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Investigation of cytokine changes and deiminated proteins in LPS-induced inflammation in BV2 microglial cells

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ABSTRACT

Background: Recent studies show that, deimination, one of the post-translational modifications, is associated with the neurodegenerative disease process. Peptidyl arginine deiminases (PADs) catalyze deimination, PAD2 is particularly active in the central nervous system. This study aimed to examine the changes in proteins regarding deimination by inducing inflammation with lipopolysaccharide (LPS) in the BV2 microglial cell line and observe the changes in cytokines.

Methods: LPS was applied to the microglial cell line. The change in Interleukin-1β (IL-1β) was observed by enzyme linked immunosorbent assay (ELISA). Western blotting with F95 antibody was performed to identify deimine proteins. To determine whether C-reactive protein (CRP) was changed, immunoprecipitation with anti-CRP antibody or not was followed by western blotting with F95 antibody. Real-time polymerase chain reaction (RT-PCR) was performed to determine the change in PAD2 and CRP expression levels.

Results: A significant increase in IL-1β due to inflammation was observed in microglia. An increase in the proteins subjected to deimination was observed by Western blot method and it was determined that CRP was deiminated. A statistically significant decrease in PAD2 expression level was observed by RT-PCR.

Conclusions: In the present study, an increase in IL 1-β levels and the amount of deimination protein was observed as a result of inflammation.This result confirms that there is a connection between neurodegeneration and deimination. This study is the first to show that CRP is one of the deiminated protein candidates as a result of inflammation in microglia.

Keywords: CRP, deimination, neurodegeneration, PAD2, western blot

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INTRODUCTION

Neurodegenerative diseases are known as the most common health problems of today. An increase in the incidence of neurodegenerative diseases has been detected in direct proportion to the increase in average life expectancy in humans [1]. The development and onset of neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), stroke, traumatic brain injury, etc., are significantly influenced by microglia-induced neuroinflammation. They all result from the damage that progresses to irreversible neuron and glial cell loss [2]. Changes in microglia cellular morphology are observed in neurodegenerative diseases. Although the initial role of microglia is neuroprotective when activated, it can later evolve into a chronic neuroinflammatory response in the central nervous system (CNS) [3].

When CNS pathology is examined, changes in immune functions are observed, including the microglial response, as well as phagocytosis and the production of both cytokines and neurotrophic factors. Cytokine levels are known to increase in response to conditions such as disease, injury, and infection. They play an important role in tissue repair, especially in pathological conditions. Differences are detected in the levels of proinflammatory cytokines interleukins (such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) [4]. Microglial release of these cytokines and their role in neuronal degeneration are important [5,6]. It has been determined that the increase in cytokine levels is associated with the pathogenesis of neurodegenerative disease [7].

In neuroinflammation, the inflammation is combated by changing the secretion levels of some molecules

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(such as cytokines) to protect neurons. Changes occurring through posttranslational changes may negatively contribute to acute and chronic brain disorders. It is important to know when these changes are protective or vice versa [8,9].

Deimination (citrullination), which is one of the posttranslational modifications, is known as the conversion of arginine to citrulline in proteins. The conversion occurs through peptidyl arginine deiminase (PAD) enzymes. There are five isoforms of PADs that are known, PAD2 being the most active one in the brain [10]. Deimination results in a decrease in the net charge of the targeted proteins. This causes a structural change in the protein and alters both intra- and intermolecular ionic interactions [11].

The complement system has been demonstrated to have a function in inflammation as a defence against pathogens and homeostasis. It has also been demonstrated that the complement system is involved in pathological disorders that can be both acute and chronic [8]. According to research, astrocytes, microglia and neurons respond to the inflammatory state, while structures such as microglia can also synthesize various factors, receptors and regulators for complement proteins [12]. Likewise, it has been observed that the immune system is activated in various processes such as disruption of the blood-brain barrier in neuroinflammation [8].

Pentraxins are a phylogenetically well-conserved protein family. They create a species-specific acute phase response. Moreover, c-reactive protein (CRP) is a member of this family and it plays an active role in immune responses in inflammatory events [13]. It is thought to be important in modulating inflammation. Circulating CRP levels are already used as a marker of inflammation, while CRP in brain tissue has been linked to neurodegenerative diseases [14]. The present study aimed to observe both protein changes in terms of deimination and cytokine changes by inducing inflammation with lipopolysaccharide (LPS) in BV2 cells.

METHODS

Chemicals and reagents

The chemicals used in the cell culture stages were obtained from Life Technologies/Gibco. LPS-Sigma-Aldrich (L2630-10MG) was used. Mouse Interleukin 1 Beta ELISA kit based on the double antibody sandwich technology principle of Shanghai Coon Koon Biotech (Cat No:CK-bio-14856) company was used to detect the cytokine change. For cDNA production, a two-step RT-PCR kit (Vivantis, Malaysia/Pr No: PL8881) was used. Primers: Commercial primers for CRP (C-reactive protein), PAD2 and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) genes were obtained from Roche RT Ready Assay (Roche-Germany / Catalog No: 06720455001). Roche ProbeMaster (Catalog No: 04707494001 / Roche-Germany), which can bind to double-stranded DNA, was used as an intercalating agent. It was performed in RT-PCR strips (Axygen, USA / Catalog No: PCR-2CP-RT-C). Proteins to be used for Western blotting were isolated from BV2 cells using a lysis buffer commercial kit (Fermentas, ProteoJETTM Mammalian Cell Lysis Reagent, #K0301, USA). Primary antibodies Anti-CRP [Affinity, DF: 6027], F95 [Merc-MABN328], and housekeeping antibody β-actin [Santacruz, 1615], and secondary antibody donkey anti-mouse IgG (Abcam-6802) was provided. Immunoprecipitation kit (Merck, England). The lysis buffer used in Western blot was commercial kit Fermentas, Mammalian Cell Lysis Reagent, #K0301, Electrophoresis, spectrometer (Eppendorf BioPhotometer Spectrophotometer, Germany), TurboTM Transfer system BioRad Laboratories, Inc., #170-4155 was used for protein transfer and blocking. BioRad, Immun-Blot®, #1620177 was used as PVDF membrane, skim milk (BioRad, Non Fat Dry Milk, USA). Protein Isolation Reagents (RIPA buffer veya NP-40), Sigma-Aldrich, Thermo Fisher. BioRad Clarity Western Blot Substrate chemiluminescence peroxidase, #170-5060, USA solution was used to detect the target protein. BioRad Clarity Western Blot Substrate chemiluminescence, for detection of target protein (Peroxidase, Luminol ensahnsir #170-5060, USA) solution was used. Proteins were visualized in western blot using Licor Odyssey C-DiGit® Blot Scanner, Software USA.

Cell culture

BV2 microglial cell line was gifted by Izmir Genome Institute. We would like to thank Fatma Nihan Cankara for her contributions. The study made use of a gift of BV2 microglial cell line. Cells were grown in Dulbecco's Modified Eagle Medium (5% FBS plus 100 mg/ml streptomycin and 100 U/ml penicillin) in an incubator at 37°C under 5% CO2. Bacterial LPS is known to be recognized by the immune system, eliciting strong pro-inflammatory responses that can cause serious health problems [6]. In this investigation, LPS was used as a proxy for inflammatory mediators. One set of cells received a 24-hour treatment with LPS (1 µg/ml) [6], while the other group was maintained as the control.

Cell viability assay

The 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (MTT) was used to measure the vitality of microglia cells [9]. In 96-well plates, BV2 cells were plated at a density of 2 × 104 cells/well. The cells were treated with 0.5 mg/ml of MTT, and they were incubated for one hour at 37°C. The vessels were shaken for ten minutes following the addition of DMSO. Using a Bio-rad xMark microplate reader, absorbance was measured at 570 nm. An average was used to determine cell viability. The formula used to measure cell viability was % Viability = (OD assay/OD control) x 100%.

Enzyme-Linked Immunosorbent Assay (ELISA)

Eluates obtained from cell culture were determined quantitatively (n=3). Sterile tubes were used to collect the supernatant from cell cultures. After centrifuging it for about 20 minutes at 2000–3000 RPM, the supernatant was carefully removed. The IL-β level was ascertained in compliance with the guidelines provided by the manufacturer, Shanghai Coon Koon Biotech. Values presented are the mean ± SD of three independent experiments.

Immunoprecipitation

Using monospecific polyclonal mouse anti-CRP antibodies, immunoprecipitation was carried out using the Catch and Release v2.0 Reversible Immunoprecipitation System (Merck, UK) in accordance with the manufacturer's instructions. Western blotting was used to elute and evaluate bound CRP proteins [13].

Western Blotting

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was carried out using 5-20% Mini-Protean TGX protein gels following immunoprecipitation. With the help of the monoclonal mouse IgM pan-deimination F95 [14] antibody, which recognizes the citrulline protein, CRP was examined for post-translational protein deimination. As previously mentioned, western blotting was used to evaluate the proteins [6]. Primary antibodies anti-CRP, F95, PAD2 and housekeeping β-Actin, as well as donkey anti-mouse IgG secondary antibodies were used.

RNA isolation, quantity and quality determination

Total RNA was extracted with TRIzol (Invitrogen), and 0.5µg of RNA was used to obtain cDNA. RNA quantity and quality were measured with a spectrophotometer at 260 and 280 nm (ACT Gene, USA).

Real-time quantitative polymerase chain reaction

After obtaining cDNA, primers of selected genes were amplified in RT-PCR using cDNAs to determine gene expression differences. The data obtained as a result of optical analysis in RT-PCR was recorded as Cq. As a negative control, the same amount of ddH2O was used instead of cDNA as nonscheduled control. Data were analyzed by the 2-ΔΔCt method with normalization based on the housekeeping gene GAPDH. E =-1 + 10(-1/slope) was used to calculate the efficiency of-ΔΔCt PCR analysis. The 2-ΔΔCt technique was then employed to compute the relative gene expression analysis. For each group, Ct values were first obtained for the housekeeping gene and the target gene. The formula utilized to determine Δct was as follows: Δct = Ct values of target genes – Ct values of housekeeping gene. Their 2-ΔΔCt was computed independently.

Statistical analysis

The data of each group were obtained and statistical analysis was conducted to examine the relationship between the groups. The experimental steps were repeated three times. The difference between groups was compared by applying parametric, nonparametric tests according to the distribution of the data. Brown-Forsythe test was used for multiple groups. One-way ANOVA and Tukey's multiple comparisons test were used to analyze differences between treatment groups. For all statistical processes, the GraphPad Prism® V.6.00 (USA) software was utilized. The information is displayed as the average ± standard deviation of three separate tests. Significant differences were defined as $p<0.05$ or $p<0.01$.

Ethics Committee Approval: decision number E-12866609-300-35056 dated 07.04.2021 and numbered 09/05.

RESULTS

MTT cell viability

Cell viability at different doses of LPS was examined by MTT assay. Cell viability according to the MTT assay is shown in Figure 1. The cell line was exposed to varying concentrations of LPS ranging from 0.05 to 1 µg/ml without exhibiting any discernible reduction in viability. The viability of the cells did not significantly change. Other studies continued from the section where LPS was administered at 1 µg/ml. It has been shown that low-dose LPS (1 µg/ml and below) does not have a significant effect on cell viability. In our study, focusing on the 1 µg/ml dose is reasonable, and this dose can be selected as the optimal concentration for observing inflammatory responses.

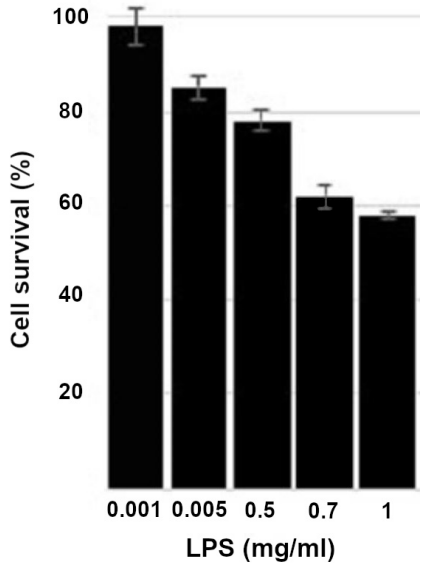

Figure 1. Cell survival analysis after LPS exposure.

IL-1β change

ELISA was applied to the proteins obtained from cell culture in order to compare the change in the level of IL-1β, one of the cytokines [15]. In our study, IL-1β levels were significantly increased in the LPS-treated cell group compared to the control group (Figure 2). This result suggests that LPS effectively induces an inflammatory response in the treated cells, as IL-1β is a key pro-inflammatory cytokine involved in initiating and amplifying the immune response. The elevated IL-1β levels highlight the activation of inflammatory signaling pathways, consistent with LPS's known role in triggering cytokine release through the TLR4 receptor. These findings align with previous studies demonstrating LPS-induced cytokine production, reinforcing the validity of our experimental model in studying inflammation.

Identification of deiminated proteins by western blot

To identify deiminated proteins, Western blotting was performed using F95 antibody after protein isolation. Different bands were observed in the range of 25-250 kDa when compared to the control group (Figure 3A). F95 western blot analysis clearly shows a wide range of increased deiminated proteins, which is a result of the inflammatory response in microglia. Western blotting of deiminated CRP with F95 antibody after immunoprecipitation with anti-CRP resulted in a CRP band at roughly 25 kDa (Figure 3B). Citrullination of CRP means the conversion of arginine residues to citrulline. This shows that it is important to study the post-translational modifications of CRP that play a role in inflammatory processes. These modifications may alter the biological activity of CRP and its role in the inflammatory response.The band in Figure 3C, which is located at about 75 kDa, shows that PAD2 has been altered by LPS. Consistent with the expression level, a decrease in PAD2 protein is seen in the Western blotting image. These findings demonstrate the dynamic changes in protein deimination and PAD2 expression, reinforcing the role of PAD2 in the regulation of inflammation and immune responses under LPS stimulation.

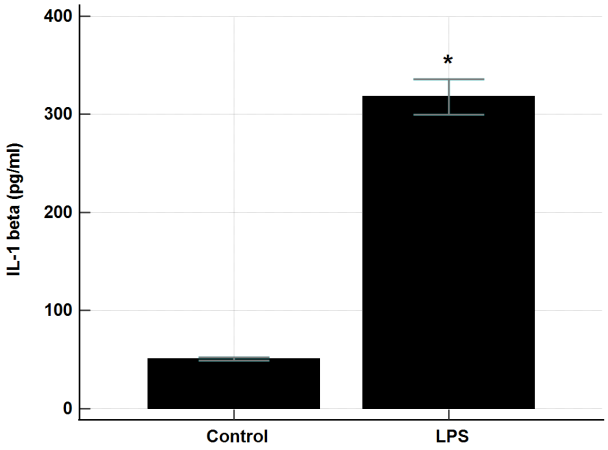

Figure 2. Secretion of IL-1β in cell cultures.

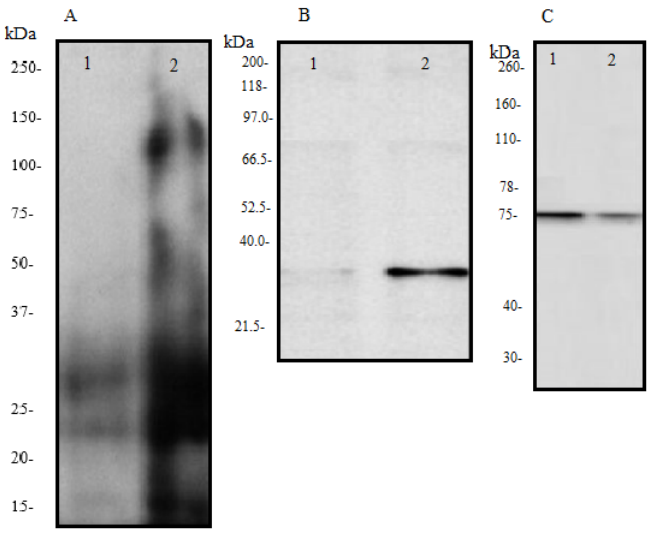

Figure 3. Western blot analysis of isolated proteins. (A) control vs stimulated; (B) deiminated CRP; (C) PAD2.

RT-PCR

The 2-ΔΔCt method was used to calculate the relative gene expression analysis. The microglial cell line's variations in PAD2 and CRP expression levels were investigated using the RT-PCR technique. The analysis of the data repeated 3 times is shown below (Figure 4). mRNA expression levels of PADI2 were significantly lower compared to the non-LPS control group ($P < 0.01$). This reduction in PADI2 expression was further validated by Western blot analysis, confirming the decrease in PAD2 protein levels, consistent with the gene expression findings. Interestingly, despite changes in PADI2 expression, no significant change in CRP expression was observed. However, given that we have shown via western blot that CRP can be deiminated, this suggests that CRP may be differentially regulated in the inflammatory response without a change in its expression. Western blot also confirmed the certain level of decrease in PAD2. These results reveal that PAD2 is responsive to LPS, whereas CRP expression remains unaffected under the same conditions.

DISCUSSION

Neurodegenerative illnesses like AD, PD, MS, and traumatic brain injury are significantly impacted by chronic inflammation in the brain. Microglia appears as the main effector when the pathological process is considered [16]. Numerous studies have employed the BV2 microglial cell line to identify cellular alterations in neurodegeneration. [17–20]. In microglia, post-translational changes of proteins that take place during life, such as methylation, acetylation, glycosylation, and phosphorylation, have been investigated [21, 22]. Protein deimination has been studied in many areas including

neurodegenerative diseases, cancer and autoimmune diseases [23]. This study examined the effects of inflammation on deiminated protein in BV2 microglia cells and found that there was an increase in the quantity of deiminated protein. Immunoprecipitation was used to study CRP deimination, and the results showed that CRP was deiminated after the inflammatory process. Previously, investigations have employed the F95 antibody, which we used in our investigation to discover deiminated proteins [11,24]. Deiminated proteins are generally not seen in the normal brain, but when detected in the brain, they have been associated with Parkinson's disease [23,24], AD [25–28]. For instance, the presence of misfolded α-synuclein protein in PD has been associated with increased protein deimination [16]. In many studies conducted in PD [16,24] and different brain areas, these have been found to have proteins that undergo deimination in conjunction with F95 labeling. Numerous fields have examined the deimination process, including astrocyte, oligodendrocyte, and neuron. However, detailed research has not been done on microglia. Our study's observation of an increase in deiminated protein in microglia may be related to the processes underlying neurodegenerative diseases. It may be an option in developing treatment options during the disease process.

Increases in CRP structural changes due to neuroinflammation have been observed in different parts of the brain [13,14]. It has been proposed that patients with Parkinson's disease have higher plasma CRP levels and that this illness is linked to a higher chance of developing Parkinson's disease. A different study showed the association between beta-amyloid (Aβ) plaques of different forms of CRP in AD patients. There is not much data regarding CRP deimination. It has been shown that CRP can undergo deimination in mucus, serum and brain samples from different living groups [26]. CRP has been linked to plasma deimination candidates in animal models of pre-motor Parkinson's disease [24]. Nevertheless, a direct connection to microglia has not been proven. In our study, we aimed to examine the deimination of CRP protein depending on enzyme activity in microglia. No change was detected in CRP expression levels. However, to determine the deimination ability of CRP, we detected an approximately 25 kDa CRP band in our western blot analyzed with anti-CRP and F95 antibodies. Deimination of CRP occurs in microglia due to inflammation and this result overlaps with studies in the field. LC-MS/MS analysis is required to fully identify other proteins.

PAD enzymes convert arginine residues on target proteins to citrulline residues in the presence of calcium ions and are associated with deimination in the brain. The presence of PAD2 has been demonstrated in studies conducted in different regions of the brain. Increased protein deimination and dysregulated PAD activity have been observed in neurodegenerative disease [10]. In studies conducted to examine deimination, PAD2 was detected in macrophages and it has been shown that PAD2 is especially expressed in monocytes and monocyte-derived macrophages [29]. For example, in a study on microglia [30], they found that PAD2 immunoreactivity was present in both astrocytes and microglia. In many studies, PAD2 has been thought to be concentrated mainly in astrocytes [29,30]. In another study, numerous deiminated proteins were detected when different regions of the brain were labeled with F95, without a macroscopic increase in PAD2 in tissue analysis following

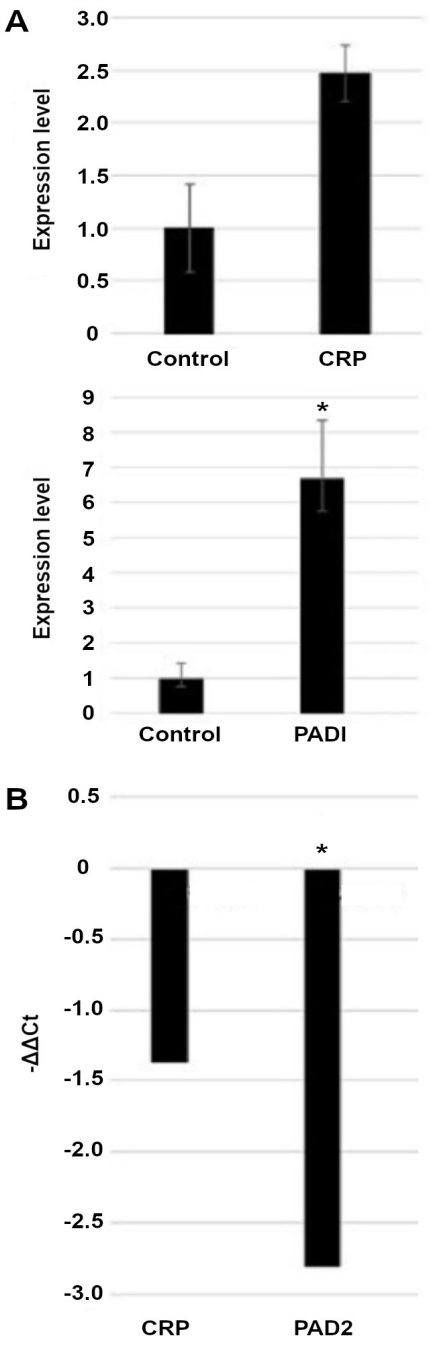

Figure 4. Relative gene expression analysis by RT-PCR. (A) Control vs CRP and PADI, respectively; (B) CRP vs PAD2 relative expression.

neuroinflammation [31]. This confirms that the activity of PAD2 changes following inflammation. In a study [32] conducted in the rat brain, they detected an increase in the PAD2 expression level and the amount of deiminated protein due to inflammation. They detected a large number of deiminated proteins following neuroinflammation in different brain cell groups, but stated that they did not observe a macroscopic increase in PAD2 in these regions [25]. In later years, the same researcher examined the inflammation process, especially in microglia, and measured the PAD2 level on different days. It was stated that the amount of PAD decreased on the 1st day and then increased until the 7th day [33]. In the present study, we found that the level of PAD2 expression has decreased statistically significantly. According to data from past investigations, PAD2 reacts to inflammation, and this enzyme activity is linked to deimination. The alteration in PAD2 expression could potentially enhance comprehension of inflammation-associated pathways and their impact on the progression of illness. Future research on the function of PAD2 and the pathogenic processes connected to this enzyme will be crucial.

Receptors on the surface of neurons are activated by stimulation of inflammatory cells and proinflammatory cytokines are released [21]. A study carried out on peripheral blood in AD revealed significant associations between neurodegenerative illnesses and elevated levels of cytokines including IL-1β and tumor TNF-α. When Parkinson's patient samples were examined, significant relationships were seen between the course of the disease and high levels of IL-1β, CRP, IL-6, TNF, IL-2, IL-10 [5]. In this study, in agreement with previous data, we detected a significant increase in IL-1β levels in LPS-treated cell lines compared to control groups. This increase shows the effectiveness of LPS on microglia.

Limitations of the study: In our study, we looked at the deimination of proteins in microglia following neuroinflammation and observed an increase in the deimination of proteins by Western blotting. However, LC-MS/MS is required for detailed protein identification.

CONCLUSIONS

LPS to the BV2 microglial cell line was applied to induce neuroinflammation. In our study, a significant increase in IL-1β levels was found as a result of inflammation. PAD enzymes work in deimination, which is one of the posttranslational modification (PTMs). PAD2, in particular, is responsible for activity in the brain. It has been shown that irregularities in PAD expression occur in brain tissue due to inflammation and this is associated with deimination. We found a significant decrease in PAD2 expression levels in our investigation. A deeper comprehension of inflammation-related pathways and how they impact the progression of disease may be facilitated by alterations in PAD2 expression. It is imperative to conduct additional research on the function of PAD2 and the pathogenic mechanisms linked to this enzyme. The deiminated proteins were identified using the F95 antibody. Western blotting revealed a rise in the quantity of deiminated proteins. Despite the decrease in PAD level, a deimination in protein groups was identified. This condition is primarily observed in relation to the enzyme's activity. Consistent with other studies, the amount of deiminated protein appears to be associated with inflammation. It is seen that CRP protein undergoes deimination. The present investigation is the first to demonstrate that CRP is one of the deiminated protein possibilities resulting from microglia neuroinflammation.

ABBREVIATIONS

Aβ – beta-amyloid
PD – Parkinson's disease
AD – Alzheimer's disease
ALS – amyotrophic lateral sclerosis
CNS – central nervous system
CRP – c-reactive protein
ELISA – enzyme-linked immuno sorbent assay
GAPDH – glyceraldehyde-3-phosphate dehydrogenase
IFN-γ – interferon-γ
IL1-β – interleukin-1 beta
LPS – lipopolysaccharide
MTT – 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide
OD – optical density (absorbance reading)
PAD – peptidyl arginine deaminase
PTM – posttranslational modification
RT-PCR – real-time polymerase chain reaction
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNF – tumor necrosis factor

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AUTHORS' CONTRIBUTION

GD – methodology, investigation, methodology, writing (review and editing)
MG – validation
NK – writing (original draft), visualization
EMÖ – data curation and management
ÇDD – formal analysis

CONFLICT OF INTEREST

None to declare.

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