Research Article

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Effects of kynurenic acid and choline on lipopolysaccharide-induced cyclooxygenase pathway

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Abstract

Objectives: Inflammation can be endogenously modulated by the cholinergic anti-inflammatory pathway via calcium (Ca^{2+})-permeable alpha-7 nicotinic acetylcholine receptor (α7nAChR) ion channel expressed in immune cells. α7nAChR agonist choline and tryptophan metabolite kynurenic acid (KYNA) produces immunomodulatory effects. This study aimed to determine the effects of the choline and KYNA on the lipopolysaccharide (LPS)-induced cyclooxygenase (COX)-2 pathway.

Methods: *In vitro* inflammation model was produced via LPS administration in macrophage cells. To determine the effective concentrations, choline and KYNA were applied with increasing concentrations and LPS-induced inflammatory parameters investigated. The involvement of nAChR mediated effects was investigated with the use of non-selective nAChR and selective α7nAChR antagonists. The effects of choline and KYNA on COX-2 enzyme, PGE_2, TNFα, NF-κB and intracellular Ca^{2+} levels were analyzed.

Results: LPS-induced COX-2 expression, PGE_2 TNFα and NF-κB levels were decreased with choline treatment while intracellular calcium levels via α7nAChRs increased. KYNA also showed an anti-inflammatory effect on the same parameters. Additionally, KYNA administration increased the effectiveness of choline on these inflammatory mediators.

Conclusions: Our data suggest a possible interaction between the kynurenine pathway and the cholinergic system on the modulation of LPS-induced inflammatory response in macrophages.

Keywords: α7nAChR; choline; COX-2; intracellular calcium; kynurenic acid; prostaglandin E_2.

Introduction

Bacterial endotoxin lipopolysaccharide (LPS) initiates an inflammatory response that produces pro-inflammatory cytokines by activating a member of toll-like receptors, TLR4, among “pathogen-associated molecular pattern (PAMP) recognition receptors” expressed in immune cells [1]. Although acting against infections, the uncontrolled release of cytokines causes widespread inflammatory response (as in the case of cytokine storm).

The cholinergic anti-inflammatory pathway (CAP) can control inflammatory response endogenously. Alpha-7 nicotinic acetylcholine receptors (α7nAChRs) expressed on immune cells, including macrophages, are highly permeable to calcium (Ca^{2+}) and, upon activation, the increased intracellular Ca^{2+} levels trigger a variety of downstream signalling cascades which provides interactions between parasympathetic and the immune systems leading to activation of CAP [2, 3]. Evidence suggests that α7nAChR-activating agents modulate LPS-induced cytokine release in different *in-vivo* and *in-vitro* experimental models [4] and produce anti-inflammatory effects [5, 6]. Choline, a precursor of ACh, enhances cholinergic activity by directly interacting with cholinergic receptors at high concentrations [7]. Several studies showed that activation of CAP by choline via α7nAChRs reduces the levels of pro-inflammatory factors

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from endotoxin-induced mononuclear cells via nuclear factor kappa B (NF-κB) pathway [8].

The majority of tryptophan, a precursor of serotonin and kynurenic acid (KYNA), is metabolized via the kynurenine pathway while the remaining 5 % participates in the serotonin/melatonin pathway [9]. Kynurenic acid produces prophylactic effects in different neurodegenerative disorders [10]. Moreover, KYNA has been shown to activate G-protein-coupled receptor 35 (GPR35) to produce anti-inflammatory and antioxidant actions [11, 12]. Regarding the interaction with the cholinergic system, there is controversy about the action of KYNA on α7nAChRs as KYNA was reportedly defined as an α7nAChR antagonist earlier [13]. However, its antagonistic property has never been confirmed [14].

Prostaglandins (PGs) are produced from arachidonic acid by two cyclooxygenase (COX) enzymes. The COX-1 enzyme is mainly associated with homeostasis, whereas COX-2, the inducible isoform, is stimulated by cytokines in inflamed tissues, eventually contributing PG synthesis in inflammation [15]. We previously showed that choline downregulates LPS-induced COX pathway along with PG levels in central and peripheral tissues of endotoxemic rats [16]. However, the interaction between endogenous choline and KYNA on inflammatory pathways is unclear. Thus, this study aimed to examine the role of choline and KYNA in the LPS-induced COX-2 pathway via α7nAChR along with changes in intracellular Ca^{2+} levels, pro-inflammatory mediator TNFα and NF-κB.

Materials and methods

Cell culture

RAW 264.7 murine macrophage cells (ATCC TIB-71, Manassas, VA, USA) at passage #5 were obtained and produced in DMEM (Sigma Aldrich D6429) with FBS (10 %, Sigma Aldrich F7524) and penicillin/streptomycin (Gibco, Carlsbad, 15140122) at 37 °C in 5 % CO_2. Macrophages were seeded in culture plates (48-well, 500,000 cells/well) and maintained in serum-free culture media for 24 h before the drug administration.

Choline and KYNA treatments and cell viability assay

The effects of drug treatments on cell viability were examined by applying increasing concentrations of choline and KYNA by MTT assay (n=5, A015, ABP Biosciences, China). Firstly, increasing concentrations of LPS (*Escherichia coli*, Sigma Aldrich L4130 0111: B4; in μg/mL 1–3 and 10) were applied, and COX-2 expression and PGE_2 levels were analyzed to establish the effective concentration [8]. Secondly, pretreatment of increasing concentrations of choline chloride (Sigma-Aldrich C7017) (in μM: 1–3–10–30) 30 min before LPS administration applied to establish the effective concentration of choline on LPS-induced parameters. Thirdly; a non-selective nAChR antagonist mecamylamine hydrochloride (MEC, 50 μM, Sigma Aldrich M9020) and a selective α7nAChR antagonist methyllycaconitine citrate (MLA, 1 μM, Sigma Aldrich M168) were applied 30 min before choline and LPS to investigate the involvement of nAChRs [17]. After that, cells were pretreated with increasing concentrations of kynurenic acid (KYNA, Sigma-Aldrich K3375, in μM: 1–3–10–30) 30 min before LPS administration to determine its effective concentration. Finally, in the fifth group, cells were pretreated with increasing concentrations of KYNA (in μM: 1–3–10–30) 30 min before choline (10 μM) and LPS (1 μg/mL) administration to analyze the effects of KYNA and choline treatment.

Reverse transcription polymerase chain reaction (RT-PCR) analyses

Cells were harvested 24 h after the LPS administration. RNA was isolated by an extraction kit (K0731, Thermo Scientific) and each sample was converted into complementary DNA (cDNA) using a standardized kit (K1622, Thermo Scientific). Samples of cDNA samples were amplified by using RT-PCR device (Biorad CFX Connect) with forward and reverse primers of COX-2 gene (n=5). Thermal cycle conditions were 95 °C for 10 min for polymerase activation/denaturation and 40 cycles (95 °C for 15 s, and 60 °C for 60 s for COX) for amplification, followed by a denaturation stage (65 °C for 5 s, then rising the temperature from 65 to 95 °C with 0.5 °C increments-each for 5 s). Housekeeping gene *β-actin* was used as an internal control for normalization (Supplementary Table 1). $2^{-\Delta\Delta Ct}$ method was employed for the relative quantification of mRNA expression [18].

Enzyme-linked immunosorbent assay (ELISA)

Levels of COX-2 product PGE_2 (EM1503, Wuhan Fine Biotech, China, range: 31.25–2,000 pg/mL, sensitivity:<18.75 pg/mL, Intra-Assay: CV<8 %, Inter-Assay: CV<10 %) and TNFα (BMS607-3 Invitrogen, Carlsbad, CA, range: 31.3–2,000 pg/mL, sensitivity: 3.7 pg/mL, Intra-Assay: CV<5.7 %, Inter-Assay: CV<6.5 %) released into media were determined by Enzyme-Linked Immunosorbent Assay (ELISA) according to the standardized instructions (n=5).

Western blot

To evaluate the effects of choline and KYNA on COX-2, cell lysates were prepared with a lysis buffer and run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes and immunoblotted with primary antibodies of COX-2, NF-κB p65 and β-actin (Bioss, bs-0732R, Cell Signaling 8242S and Bioss, bs-0061R, respectively). Goat anti-rabbit IgG antibody-HRP conjugate (111-035-045, Jackson ImmunoResearch Laboratories) was used as a secondary antibody. Protein bands were visualized (n=3) by using enhanced chemiluminescence (ECL) reagent (R-03025, Advansta, USA).

Intracellular calcium (Ca^{2+}) analysis

Changes in intracellular Ca^{2+} concentration ($F_{340/380}$) were monitored via spectrofluorometric fura-2 chemistry (n=4, ab176766, Abcam, Cambridge, UK) as described by the manufacturer.

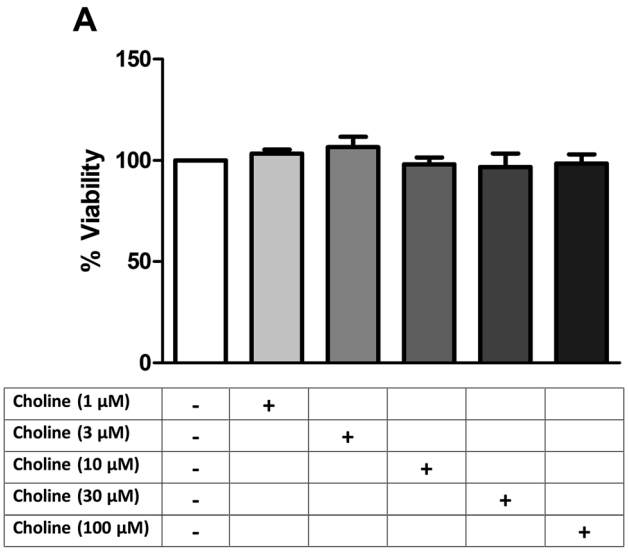
Statistical analysis

One-way analysis of variance analysis (ANOVA) with Tukey-Kramer tests and Student's t-test (GraphPad Prism 5, La Jolla, CA) were employed for analyses. Data were expressed as mean ± standard error of the mean (S.E.M). Significance was accepted at $p<0.05$.

Results

Effects of choline and KYNA on cell viability

RAW 264.7 cells were incubated with increasing choline and KYNA (1–3–10–30–100 μM) for 24 h, and results were normalized against the control (n=5). The lack of cytotoxicity confirmed the suitability of the concentration range for the following experiments (Figure 1).



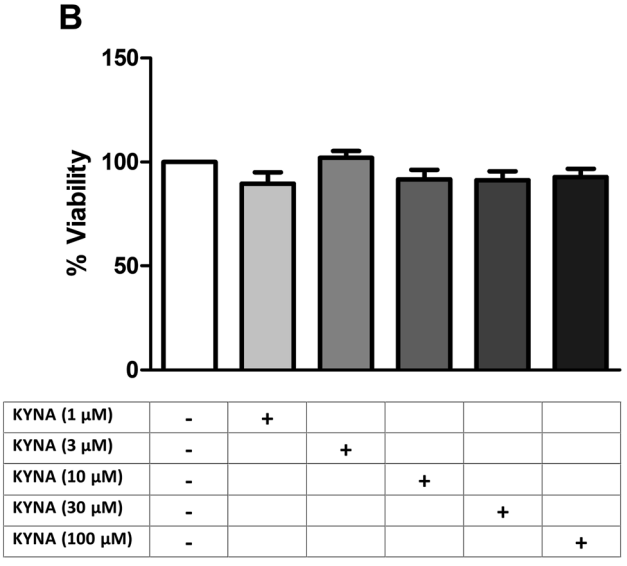


Figure 1: Effects of choline and KYNA on viability of RAW264.7 cells. Shown are the effects of choline (A), KYNA (B) on cell viability assessed by MTT assay, n=5. KYNA, kynurenic acid.

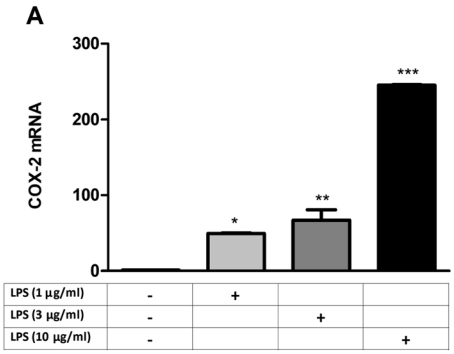
Effects of choline and KYNA on LPS-induced COX-2 levels

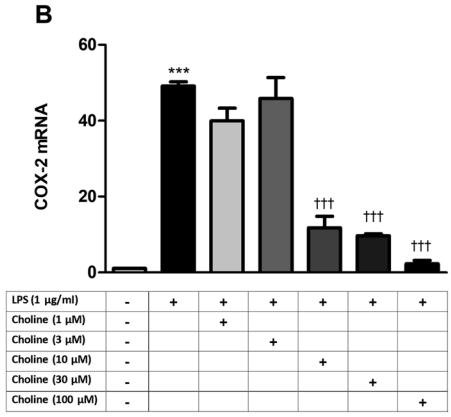
COX-2 mRNA expressions concentration-dependently increased in LPS-treated groups ($p<0.001$, n=5) comparable to control (Figure 2A). The minimally effective LPS concentration, which induced COX-2-mediated response (both COX-2 and PGE_2) was determined as 1 μg/mL (n=3, Figure 3).

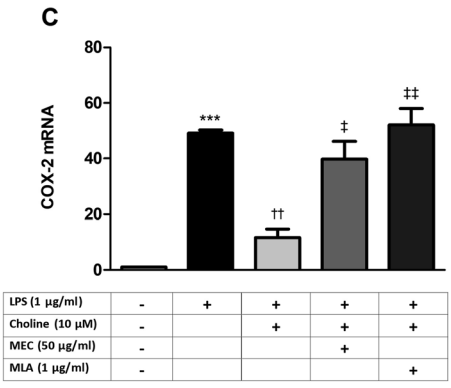
Choline decreased LPS-induced *COX-2* mRNA ($p<0.001$, n=5, Figure 2B) and COX-2 protein concentration-dependently (at 10 μM or higher concentration) comparable to LPS control group (n=3, Figure 3). *COX-2* mRNA and protein expressions increased in MEC and MLA-treated groups ($p<0.05$ and 0.01, respectively) comparable to choline-treated group (n=5, Figure 2C and n=3, Figure 3). KYNA decreased LPS-induced *COX-2* mRNA in a concentration-dependent manner ($p<0.001$, n=5, Figure 2D) which were also confirmed by WB data (n=3, Figure 3). *COX-2* mRNA expression levels decreased significantly in KYNA- and choline treated groups ($p<0.01$ in each, n=5) comparable to choline-only treatment (Figure 2E). Equieffective concentrations of KYNA- and choline (10 μM) treatment decreased COX-2 protein levels comparable to choline- and LPS-treated groups (n=3, Figure 3).

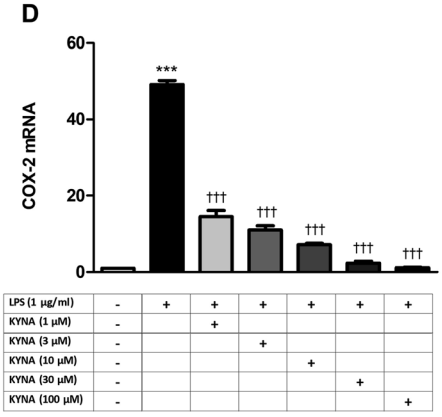
Effects of choline and KYNA on LPS-induced PGE_2 levels

PGE_2 levels concentration-dependently increased in LPS treated groups ($p<0.01$, n=6, Supplementary Figure 1A) which decreased significantly in choline-treated groups ($p<0.001$, n=6, Supplementary Figure 1B). LPS-induced PGE_2 levels increased significantly in MEC- and MLA-treated groups ($p<0.001$, n=6) comparable to choline-treated group (Supplementary Figure 1C). LPS-induced PGE_2 levels decreased significantly in the presence of KYNA ($p<0.001$, n=6, Supplementary Figure 1D). KYNA appeared to be maximally effective at 1 μM concentration. PGE_2 levels decreased significantly in KYNA and choline-treated groups ($p<0.001$, n=6) comparable to choline- and LPS-treated groups (Supplementary Figure 1E).









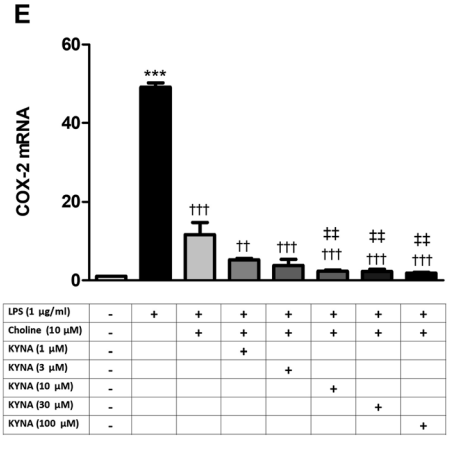


Figure 2: Effects of choline and KYNA on LPS-induced COX-2 mRNA expressions. Shown are the effects of LPS (A), choline (B), nicotinic receptor antagonists (C), KYNA (D), choline + KYNA (E) on COX-2 expressions. ***p<0.001, vs. control; ††p<0.01, †††p<0.001 vs. LPS group; ‡p<0.05, ‡‡p<0.01 vs. LPS + choline, one-way ANOVA with post-hoc Tukey-Kramer multiple comparison test or Student's t-test, n=5. MEC, mecamylamine; MLA, methyllycaconitine; KYNA, kynurenic acid.

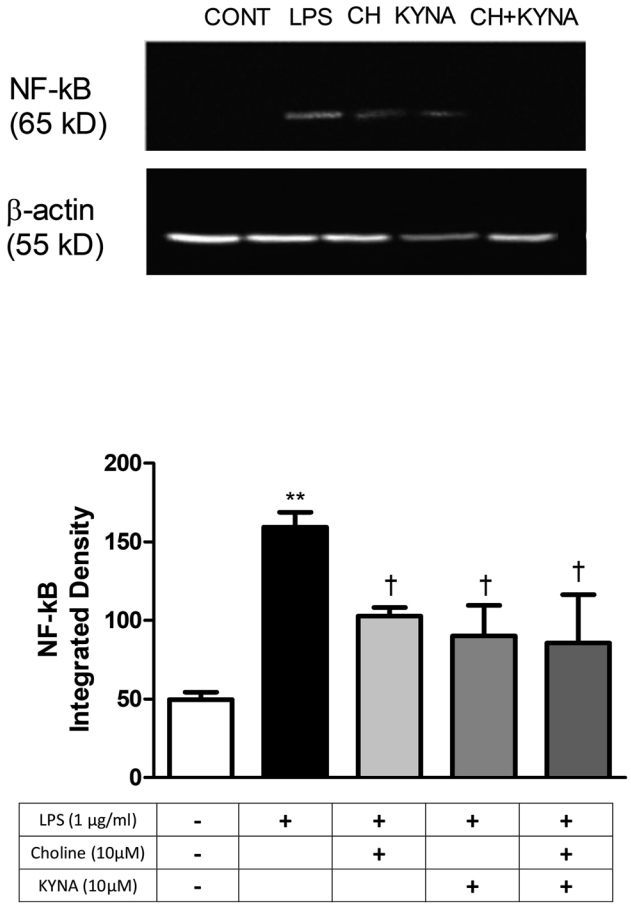


Figure 3: Effects of choline and KYNA on LPS-induced COX-2 protein expressions. Shown are the effects of LPS, choline, nAChR antagonists, MEC and MLA, KYNA, choline + KYNA on COX-2 protein expressions. **p<0.01, vs. control; †p<0.05 vs. LPS group, one-way ANOVA with post-hoc Tukey-Kramer multiple comparison test or Student's t-test, n=3. MEC, mecamylamine; MLA, methyllycaconitine; KYNA, kynurenic acid; CH, choline.

Modulation of intracellular Ca^{2+} levels by choline and KYNA

Intracellular Ca^{2+} levels increased significantly upon choline treatment (p<0.01, n=4, Supplementary Figure 2B), decreased in the presence of either MEC or MLA (p<0.05, n=4, Supplementary Figure 2C) and by >3 μM KYNA (p<0.05 and 0.01, n=4, Supplementary Figure 2E). Ca^{2+} levels were not altered significantly by LPS or KYNA *per se* (n=4, Supplementary Figures 2A and 5D).

Effects of choline and KYNA on LPS-elevated TNFα and NF-κB protein levels

Choline (10 μM) treatment suppressed the LPS (1 μg/mL)-elevated TNFα (p<0.001; n=5, Supplementary Figure 3) and NF-κB protein levels (n=3, Supplementary Figure 4) comparable to LPS group. Selective nAChR antagonist MLA antagonized the choline's inhibitory effect while the non-selective antagonist (MEC) increased LPS-induced TNFα levels even further (p<0.01 and 0.001, respectively, n=5) comparable to choline and LPS treatment (Supplementary Figure 3). KYNA (10 μM) treatment showed a trend for decreased TNFα without reaching statistical significance (Supplementary Figure 3) and NF-κB levels (n=3, Supplementary Figure 4) comparable to LPS group.

Treatment of KYNA- and choline (10 μM) additively decreased the LPS-elevated levels of TNFα (p<0.001, n=5, Supplementary Figure 3) and NF-κB (n=3, Supplementary Figure 4) comparable to choline or KYNA treatment *per se*.

Discussion

The present study shows that choline significantly inhibited LPS-induced COX-2 and PGE_2 levels by modulating the cholinergic anti-inflammatory pathway (CAP) via α7nAChR activation. Additionally, the effects of choline were potentiated in the presence of a tryptophan metabolite, KYNA, suggesting its possible interaction with cholinergic control of LPS-induced response in macrophages.

The anti-inflammatory effects α7nAChR agonists have been widely investigated in LPS-stimulated RAW264.7 cells *in vitro* [5, 8]. Earlier, significant decrease in pro-inflammatory cytokine levels including TNF-α, interleukins (IL-1β, -6, -18) and HMGB1 by α7nAChR-mediated activation of CAP in LPS-activated macrophages suggested that α7nAChRs would be identified as target receptors in the treatment of inflammatory conditions [6]. Furthermore, COX-2 expression in macrophages has been shown to be increased in response to LPS [16, 19] besides elevated pro-inflammatory cytokine, TNF-α, which also induces PLA_2 (phospholipase A2) and COX-2 activities [20, 21].

Earlier studies showed conflicting results regarding the role of cholinergic agonists and COX-2 pathway in several cell types. In one study, tacrine, an ACh esterase inhibitor, has been shown to decrease LPS-induced COX-2 and PGE_2 levels in RAW 264.7 cells [22], while in the other, nicotine, an α7nAChR agonist, increased the same parameters in microglial cells [23]. Choline and a non-selective COX inhibitor, acetylsalicylic acid, showed potentiated anti-inflammatory effects by decreasing PG and cytokine levels via α7nAChR activation in an acute inflammation model in mice [24]. Our previous study showed that intraperitoneal administrations of CAP-acting agents, choline and CDP-choline, decreased LPS-stimulated COX-2, PGE_2, PGI_2, TXA_2 along with the TNF-α levels in the endotoxemic rats [16]. In the present study,

choline administration significantly reduced COX-2 expression and the PGE_2 levels via α7nAChR activation in LPS-activated murine macrophage cells indicating that α7nAChR-mediated activation of CAP has a major role in choline' effectiveness on COX pathway under inflammatory conditions.

Changes in cytosolic Ca^{2+} levels may affect different cellular sensors including Ca^{2+}/calmodulin-dependent protein kinase II, protein kinase C (PKCs), and the p21ras/phosphatidylinositol 3-kinase (PI3K)/Akt pathways, which are involved in NF-κB activity [25]. Activation of Ca^{2+}-permeable α7nAChRs and the changes in downstream signalling pathways alters inflammatory response. Studies showed that α7nAChR activation elevates intracellular Ca^{2+} concentration, which leads to secondary Ca^{2+} release from intracellular stores (Ca^{2+}-induced Ca^{2+} release, CICR) and activates ERK/MAPK cascade in neurons and astrocytes [26]. Additionally, α7nAChR activation with AChEI donepezil mediated elevation of the phosphorylation of Akt, effector of PI3K, has been demonstrated under inflammatory conditions [27]. On the other hand, Ca^{2+}-dependent protein phosphatase, calcineurin, dephosphorylates calcium-dependent transcription factor, nuclear factor of activated T cells (NFAT)c1, then, NFAT and the NFAT:calcineurin complex is rapidly imported to the nucleus where it is transcriptionally active [28]. The level of activated NFAT can be altered by oscillating concentrations of intracellular Ca^{2+} [29]. Dual roles of α7nAChRs proposed by recent studies suggest that G proteins might also be involved in inositol-triphosphate(IP_3)-induced Ca^{2+} release and metabotropic actions of this receptor in immune system cells; which may also account for their immunomodulatory actions [30–32]. In addition, Ca^{2+} elevation by α7nAChRs may be categorized to couple Ca^{2+} to downregulation of NF-κB by activating Ca^{2+}-dependent phosphatases within these restricted subsarcolemmal areas similar to that we proposed earlier for vascular smooth muscle cells [33]. Choline treatment downregulated NF-κB and cytokine levels via α7nAChR [8]. Consistent with previous data, our results showed that intracellular Ca^{2+} concentration was significantly elevated by choline (>3 μM) via α7nAChR which downregulated LPS-induced NF-κB and COX-2 expressions along with that of PGE_2 and TNFα.

KYNA has been shown to activate G-protein-coupled receptor 35 (GPR35) to produce anti-inflammatory actions [11, 34]. Studies demonstrated that under inflammatory conditions, KYNA decreases the levels of cytokines, including TNF, interleukins and HMGB1 in different inflammatory cells [11, 35]. GPR35-mediated action of KYNA has been shown to reduce LPS-induced TNF-α levels in mononuclear cells obtained from human peripheral blood samples [11]. The present study shows that KYNA decreased LPS-induced TNF-α levels leading to decreased COX-2 expression and PGE_2 levels. However, co-administration of equieffective concentrations (10 μM) of KYNA showed only an additive interaction on COX-2 inhibition as this combination did not yield any further downregulation. Induction of PLA_2 and COX-2 activities by pro-inflammatory cytokines produced by macrophages in response to an inflammatory stimulus may counteract the inhibitory effects of choline and KYNA [20, 21]. Therefore, a possible involvement of GPR35 in the anti-inflammatory effects of KYNA on COX pathway should also be investigated further as a decrease in TNF-α levels might mediate interaction between KYNA and choline on COX-2 pathway based on our current observations.

α7nAChR antagonistic property of KYNA has been extensively debated as the data was not reproduced [13, 14]. In another study, KYNA did not antagonize ACh on nicotinic receptors [14]; however, it upregulated central nAChR expression [13]. Consistent with previous findings, our data showed that elevated intracellular Ca^{2+} followed by choline-induced α7nAChRs activation decreased partly in the presence of KYNA (>10 μM), suggesting an interaction between KYNA and choline on α7nAChR only at higher concentrations. The reversal of the antagonistic effect of KYNA on choline-induced Ca^{2+}-elevation at the highest KYNA concentration (100 μM) might be due to the mobilization of Ca^{2+} from internal Ca^{2+} stores or to an additional Ca^{2+} influx from extracellular space.

Limitations

This study suggests a possible interaction between two endogenous molecules, KYNA and choline, on the LPS-induced COX pathway via α7nAChRs. However, the nature of the interaction for KYNA and choline on inflammatory response and the role of GPR35 in the anti-inflammatory effects of KYNA was not investigated in the present study.

Conclusions

Inflammation comprises complex reactions which require treatment strategies that produce comprehensive effects. This study demonstrates that choline exerts its anti-inflammatory effect on COX pathway via activating α7nAChRs and downregulating NF-κB signaling pathway in LPS-induced inflammation model in murine macrophage cell lines. Additionally, KYNA affects choline's actions on COX pathway, suggesting a possible interaction of kynurenine and cholinergic pathways under inflammatory conditions. In addition to choline's effects on different systems affected by inflammatory

response via nicotinic receptors, its downregulating effects on COX-2 pathway also increase its potential value as a treatment option. Moreover, this study demonstrated for the first time that the choline supplements may enhance anti-inflammatory effects of endogenous KYNA, possibly affecting neuro-inflammatory disorders. Further studies investigating the possible interaction between choline and KYNA are required for the development of novel anti-inflammatory strategies.

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Author contributions: EB, ABD and MT designed the project and experimental groups. EB, OS and OUY performed the experiments and collected the data. EB and MT analyzed and visualized the data. EB and MT wrote the draft, and all the authors critically reviewed the manuscript.

Competing interests: The authors declared no conflicts of interest.

Informed consent: Not applicable.

Ethical approval: Not applicable.

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