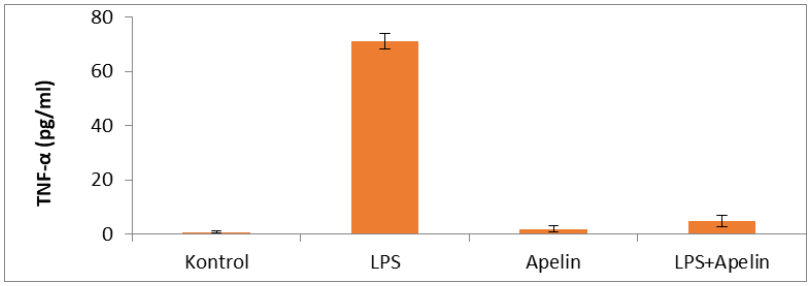

Figure 3. Distribution of TNF-alpha levels by groups (ELISA)

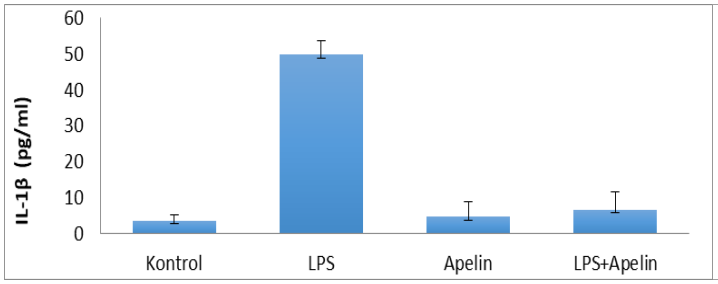

Figure 4. Distribution of L-1β level in groups by ELISA method

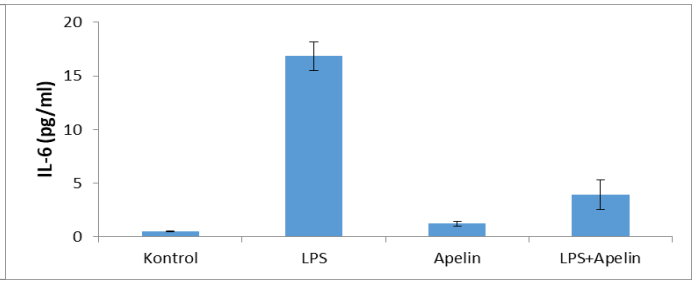

Figure 5. Distribution of L-1β levels in groups by ELISA method

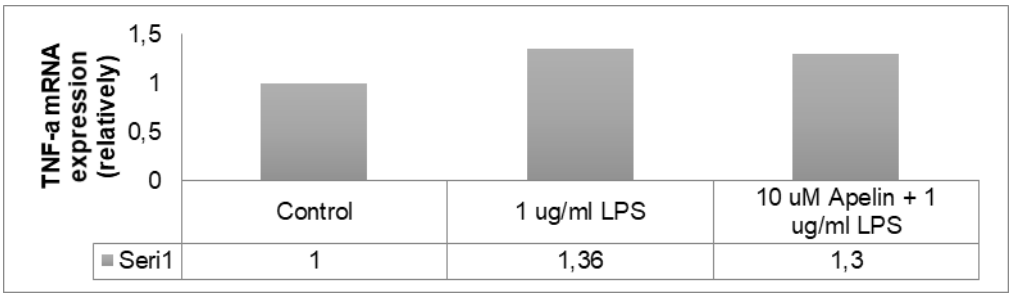

Figure 6. The results of TNF-α mRNA RT-qPCR expression obtained after 24 hours incubation in astrocytes in which LPS, apelin, or combinations were administered.

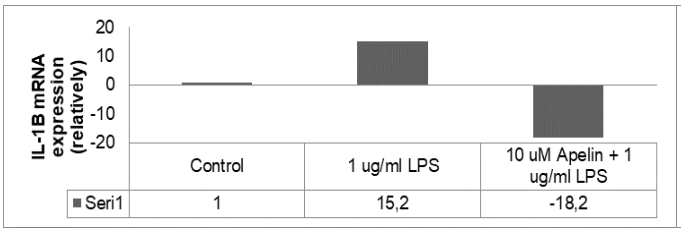

Figure 7. The result of IL-1β mRNA RT-qPCR expression after 24 h incubation in astrocytes in which LPS, apelin, or combinations were administered

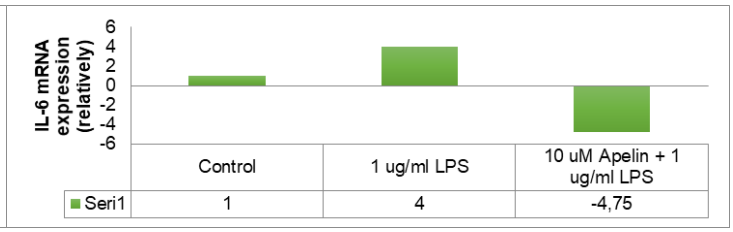

Figure 8. The Results of RT-qPCR expression of IL-6 mRNA obtained after 24 hours of incubation in astrocytes in which LPS, apelin, or combinations were administered.

From these supernatants inflammatory markers, TNF-α, IL-1β, IL-6 protein levels were analyzed in a multi-plate reader (Multi Scan GO) by using commercial kits (Figures 3, 4, 5).

The ELISA kits we used were mouse IL-1 Beta, mouse TNF-alpha, mouse IL-6, mouse IL-10, Mouse malondiadehit manufactured by Boster Immunoleader company.

2.1.4. RT-qPCR analysis:

TNF-α, IL-1β, IL-6, and IL-10 mRNA transcription levels were determined by the qRT-PCR method. Total RNA samples used in the Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) method were obtained using the ready commercial kit (Trizol, Sigma). At the end of the experiment (24 hour incubations), total RNAs of cell samples taken from the control and test groups were obtained according to the protocol of the RNA isolation kit, and the purity levels of the RNAs were determined by spectrophotometer (OD 260 nm and 280 nm) and that RNA/DNA ratio in 1.8- 2.0 range were used in the study .

In order to be used as a template in the PCR reaction, 1 mcg of RNA from each sample was used for complementary DNA (cDNA) synthesis with reverse transcriptase (RT), then 1 mcl of each sample from the cDNA was taken and a pair of primer (oligonucleotide) and SYBR Greenmaster was added. The primers were specific for each transcription assay and were obtained by synthesis of the base sequences previously mentioned in the

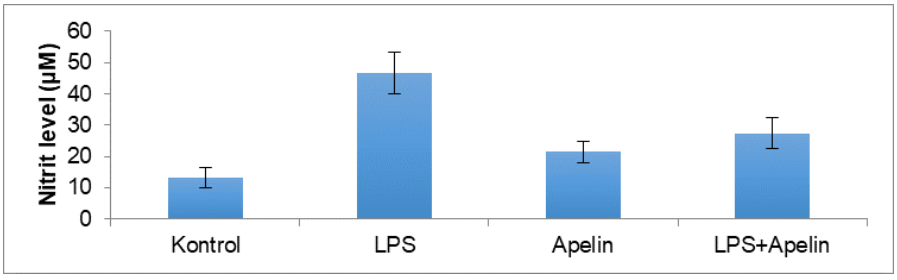

Figure 9. NO values obtained in cell culture media collected after 24 h incubation with LPS, apelin or combinations

Table 1. Primary sequences used in mRNA expression analysis by PCR

Gene	Forward primary	Reverse primary
TNF-α	5'-AGCCGATGGGTTGTACCTTGTCTA-3'	5'-TGAGATAGCAAATCGGCTGACGGT 3'
IL-1β	5'-TTGTGGCTGTGGAGGGGCTGT-3'	5'-AACGTCACACACCAGCAG TT-3'
IL-6	5'- ATCCAGTTGCCTTCTTGGGACTGA-3'	5'- TAAGCCTCCGACTTGTGAAGTGGT-3'
iNOS	5'- GCCCCACGGAGAACAGCAGAG-3'	5'-GGGCGGGTCGATGGAGTCAC-3'
β-actin	*House keeping gene*	
	5'-ATGGATGACGATATCGCTGCG-3'	5'-AGGGTCAGGATGCCTCTCTT-3'

literature and used about 100 ng in the reaction. The primers used during RT-qPCR are given in Table 1 (Pico Real Time PCR, ThermoScientific). The expression levels of the analyzed genes were normalized with the β-actin gene (Figure 6,7,8).

2.1.5. NO Analysis:

NO, released from astrocyte cells and converted into nitrite, was analyzed according to the Griess method. The cell culture medium was collected after incubations as in the ELISA assay, and the reaction was carried out by the addition of Griess reagent (sulfanilamide 1%, alpha-naphthyl ethylene diamine dihydrochloride 0.1%, phosphoric acid 2.5%). Absorbance was read on the spectrophotometer at 540 nm and sodium nitrite was used as the standard (Figure 9).

3. RESULT AND DISCUSSION

The level of TNF-alpha (inflammatory cytokine) was investigated by ELISA method and LPS application was observed to stimulate the cells and cause inflammation. However, apelin treatment decreased the level of TNF-alpha to control levels ($p < 0.001$) (Fig. 3). Figure 4 shows the IL-1beta (inflammatory cytokine) level detected by ELISA. When the groups were compared, it was shown that the highest cytokine level was in the LPS stimulated group but the cytokine level decreased significantly in the apelin treated groups. In Fig. 5, it was found that LPS increased the release of IL-6 and the addition of apelin suppressed cytokine release. The 24-hour incubation of cells with 1 mcg/ ml LPS increased TNF-α transcription by about 1.36 fold compared to control, while the addition of 10 nM apelin brought this increase to a very low level (0.06 fold) (Figure 6). In astrocytes exposed to 1 mcg/ ml LPS for 24 hours, IL-1β mRNA expression increased by 15.2 fold compared to the control group; however, 10 nM apelin combined with LPS with for the same concentration and duration had significantly decreased the IL-1β level by 33.4 fold (Figure 7). qRT -PCR analyses showed that IL-6 transcription was increased approximately 4-fold in the incubation of astrocytes with 1 mcg/ ml LPS for 24 hours compared to the control group (Figure 8) In Figure 9, NO amounts of culture media are given. LPS in astrocytes significantly stimulated NO production, while apelin administration was found to significantly reduce this effect.

Alzheimer's, Parkinson's and Huntington's Diseases and Multiple and Amyotrophic Lateral Sclerosis are diseases accompanied by cognitive disorders with slow and progressive neuronal loss in the central nervous system. These diseases affect millions of people around the world and they have an important place due to the expenses made for the treatment of the disease in the period following diagnosis. Since the underlying causes of these diseases have not yet been fully elucidated, specific therapies are still not available today. According to scientific researches, free radicals that have accumulated as a result of neuroinflammation and OS in CNS have an important place in the pathogenesis and prognosis of these diseases. However, there is a need for new effective alternative agents to prevent inflammation and stress-induced degeneration of the CNS for both preventive and therapeutic purposes (12). There are some findings that the apelin molecule, which is naturally produced in the human body and is known to have an active role in many physiological events, may be an alternative for this purpose. The functions of apelin-13, which is known to have immunomodulatory effects and have receptors in

neurons and astrocytes, have not yet been elucidated. Therefore, this study may be a Pioneer in this field.

Although it is known that apelin-13 is expressed in many regions of the brain, its role in neuroinflammation and related OS in the CNS has not yet been fully investigated (13).

In our study, we aimed to determine the effect of apelin on the changes in cellular viability that would develop with the cytotoxic and/or inflammatory effect of LPS. Kim et al. in 1999, performed MTT analyses to determine the LPS dose in the 264.7 macrophage-derived RAW cell line and determined 1000 ng (1 microgram/ ml) dose as a result of these analyses . Go HS et al. in 2009 (14) have shown the proliferative effect of LPS at low concentrations. In our study, a significant difference could not be obtained between the doses and the analyses durations , the most appropriate time and dose of 1000 ng/ml was used in accordance with the literature. Zou et al. in 2015 (15), performed MTT analyses to determine the effective dose of apelin-13 in the PC12 cell line, and there was no significant difference in apelin-13 doses on cell viability. In our study, LPS dose and duration were determined by MTT analysis and in order to determine the appropriate dose for apelin, in addition to 1000 ng LPS, doses of 1-2.5-5-10-25-50-100 nM of apelin were added and after 24 hours effects on cell viability were evaluated by MTT analysis. Since no significant difference was observed between doses, a dose of 10 nM was selected, in accordance with the literature, to be used in the remaining stages of the study.

Astrocytes and microglia are also responsible for the secretion of many neurotrophic factors (16). These cells produce a proinflammatory signaling molecule after activation with various stimuli such as LPS. The common of these molecules are proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12, etc.), macrophage inflammatory protein (MIP)-1, monocyte chemo-attractant protein (MCP)-1, growth factors, complement molecules, chemokines, and cell adhesion molecules (17). These cells are also among the primarily responsible cells in the pathogenesis of neurodegenerative diseases (18). In our study, the effects of C8D1-A astrocyte cells with LPS stimulation and the effects of apelin administration were investigated by ELISA method for protein levels of TNF-α, IL-1β, IL-6, and IL-10. At the end of the study, TNF-α, IL-1β, IL-6, protein levels of proinflammatory cytokines were found to be significantly higher in LPS-treated groups and lower in LPS+ apelin group. The anti-inflammatory cytokine, IL-10, was found to be low in the LPS group and high in LPS+ apelin group.

Cytokines are polypeptides and the most important ones in inflammation are interleukins (IL) and tumor necrosis factor-alpha (TNF-α). Particularly, IL-1β and TNF-α share many common biological characteristics. Both are secreted by activated macrophages, lymphocytes, and other cell types and are called proinflammatory cytokines (19). IL-6, IL-1β, and TNF-α are also in the proinflammatory cytokine group. In our study, mRNA expression of TNF-α, IL-1β, IL-6, and IL-10 produced by the stimulation of C8-D1A astrocytes with LPS and the addition of apelin was investigated by RT-PCR. As a result of the study, only the LPS stimulated group showed increased proinflammatory cytokines- TNF-α, IL-1β, IL-6- levels than the control group. On the other hand, TNF-α, IL-1β, and IL-6 mRNA levels were significantly lower in the apelin+ LPS group than the control group. The results we found with both RT-PCR and ELISA method strongly support the hypothesis that apelin does anti-inflammatory effects in the CNS.

Apelin reduces the production of OS and ROS by inducing enzyme activity and level of catalase (20). In our study, NO level was investigated by the Griess method. The nitrite level in the LPS group was higher than the LPS + apelin group. These findings indicate that apelin may suppress OS and its products in both CNS and peripheral tissues.

4. CONCLUSION

The results of this study revealed the antiinflammatory, antiapoptotic and antioxidant effects of apelin on the development of neuroinflammation and oxidative cell damage, which play a role in the development of the immune response in the CNS. Thus, this molecule may have a role in the treatment of neurodegenerative diseases with inflammation. These results will give some insight in to the studies to be carried out on this subject and for new agents to be used in the treatment of neurodegenerative diseases

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