Kynurenic acid and its analog SZR104 exhibit strong antiinflammatory effects and alter the intracellular distribution and methylation patterns of H3 histones in immunochallenged microglia-enriched cultures of newborn rat brains

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1. INTRODUCTION

Several recent experimental and clinical studies have found that endogenous tryptophan metabolites, including kynurenic acid (KYNA), are involved in a number of neurophysiological and neuropathophysiological mechanisms and play a crucial role in the regulation of the immune response. However, since KYNA does not pass the blood–brain barrier, the synthesis of KYNA analogs (for example, SZR104; N-(2-(dimethylamino)ethyl)-3-(morpholinomethyl)-4-hydroxyquinoline-2-carboxamide) that can penetrate this barrier could lead to possible treatments for neurodegenerative or neuroinflammatory disorders.

Microglial cells are the intrinsic immune cells of the central nervous system (CNS). They possess complex cellular and molecular mechanisms that detect deviations from homeostasis in nervous tissue. At the site of damage or inflammation, activated microglia change their morphology, express increased levels of major histocompatibility antigens, and become phagocytic. They also release inflammatory cytokines and other potentially cytotoxic substances that can amplify inflammatory responses by activating and recruiting other cells to a lesion or infection. For example, inflammation can be exacerbated by the secretion of the C–X–C motif chemokine ligand 10 (CXCL10) from microglia or other cell types. CXCL10 induces chemotaxis, apoptosis, the inhibition of cell growth, and angiostasis. Both CXCL10 and its receptor (the C–X–C motif chemokine receptor 3) are crucial for leukocyte trafficking and homing to inflamed tissues, as well as for the perpetuation of inflammation that leads to tissue damage or involved in the pathophysiology of multiple sclerosis. Similarly, the C–C motif chemokine receptor 1 (CCR1) and its ligands may play a role in the pathogenesis of multiple sclerosis.

DNA in the nucleus is wrapped around proteins known as histones, which form the chromatin structure. The capability of eukaryotic cells to maintain their diverse phenotypes is ensured by the chemical modifications of the DNA molecule, the activities of chromatin-associated proteins, and numerous posttranslational modifications (PTMs) of the histone proteins. Although histones are typically located inside the nucleus, where they regulate transcription, they have a wide range of functions in various cellular and extracellular locations as well. When they are in the extracellular milieu, they become damage-associated molecular patterns that promote inflammation, cytotoxicity, coagulation, and apoptosis. Histone modifications (acetylations, methylations, phosphorylations, ubiquitinations, etc.) are PTMs made in the nucleus by the appropriate enzymes. Consequently, transcription often becomes altered because modified histones and the DNA will interact differently. The cytoplasmic accumulation of such modified histones might indicate that they were released/transported from

the nucleus, perhaps because of distress. Histone modifications are key epigenetic regulatory features that govern many cellular functions. Specific histone PTMs can direct site-specific activation or silencing of transcription; hence, they are the principal players that regulate gene expression. Histone methylations at lysine (lys (K)) and arginine residues are relatively stable and considered potential marks for carrying the epigenetic information present in specific regions of the genome. For example, several monomethylations (i.e., H3K9me1, H3K27me1, and H3K79me1) and some dimethylations of histone H3 proteins (H3K36me2) are linked to active transcription, while other dimethylations (H3K9me2) and most trimethylations of this core histone (i.e., H3K9me3, H3K27me3, and H3K79me3) are linked to gene repression.

In this study, we investigated the relationships of KYNA and its analog SZR104 toward (a) the change in phagocytotic activity of the microglial cells, (b) the inflammatory mechanisms that give rise to epigenetic changes *via* histone methylations, and (c) the intracellular distribution/localization of unmethylated and methylated histones in microglial cells. Besides the inflammatory markers CXCL10 and CCR1, we quantitatively analyzed the levels of unmodified core histone H3 and histone H3 lys methylations at the H3K9me3 and H3K36me2 sites, marks that are considered contrary in regulating gene expression and also involved in immunomodulation, using western blots and multicolor light microscopic immunofluorescence.

2. SPECIFIC AIMS

In the CNS, the kynurenine pathway is involved in several physiological and -pathophysiological mechanism and its immunomodulatory properties in vertebrates are all well-established. As KYNA was shown to mediate immunosuppressive effects, we felt it was important to study inflammatory mechanism and microglia function using an analog that could penetrate the blood-brain barrier and hence has the potential to provide possible treatments for neurodegenerative or neuroinflammatory disorders.

Microglia possess complex cellular and molecular mechanisms that detect deviations from homeostasis in nervous tissue. In activated state, they release inflammatory cytokines and other potentially cytotoxic substances, such as CXCL10 or CCR1 and its ligands, that can amplify inflammatory responses by activating and recruiting other cells to a lesion or infection. Histone modifications are key epigenetic regulatory features that govern many cellular functions (for example, inflammation, coagulation, apoptosis).

In this study, our specific aims were:

1) to determine whether KYNA and its analog SZR104 alter microglial function (e.g., phagocytotic activity);
2) to determine whether KYNA and SZR104 induce the inflammatory mechanisms that give rise to epigenetic changes *via* histone methylations in microglial cells;
3) to determine whether KYNA and SZR104 induce changes in intracellular distribution/localization of unmethylated as well as methylated histones in microglial cells.

3. MATERIALS AND METHODS

In this study, we used purity-checked secondary microglial cultures prepared from the forebrains of newborn Sprague-Dawley rats. On subDIV6, the expanded microglia-enriched secondary cultures were treated for 24 h with bacterial lipopolysaccharide (LPS) alone (20 ng/mL final conc., dissolved in Dulbecco's Modified Eagle's Medium (DMEM)), KYNA alone (1 μM final conc., dissolved in DMEM), SZR104 alone (1 μM final conc., dissolved in DMEM), or with a combination LPS + KYNA or LPS + SZR104. LPS treatment served as an immunochallenge. The following six culture types were used: (a) control (unchallenged and untreated) cultures, (b) 20 ng/mL LPS-stimulated cultures, (c) 1 μM KYNA-treated cultures, (d) 1 μM SZR104- treated cultures, (e) LPS-challenged + KYNA-treated cultures (at indicated doses), and (f) LPS-challenged + SZR104-treated cultures (at indicated doses). The secondary cultures were assessed using immunocytochemical and western blot techniques employing antibodies against a microglia-specific protein (CD11b/c), two inflammation markers (CXCL10 and CCR1), one unmodified core histone (H3) and two specific histone H3 lys modifications (H3K9me3 and H3K36me2). The fluid-phase phagocytotic capacity of the microglial cells was determined *via* the uptake of fluorescent microspheres (2 μm diameter; 2 μL 2.5% aqueous suspension of fluorescent microspheres in 2 mL DMEM; incubation lasted for 60 min).

Grayscale digital images of the blots were acquired by scanning the autoradiographic films with a desktop scanner (Epson Perfection V750 Pro). Images were scanned and processed at identical settings to allow comparisons to be made of the blot results obtained from different samples. The densities of immunoreactive lanes equally loaded with protein aliquots were quantified, and data values were presented as a percentage of the control. For statistical comparisons, a one-way analysis of variance (ANOVA) or Mann–Whitney rank sum test was used and a p value of < 0.05 was considered significant. Values are presented as the mean ±

standard error of the mean (SEM) from at least five immunoblots, one from each independent experiment.

Digital images were captured using a Leica DMLB epifluorescence microscope equipped with a Leica DFC7000 T CCD camera and *via* the LAS X Application Suite X (Leica). A quantitative microscopic analysis of cell specific and histone immunofluorescence and fluorescent microbeads was conducted in ImageJ (version 1.47). For the intracellular localization of histone immunolabels, the densities of the whole cell and the nucleus (mean gray values), their areas, and their integrated optical densities (fluorescence per area) were measured. The corrected total cell fluorescence (CTCF) values ($CTCF_{whole\ cell}$ and $CTCF_{nucleus}$) were then computed. Statistical comparisons were made using SigmaPlot and data were analyzed with a Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison procedures for statistically significant differences between the groups.

4. RESULTS

4.1. KYNA and SZR104 downregulate LPS-induced CXCL10 levels while differentially altering CCR1 levels

In CD11b/c-labeled microglial cells taken from unchallenged (control) and treated microglia-enriched secondary cell cultures (subDIV7), there was a robust increase in the amount of immunoreactivity of the inflammation marker CXCL10 in LPS-treated microglia. Following an LPS immunochallenge, typical ameboid morphology was observed with CXCL10 immunoreactivity localized in the cytoplasm. Treatments with KYNA or SZR104, either alone or in combination with LPS, reduced CXCL10 immunoreactivity to unchallenged levels. Moreover, quantitative light microscopic microdensitometric analysis of CXCL10 protein expression revealed that LPS challenge significantly elevated CXCL10 immunoreactivity (approximately fourfold) in microglial cells, whereas KYNA alone, SZR104 alone, or either combined with LPS significantly depleted the CXCL10 immunoreactive signal to control (unchallenged) levels. A similar but weaker response was recorded when the immunoreactivity of CCR1, another inflammation marker, was tested in control and treated cells. A localization analysis in CD11b/c-labeled microglial cells revealed slightly increased CCR1 immunoreactivity in LPS-challenged cultures relative to that observed in the control. A quantitative light microscopic microdensitometric analysis of the cellular CCR1 levels of the cultures showed that an LPS challenge significantly elevated CCR1 immunoreactivity by 48%, whereas KYNA or SZR104 alone, or the combined treatment with LPS + KYNA, proved

ineffective. Interestingly, the combined treatment of LPS + SZR104 significantly lowered CCR1 immunoreactivity as compared to LPS-treated cultures, and it reverted to a level observed in unchallenged (control) cultures. A quantitative western blot analysis revealed that cytoplasmic CCR1 immunoreactivity was significantly increased after LPS treatment. KYNA or SZR104, either alone or in combination with LPS, did not noticeably affect CCR1 levels.

4.2. KYNA and SZR104 inhibit microglial phagocytosis

The treatment of the secondary microglial cultures with LPS increased the phagocytotic activity of the microglia cells significantly. The unstimulated cultured microglia cells display a basal phagocytotic activity: the number of microbeads in a single cell is typically two. Following the LPS challenge, this number rises sharply up to nine per cell. Treatment of the cell culture with KYNA and SZR104 before the LPS challenge prevented the increase in the number of phagocytosed microbeads; the phagocytotic activity of the microglia in these cases remained at the control level. The inhibitory effects of KYNA and SZR104 were statistically significant. Furthermore, quantitative western blot analysis of Iba1 expression demonstrated that the Iba1 content of the secondary cultures did not change significantly following the different pharmacological (LPS, KYNA and SZR104) treatments.

4.3. KYNA and SZR104 alter the intracellular histone H3 distribution and H3 lys methylation patterns

Unmodified histone H3 proteins form a pool for further PTMs. H3 immunoreactivity was detected in both the cytoplasm and the nucleus of unchallenged microglia; that is, after nuclear import, histone H3 mostly accumulated in the nucleus. Interestingly, most of the experimental manipulations of the cells, except for the LPS + SZR104 treatments, resulted in the increased accumulation of histone H3 in both the nuclear and the cytoplasmic compartments, indicating that both compounds elevated *de novo* synthesis and increased nuclear import. Of these treatments, KYNA produced the strongest nuclear accumulation of unmodified H3 immunoreactivity. When LPS and SZR104 treatments were combined, however, neither the nuclear nor the cytoplasmic H3 immunoreactivities were different from the controls. Quantitative densitometry of H3 immunosignals on cultured microglia corroborated these findings. The CTCF values for nuclear localization increased significantly only after LPS or KYNA treatments, compared to controls, but decreased significantly after the combined treatments of LPS + KYNA and LPS + SZR104, as compared to LPS-challenged cultures. In contrast, CTCF values for cytoplasmic localization were higher in all experimental groups

except for the LPS + SZR104 treatment case. Moreover, cytoplasmic H3 levels were affected differently by the combined treatments, i.e., LPS + KYNA increased, while LPS + SZR104 returned cytoplasmic H3 levels to controls. The nuclear and cytoplasmic histone H3 distributions elicited by the LPS + SZR104 treatment were rather like those of unchallenged microglia.

However, when the amount of unmodified cytoplasmic histone H3 was quantitatively analyzed *via* western blots, only the LPS + KYNA treatment showed a significant increase. This might be due to denaturing conditions in detecting histone H3 proteins in westerns that concealed the changes observed in multicolor immunocytochemistry when individually identified microglia were analyzed. Our analysis of the intracellular localization of H3K9me3 immunoreactivity in CD11b/c labeled microglia in unchallenged (control) and variably treated microglia-enriched secondary cell cultures (subDIV7) demonstrated that LPS challenge increased H3K9me3 immunopositivity relative to that observed in the unchallenged control or other treatments.

Strong histone H3K9me3 immunoreactivity was detected in both the nucleus and cytoplasm after the LPS challenge but decreased when LPS treatment was combined with KYNA or SZR104. Quantitative fluorescent microdensitometry revealed that the nuclear accumulation of H3K9me3 protein increased significantly after LPS treatment but that the LPS + KYNA or LPS + SZR104 treatments reduced this accumulation. However, the effect of KYNA alone did not differ significantly from that of the control. By contrast, SZR104 had a greater inhibiting effect on the extranuclear translocation of H3K9me3. Similar values and tendencies were noted when cytoplasmic H3K9me3 was analyzed: KYNA or SZR104, either alone or in combination with LPS, was able to recover LPS-induced cytoplasmic accumulation of H3K9me3 protein. Furthermore, SZR104 was found to be more potent than KYNA in inhibiting H3K9me3 translocation to the cytoplasm.

When the intracellular distribution of H3K36me2 immunoreactivity in CD11b/c labeled microglia was measured, a marked increase, relative to unchallenged control levels, was seen exclusively within the nuclei of the microglia after LPS challenge. By contrast, the other treatments did not affect H3K36me2 immunoreactivity appreciably. For example, LPS + SZR104 treatments returned the nuclear levels of H3K36me2 to the control levels. A quantitative microdensitometric analysis of H3K36me2-immunopositive signals revealed that LPS treatment dramatically increased the amount of H3K36me2 signal in the nuclei of CD11b/c labeled microglial cells, whereas the other treatments had only a minor effect. Moreover, KYNA or SZR104 alone, or the combination treatments, had significantly lower H3K36me2

signals compared to LPS-challenged levels. No extracellular histone accumulation was detected in these studies.

5. DISCUSSION

The endogenous kynurenine system is implicated in the functioning of the immune system. Recent studies showed that the amounts of several inflammation-related marker proteins decreased after treatments with KYNA or its analogs. Interestingly, a possible role for IDO, a key kynurenine pathway enzyme, in immunity has recently emerged, further emphasizing the crucial role this system plays in immunomodulatory functions. As KYNA is a metabolite of the endogenous kynurenine system with proven antiinflammatory properties, we wanted to know whether its effect went beyond conventional targets in the intermediary metabolic or intracellular signaling pathways and perhaps had effects on phenomena such as epigenetics elicited through histone metabolism and/or intracellular transport. We sought to investigate (a) how KYNA and SZR104, a brain-penetrable analog of KYNA, behaves in our systems; (b) how phagocytic activity change in microglia cells during our experimental conditions; (c) how pro- and antiinflammatory signals affect histone methylations and, consequently, epigenetic changes; and (d) how the intracellular localization of unmodified and methylated histones change during inflammation or amelioration of inflammation.

For the first time in the present study, KYNA and SZR104 were shown to exhibit strong antiinflammatory properties, as demonstrated by their effective amelioration of LPS-challenged CXCL10 and CCR1 production in microglial cultures. Our results agree with those of other studies that found similar CXCL10 reduction in activated microglia after treatment with other antiinflammatory drugs. While KYNA and SZR104 were both effective in ameliorating the LPS-induced elevation of CXCL10 immunoreactivity, only the combined treatment with LPS + SZR104 was effective in significantly inhibiting CCR1 immunoreactivity in these tests. Structural differences between KYNA and SZR104 might be the reason these compounds produced somewhat different responses in immunocytochemical and western blot analyses. As we used high-purity microglia, this was also the first time that we could identify microglia-specific immune responses to these compounds.

The number of internalized microbeads increased more than four-fold in the LPS-stimulated cells probably due to stimulation through Toll-like receptor 4 (TLR4) and transient receptor potential ankyrin 1, 4 receptors (TRPA1, TRPA4). These receptors mediated those membrane and cytoplasmic processes which lead to phagocytosis; they increase Ca^{++} permeability of the membrane and increase the cytoplasmic cation concentrations (TRPA

effects), and exert regulatory effects on gene expression (TLR4 effect). Treatments of the cells with KYNA and SZR104 prevented the increase in phagocytotic activity elicited by LPS. We speculate that during *in vitro* exposure, KYNA and SZR104 prevented the actions of LPS mainly through the repression of TLR4 by the deregulation of inflammation-related genes, consequently preventing phagocytosis. Furthermore, KYNA and SZR104 directly facilitated the expression of antiinflammatory tumor necrosis factor-stimulated gene-6 (TSG-6) and, at the same time, attenuated the tumor necrosis factor-α (TNF-α) production in monocytic cell cultures. These actions are probably mediated through the cytoplasmic aryl hydrocarbon receptor as experimental evidence suggests that KYNA is an endogenous ligand of the aryl hydrocarbon receptor-associated (AHR)-signaling pathways and inhibited phagocytosis in myeloid cells through AHR signaling.

The strong antiinflammatory action of KYNA and SZR104 on inflammatory marker proteins was accompanied by a similarly favorable regulation of histone methylation marks in microglia-enriched cultures. We demonstrated that KYNA and SZR104 reverted the H3K9me3 and H3K36me2 immunoreactivities toward levels observed in the control, i.e., unchallenged values, after immunochallenge by LPS treatment. Our findings also indicated that the cytoplasmic translocation of methylated H3K9 proteins from the nucleus after the LPS challenge was a cellular response to immunological distress. Cytoplasmic translocation from the nucleus of these methylated histones could be ameliorated or inhibited by KYNA and SZR104, which confirms the antiinflammatory nature of these drugs in our experimental setup. We also observed differential effects of KYNA and SZR104 on the cytoplasmic H3 localization; that is, LPS + KYNA increased, while LPS + SZR104 returned cytoplasmic H3 levels to control values. The detailed molecular mechanisms behind these differential effects of KYNA and SZR104 are not yet understood.

Histones are essential structural and functional components of the chromatin. These proteins are typically located in the nucleus, but they have functions at extranuclear or even extracellular sites. For instance, extracellular histones released in response to a bacterial challenge contribute to endothelial dysfunction, renal failure, and death during sepsis. Observations on cytoplasmic accumulation of histones have been made in certain pathologic states by previous studies. During microglia activation by LPS, DNA damage and genome instability were observed. The cytoplasmic localization of histones is also of pathophysiological importance.

Alterations in histone PTMs are viewed as an important process by which various cellular functions, including transcription, gene silencing, and immunity, are regulated. For

example, methylation sites can influence the binding of epigenetic factors to histone tails, which alters the extent to which DNA is wrapped around histone proteins and the availability of genes in the DNA to be activated. In neuronal cultures, previous studies found three metabolites (kynurenine, 3-OH-kynurenine, and anthranilate) from the tryptophan pathways that increase H3K4 trimethylation, resulting in upregulated gene expression at hippocampal linked loci (except those encoding pan-neural markers). Dimethylated and trimethylated H3K9 sites, i.e., transcriptionally repressive marks, are both found more often at silenced genes and are typical of heterochromatic regions. H3K9me3 has been implicated in the opening of chromatin on inflammatory gene promoters, and it is seen at significantly increased levels in treatment-resistant tumors. Additionally, macrophages cultured in high-glucose conditions display increased expressions of cytokine genes and decreased H3K9me3 levels when compared with cells incubated in a normal glucose culture. Our data suggest that while the expressions of unmodified histone H3 proteins and inflammatory marker proteins such as CXCL10 and CCR1 are probably regulated independently from each other by pro- and antiinflammatory agents, the subcellular localization of this protein and its methylated forms could be affected by both pro- and antiinflammatory agents through yet unidentified mechanisms.

Our results could be corroborated by previous studies from the literature as follows:

1) The antiinflammatory response by KYNA and SZR104 could attenuate TNF-α production and increase TSG-6 expression while elevate the interleukin-1β in sepsis. Interestingly, a possible role for IDO, a key kynurenine pathway enzyme, in immunity has recently emerged, further emphasizing the crucial role this system plays in immunomodulatory functions.

2) The inhibition of microglia phagocytosis by SZR104 and KYNA could also utilize AHR pathways, causing the decrease in TLR-4 expression on the cell surface, the induction of cytoskeletal changes or the deregulation of the nuclear factor kappa-light-chain-enhancer of activated B cell pathway.

3) The alterations in histone methylation modifications could be viewed as important processes in the regulation of inflammatory responses because they supress inflammatory protein synthesis. Cytoplasmic accumulation of histones was observed in certain pathologic states (as a precursor of apoptosis, occurring in parallel with the initial phagocytosis signals). For example, DNA damage and genome instability were observed during microglia activation by LPS.

In summary, methylations of the histone H3 lys sites seem to be essential epigenetic marks for inflammation. KYNA and its analog SZR104 might act on KYNA signaling pathways

that potentially ameliorate neuroinflammation through the facilitation of antiinflammatory actions. Our findings corroborate previous studies on the antiinflammatory properties of endogenous KYNA and raise the possibility that some of the newly designed KYNA analogs that can penetrate the blood–brain barrier may alter gene expression epigenetically to activate antiinflammatory mechanisms. Hence, our findings may lead to the development of antiinflammatory medications targeting the CNS.

6. THE MAIN FINDINGS OF STUDY

1) KYNA and the KYNA analog SZR104 exhibited strong antiinflammatory properties, as demonstrated by their effective amelioration of LPS-challenged CXCL10 and CCR1 production in microglia-enriched cultures. While KYNA and SZR104 were both effective in ameliorating the LPS-induced elevation of CXCL10 immunoreactivity, only the combined treatment with LPS + SZR104 was effective in significantly inhibiting CCR1 immunoreactivity in these tests.

2) KYNA and SZR104 displayed further antiinflammatory effects as they inhibited the LPS-induced in phagocytotic activity of microglia cells *in vitro*.

3) KYNA and SZR104 reverted the H3K9me3 and H3K36me2 immunoreactivities toward levels observed in the control, unchallenged cultures, after immunochallenge by LPS treatment.

4) Cellular response to LPS challenge resulted in a cytoplasmic translocation of methylated H3K9 proteins from the nucleus; this could be ameliorated or inhibited by KYNA and SZR104.

5) KYNA and SZR104 exerted differential effects on the cytoplasmic H3 localization: LPS + KYNA increased, while LPS + SZR104 returned cytoplasmic H3 levels to control values.

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8. CO-AUTHORS' STATEMENT

We, the Co-Authors, hereby state that data presented in this Thesis from the journal article specified below* is largely the work of the Author of this PhD Thesis, and none of the Co-Authors have used or will ever use those data to obtain a PhD degree.

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