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Effect of creatine, creatinine, and creatine ethyl ester on TLR expression in macrophages

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Abstract

Despite the widespread availability and use of dietary supplements, minimal work has been performed to assess the potential dangers many of these supplements may have on the host's well-being, in particular the host's ability to respond to infection. One supplement extensively used by both adolescents and adults is creatine. Using Real-time PCR, we examined the impact of short-term exposure of a mouse macrophage cell line (RAW 264.7 cells) to two readily available forms of creatine used in supplements – creatine monohydrate (CR) and creatine ethyl ester (CEE) as well as the end product of creatine metabolism, creatinine (CRN), on expression of toll-like receptor-2 (TLR-2), TLR-3, TLR-4, and TLR-7. CR down-regulated TLR-2, TLR-3, TLR-4 and TLR-7 mRNA levels in RAW cells. Similar results were observed following exposure of RAW cells to CRN. Conversely CEE appears to possess immunostimulatory properties and increases expression of TLR-2, TLR-3, TLR-4, and TLR-7 in RAW cells. These data are supported by immunostaining using antibodies specific for the individual TLRs before and after exposure of RAW cells to CR, CRN, or CEE. To extend these findings, we isolated murine splenocytes and exposed the cells to CR, CEE, or CRN for 24 hours and performed immunofluorescent staining for TLR-2, TLR-3, TLR-4 and TLR-7. The results obtained from this study with primary splenocytes were consistent with the studies using RAW cells. Together, these data suggest that creatine and creatine derivatives may impact the ability of immune cells to sense a wide array of viral and bacterial pathogens. Of great interest, CRN - largely considered to be a waste product of the arginine biosynthesis pathway may also have immunosuppressive properties similar to those of CR.

Keywords

creatine; creatine ethyl ester; creatinine; toll-like receptors

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1. Introduction

Toll-like receptors (TLR) are expressed by many cell types including phagocytes and are involved in sensing the presence of foreign antigen in a host via binding of pathogen-associated molecular patterns (PAMPs) on the microbe. Binding of the PAMPs to the TLRs results in a cascade of events resulting in the generation of inflammation. Reactive oxygen intermediates generated during respiratory burst by neutrophils and macrophages are key mediators of the inflammatory process [1-4]. Oxidative stress and inflammation have been implicated as participants in a variety of disease processes including Alzheimer's disease [4,5], cardiovascular disease [4,6], type 2 diabetes [4,6,7], and Parkinson's disease [4,5]. Increased generation of reactive oxygen species by the mitochondria also contributes to the normal aging processes [5,8,9]. The mechanisms by which reactive oxygen species enhance inflammation is via the activation of NF κ B [10], resulting in the upregulation of cytokine gene transcription including interleukin-8 (IL-8) [3,11] and tumor necrosis factor- α (TNF- α) [12,13], and altering vascular tone in the host [9]. The signal generated by binding of the PAMPs to the TLRs results in activation of the NF κ B via the MyD88-dependant pathway.

Certain amino acids possess anti-inflammatory properties [14-19]. A variety of mechanisms are responsible for confirming these anti-inflammatory properties including myelotoxicity [17], an ability to recruit lymphocytes and neutrophils [17,18], altering cytokine production [20], and inhibiting T cell activation [17,21,22]. Histadine, an essential amino acid, has been postulated to reduce mucosal inflammation with Crohn's disease via mechanism that reduces activation of NF κ B [20,23]. Another amino acid reported to possess anti-inflammatory properties is creatine [16,24-26], which is produced by the kidneys and liver. It is postulated that creatine stabilizes mitochondrial membranes and functions as an anti-oxidant [27].

In vivo, creatine is a product of arginine biosynthesis and metabolizes to creatinine [8]. Creatine is commonly used by athletes as a non-vitamin, non-mineral supplement that enhances athletic performance, reduces muscle inflammation, and speeds recovery following strenuous training [28-35]. Despite its extensive use as an unregulated nutritional supplement, relatively little work examining the impact of creatine on other host functions has been done, although there have been a few reports that suggest that immune function is altered by creatine supplementation [26,36]. Multiple forms of creatine are currently utilized by athletes and one form, creatine ethyl ester (CEE) is purported to contain the activity required to build strength without side effects such as water retention. In aqueous solutions (including plasma) [37], CEE is rapidly converted to creatinine (CRN), a compound that is generally viewed as inert. The rapid conversion of CEE to CRN brings into question the utility of CEE as an anti-inflammatory agent [37]. However, one study [16] suggests that CRN (at least under some circumstances) may also contain some anti-inflammatory properties.

We examined the impact of creatine monohydrate (CR) and some creatine derivatives on transcription of genes known to be involved in the sensing phase of the immune response. Specifically, we compared the ability of CR, CEE, and CRN to suppress transcription of the genes encoding toll-like receptor 2 (TLR-2), TLR-3, TLR-4, and TLR-7. Results were further confirmed using immunohistochemistry. These particular receptors are triggered by a wide array of pathogens including gram positive (TLR-2) and gram negative (TLR-4) bacteria, and viruses (TLR-3, TLR-7). Overall, these data support the hypothesis that CR and CRN possess anti-inflammatory properties that impact the sensing arm of the innate immune system.

2. Materials and methods

2.1. Cell culture

RAW 264.7 (American Tissue Type Culture Collection; Manassas, VA) were maintained in Dulbecco's media supplemented with 10% FBS (Invitrogen; Carlsbad, CA), 100 µg/ml streptomycin (Invitrogen), and 100 U/ml penicillin (Invitrogen) at 37°C and 5% CO₂. Dose response experiments were performed following exposure of the RAW 264.7 cells to varying doses of creatine ethyl ester hydrochloride (MW 195; Vireo Systems Inc, Madison TN), creatinine hydrochloride (MW 149.58; Sigma-Aldrich; St. Louis, MO) and creatine monohydrate (MW 131.13; Creapure, Degussa AG, Germany). Control cultures were treated with media. A dose of 0.1 mM CEE, CRN, and CR were chosen for use in these studies based on results from the dose response studies, and are within the normal physiological levels of creatine in the blood [38]. CR, CEE, and CRN were added immediately to the cell cultures upon solubilization. No significant differences in pH of the media were detected between treatments.

Spleens were harvested from B6 × 129 mice, placed in cold HBSS, and dissociated into a single cell suspension. Cells were washed and resuspended at a concentration of 1 × 10⁶ cells/ml in RPMI 1640 (Sigma-Aldrich), 5% FCS, 100 µg/ml streptomycin, 100 U/ml penicillin, and 0.1 mM CEE, CRN, or CR. Control splenocytes received only media. Cultures were incubated at 37°C and 5% CO₂ for 24 hours. After 24 hours, cells were collected, washed in HBSS, resuspended to a concentration of 1 × 10⁶ cells/ml in culture media. Cytospins were prepared and the slides fixed in acetone and air-dried. Slides were stored at -80°C until the time of immunofluorescent staining.

2.2. RNA isolation

Following treatment, RAW cells were lysed in Trizol (Invitrogen, Carlsbad, CA). After homogenization, samples were extracted with chloroform, centrifuged, and the aqueous phase was collected. RNA was precipitated with isopropyl alcohol. Following centrifugation, the pellets were washed with 70% ethanol and air dried. RNA was resuspended in dH₂O. Residual DNA was removed from the samples by treatment with RQ1 DNase (Promega, Madison, WI) per the manufacturer's protocol. After incubation with DNase at 37°C for 1 hr, samples were extracted with phenol:chloroform:isoamyl alcohol (25:24:1). RNA was precipitated with 1/10 volume of 3M sodium acetate (pH=5.2) and 3 volumes of 100% ethanol. Samples were stored at -80 °C until use.

2.3. Real time RT-PCR

Reverse transcription (Improm-II Reverse Transcription System, Promega, Madison, WI) followed by realtime PCR analysis was performed in 20 µL volumes containing 1 µl of cDNA corresponding to 40 ng of DNase-treated RNA, gene specific internal primers (0.5 µM of each primer), and 1X SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA). Primers used were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) and have 100% sequence homology to the murine genome. Primer sequences are listed in TABLE 1. The PCR was performed using the following conditions: 95°C for 30 sec, and 40 cycles of 95°C for 5 sec and 57°C for 5 sec using a CFX96 Real-Time Detection System (Bio-Rad). GAPDH was used as a housekeeping gene to normalize mRNA levels between samples. Data were analyzed using the Bio-Rad CFX Manager V1.6.541.1028 software (Bio-Rad). The calibrator in the experiments was the sample obtained from control-treated RAW cells. Copy numbers of targeted mRNAs were expressed as ratios of GAPDH mRNA levels. The relative quantification (RQ) of gene expression in this method is determined by the formula: $RQ = 2^{-\Delta\Delta CT}$ [39,40].

2.4. TUNEL Assay

TUNEL was performed on RAW cell monolayers using the ApopTag Fluorescein *In Situ* Apoptosis Detection Kit per the manufacturer's protocol (Millipore/Chemicon; Billerica, MA). Cells were fixed with cold acetone, air-dried, and treated with Proteinase K (20 µg/µl; Qiagen). Following incubation with equilibration buffer, terminal deoxynucleotidyl transferase (TdT) was applied to the slides. TdT binding was detected using a fluorescein-conjugated anti-digoxigenin antibody. Cells were counterstained and the slides mounted using Vectashield Mounting Media with DAPI (Vector Laboratories, Burlingame, CA).

2.5 Immunostaining

RAW cells were grown in chamber slides in the presence of 0.1 mM CEE, CRN, or CR. Control cells were cultured with only media. Immunohistochemical detection of TLRs was performed as described previously with minor modifications [41,42]. After fixation in cold acetone, the slides were air-dried and rehydrated in PBS (phosphate buffered saline). Blocking was performed at room temperature for 20 minutes. Following incubation with the primary antibody the slides were washed with PBS. The following primary antibodies were used in this study: mouse anti-TLR-2 (GeneTex; Irvine, CA), goat anti-TLR-3 (R&D Systems; Minneapolis, MN), rabbit anti-TLR-4 (Abcam; Cambridge, MA), rabbit anti-TLR-7 (Abcam), hamster anti-CD3ε (BD Biosciences, San Diego, CA), rat anti-B220 (BD Biosciences), and rat anti-F4/80 (Millipore). Detection was performed using Alexa Fluor conjugated secondary antibody (Invitrogen, Carlsbad, CA). After washing with PBS, slides were mounted using Vectashield Mounting Media with DAPI (Vector Laboratories). Staining was also performed on cytopspins prepared from murine splenocytes. Images were captured using a Nikon i80 microscope (NY, USA) and a DigiFire camera and ImageSys Digital Imaging Software (Soft Imaging Systems GmbH, Munster, GER).

2.6. Statistical analysis

Data were analyzed by ANOVA with a Bonferroni's test as the post-test. p values of ≤ 0.05 were considered statistically significant.

3. Results

We examined the impact of treatment of RAW cells with creatine derivatives on TLR-2, TLR-3, TLR-4, and TLR-7 mRNA expression. These TLRs were chosen for examination based on the array of microbial components recognized by these receptors. Pattern associated molecular patterns (PAMPs) associated with these receptors include lipoteichoic acid (TLR-2), double-stranded RNA (TLR-3), bacterial lipopolysaccharide (TLR-4), and single-stranded RNA (TLR-7) (reviewed in [43]). To ensure that differences in gene expression were not due to differences in the level of cell death, we performed a TUNEL assay to determine whether there were differences in apoptosis between the groups. Furthermore, we also performed flow cytometry following propidium iodine and Annexin V staining on cells from the various treatment groups, and compared the levels of cell death between the groups. No differences in cell death were noted between the treatment groups and the control-treated cells (data not shown).

3.1. TLR-2 mRNA levels are decreased following exposure to creatine monohydrate or creatinine hydrochloride

RAW cells were exposed to 0.1 mM CR, CEE, or CRN for periods of between 10 and 60 minutes. CEE exposure had a brief immunostimulatory effect on RAW cells after 30 minutes of co-culture ($p \leq 0.01$; Figure 1), but TLR-2 transcript levels returned to baseline by 60 minutes after the addition of CEE. CR was a robust suppressor of TLR-2 mRNA in the

RAW cells. TLR-2 mRNA levels were reduced by ~70% within 10 minutes of exposure to creatine ($p \leq 0.001$; Figure 1) and remained suppressed through at least 30 minutes of exposure to CR. By 60 minutes post-exposure, mRNA levels of TLR-2 returned to control levels in the RAW cells. CRN suppressed TLR-2 transcript levels in RAW cells by approximately 50% within 10 minutes of exposure. TLR-2 mRNA levels remained decreased through at least 60 minutes post-exposure relative to control-treated cells ($p \leq 0.05$).

3.2. TLR-3 is differentially expressed following exposure to creatine ethyl ester, creatine monohydrate or creatinine hydrochloride

CEE had stimulatory effects on TLR-3 transcripts at 20 minutes post-exposure ($p \leq 0.001$), but this observation was short-lived and control transcript levels were observed by 60 minutes after exposure (Figure 2). CR exposure reduced the levels of TLR-3 mRNA by RAW cells by 80% within 10 minutes of exposure ($p \leq 0.05$). This effect lasted through at least 30 minutes post-exposure to CR. By 60 minutes TLR-3 mRNAs were similar to control levels ($p > 0.05$). Creatinine hydrochloride caused an increase in TLR-3 mRNA within 10 minutes of co-culture in the RAW cells however there was approximately an 80% reduction in TLR-3 message levels compared to the untreated controls by 30 minutes after exposure ($p \leq 0.05$), indicating a bimodal effect of the treatment on TLR-3 expression by RAW cells.

3.3. TLR-4 mRNA levels are decreased by exposure to creatine monohydrate or creatinine hydrochloride and increased by exposure to creatine ethyl ester

TLR-4, a pattern recognition receptor for LPS [43], was not significantly down-regulated by CEE at any time-point. In contrast, CEE treatment of RAW cells appeared to either stabilize or upregulate expression of TLR-4 mRNA. TLR-4 transcripts were upregulated approximately 2.7-fold by 20 minutes post-exposure to creatinine ethyl ester (Figure 3; $p \leq 0.001$). TLR-4 mRNA levels returned to control levels by 60 minutes post-exposure to CEE. CR was a potent suppressor of the TLR-4 mRNA. Within 10 minutes of exposure of RAW cells to CR, TLR-4 mRNA levels decreased by almost 70%. TLR-4 mRNA levels remained suppressed through at least 60 minutes ($p \leq 0.05$). Within 20 minutes of exposure to CRN, TLR-4 transcripts were decreased by approximately 30%. This CRN-mediated mRNA transcriptional suppression was further enhanced at 30 minutes, where a 70% decrease in mRNA levels was measured. Decreased levels of TLR-4 mRNA were observed through 60 minutes ($p < 0.001$).

3.4. TLR-7 transcripts are decreased following exposure to either creatine monohydrate or creatinine hydrochloride

TLR-7 transcript levels were negatively impacted following exposure to either CR or CRN (Figure 4). These decreased transcript levels were observed within 10 minutes post-exposure to the creatine derivative and lasted at least through 30 minutes post-exposure in the instance of CR ($p < 0.001$) and up to at least 60 minutes (CRN). While the suppressive effect on mRNA levels lasted longer in cells treated with CRN, the level of suppression observed in RAW cells was more profound following CR treatment. Interestingly, CEE was a potent upregulator of TLR-7 transcripts. Transcript levels were increased approximately 3-fold at 20 and 30 minutes of exposure, and returned to control levels by 60 minutes post-treatment ($p > 0.05$).

3.5. Immunofluorescent staining of RAW 264.7 cells reveal expression patterns reflecting alterations in mRNA levels

To assess whether changes in mRNA levels described following treatment of RAW cells with CR, CEE, or CRN resulted in altered protein expression of the TLRs, we performed

immunohistochemical staining of RAW cells following exposure to 0.1 mM CR, CEE, or CRN. As changes in protein expression manifest later than alterations in mRNA levels, we performed the staining 24 hours after addition of the creatine derivatives to the cells. Untreated RAW cells served as controls. As shown in Figure 5, RAW cells expressed all TLRs studied under control conditions. Following exposure to CRN, the intensity of TLR-2 staining was reduced over that observed in control cells (Figure 5). CR treatment also resulted in decreased expression of TLR-2 (data not shown). Minimal changes in staining were observed following RAW cell treatment with CEE (data not shown).

Immunostaining for TLR-3 suggests that there was a small decrease in protein expression of this protein following exposure to CRN (Figure 5), relative to the staining observed in the controls. Conversely, CRN and CR appear to reduce the intensity of staining for TLR-4 under similar conditions (Figure 5, data not shown). Very intense staining of RAW cells was observed using an antibody for TLR-7 (Figure 5). Staining intensity decreased upon incubation of RAW cells with CRN and dramatically decreased in the case of CR (Figure 5, data not shown), reflecting the mRNA data.

3.6 Immunofluorescent staining of primary splenocytes cultured with CEE, CR, and CRN demonstrate similar expression patterns to those observed in RAW 264.7 cells

To determine whether the impact of creatine and its derivatives on TLR expression is a RAW 264.7 cell-specific observation, we isolated splenocytes from mice and cultured the resultant cells in splenocyte media containing a 0.1 mM solution of CR, CRN, or CEE for 24 hours. Immunostaining for TLR-2, -3, -4, and -7 was performed on cytopspins made from the cells. As observed with the RAW 264.7 cells, co-culture of murine splenocytes with CR or CRN downregulated expression of TLRs-2, -3, -4, and -7 (Figure 6). Dual immunofluorescent staining studies using antibodies to CD3 ϵ , B220 and F4/80 indicate that T and B cells, as well as macrophages express TLR-2, -3, -4, and -7 as predicted (data not shown).

4. Discussion

In the past decade, the use of nutritional supplements has grown rapidly [44-46]. Creatine is a readily available supplement that is widely used by adults and juveniles [28-30]. Despite its widespread use, potential side-effects related to the use of creatine or its derivatives are largely undefined, although some studies have suggested that this supplement may have anti-inflammatory properties [24,36]. The impact of creatine supplementation on immune system function has not been well-studied. In the studies presented here, we examined the effects of low levels of creatine and other members of the arginine biosynthesis pathway on transcript levels of TLR receptors that are involved in the sensing phase of the immune response by recognizing PAMPs. TLRs, are one of the first receptors triggered in the host following infection, and can be located both on the cell (TLRs 1,2,4,5,6,10) or endosomal (3, 7, 8, 9) membranes. We examined the expression of TLRs known to be critical in sensing both bacterial (TLR-2, TLR-4) and viral (TLR-3, TLR-7) infections following short term exposure to creatine or its derivatives *in vitro* to determine whether exposure to these compounds could downregulate key molecules associated with triggering an immune response, thus having an adverse side effect on the host's ability to respond to infection.

We determined that following exposure of a mouse macrophage cell line to creatine TLR-2, TLR-3, TLR-4 and TLR-7 levels were downregulated rapidly by the cells. We expected to observe minimal effects of creatine on the mouse macrophages, due to its rapid breakdown into creatinine [47]. Instead, creatine had profound immunosuppressive effects on all of the TLRs studied. The observation that creatinine also had a significant suppressive effect on TLR expression is surprising and novel since the molecule considered to be a biologically

inert breakdown product of creatine. It has been accepted as dogma that creatinine is a pharmacologically inactive waste product of muscle metabolism devoid of biological activity which is excreted in the urine [48]. Creatinine dramatically decreased levels of TLR-2, TLR-3, TLR-4, and TLR-7 transcript levels in RAW cells. The decreased levels of mRNA levels could not be attributed to increased cell death, as apoptosis levels were similar to the level of apoptosis observed in the control cultures, regardless of the treatment (data not shown). In contrast to the observations in the cultures exposed to either CR or CRN, there were increased levels of TLRs observed at various time-points following exposure to of the RAW cells to CEE, a common over-the-counter formulation of creatine, suggesting that the precise formulation of this non-vitamin, non-mineral supplement may differentially impact the ability of the host to mount an innate immune response. Interestingly, despite decreased TLR-3 levels at 30 and 60 minutes post-exposure, TLR-3 levels were initially stimulated by the presence of CEE in the culture medium, as were TLR-2, TLR-4 and TLR-7. In fact, CEE was a potent inducer of TLR-7. This was unexpected since it was reported that CEE breaks down rapidly in aqueous solutions to CRN [49,50] which we demonstrated to be an inhibitor of TLR expression. It may be that CEE is rapidly taken up by RAW cells and/or stabilized in membranes in order to exert a stimulatory effect on TLR expression. It must be noted that the immunostimulatory effects of CEE occurred in the short-term (less than 40 minutes after exposure to CEE) and these effects are lost within 60 minutes of CEE exposure. It is likely, given the time-course data (Figs. 1-4), that longer incubation of CEE with RAW cells would demonstrate a decrease in TLR mRNA expression due increased levels of creatinine, a breakdown product of CEE in the cultures. TLRs receptor engagement is known to stimulate signaling NF- κ B-mediated signaling processes which can either directly or indirectly (via the production of cytokines) alter TLR expression levels [51]. We hypothesize that one mechanism by which TLR expression may be altered in our studies is via decreased pro-inflammatory mediator production (Leland, McDonald, and Drescher, manuscript in preparation).

The TLRs examined in this study are responsible for sensing a wide variety of pathogen components including lipoteichoic acid (TLR-2), porin derived from *Neisseria* and *Haemophilis* (TLR-2), lipopolysaccharide (TLR-4), and single- and double-stranded RNA (TLR-3 and TLR-7, respectively). These TLRs were chosen for study as they encompass sensors of a significant array of human pathogens including gram positive (TLR-2) and gram negative (TLR-4) bacteria, as well as viruses that cause significant disease in humans (i.e., influenza, coxsackieviruses, rotavirus). CR and CEE are supplements frequently used by athletes who are attempting to increase muscle mass, enhance performance, reduce recovery times, and reduce muscular inflammation [28-35]. Both short-term and long-term creatine supplementation regimens are common and often consist of a loading phase (20 grams/day for 5-7 days) followed by a maintenance phase (5 grams/day for up to 6 months). Loading protocols usually divide the 20 gram dose into four 5 gram doses/day [52,53]. Plasma creatine levels can increase up to 20-fold following ingestion of 5 grams of creatine [54]. While creatine in plasma has a relatively short half-life of approximately 3 hours [54], the increased plasma levels of creatine can be extended by ingesting multiple doses of creatine throughout the day. It is tempting to speculate that the extended elevation in creatine levels *in vivo* decreases TLR expression of cells involved in sensing infections and negatively impacts the host's ability to respond to infections. Future studies in the laboratory will focus on the impact of CR, CRN, and CEE in animal models of viral and bacterial infections.

These studies support the hypothesis that CR and its metabolite, CRN may downregulate the innate immune response by decreasing TLR expression on macrophages, a key cell involved in the early phases of the immune response. In addition, these supplements may also influence cells of the adaptive immune response based on their ability to influence

expression of TLR-2, -3, -4, and -7 on B cells, T cells, and macrophages. The observation that a derivative of CR, creatine ethyl ester, increased TLR expression is of interest and future studies need to explore whether this phenomenon is a property of other esters of CR (propyl, butyl, benzyl, etc.) or unique to CEE.

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Highlights

- The effect of creatine, creatinine, and creatine ethyl ester on
- Creatine and creatinine downregulate TLRs
- Creatine ethyl ester upregulate TLRs over the short-term

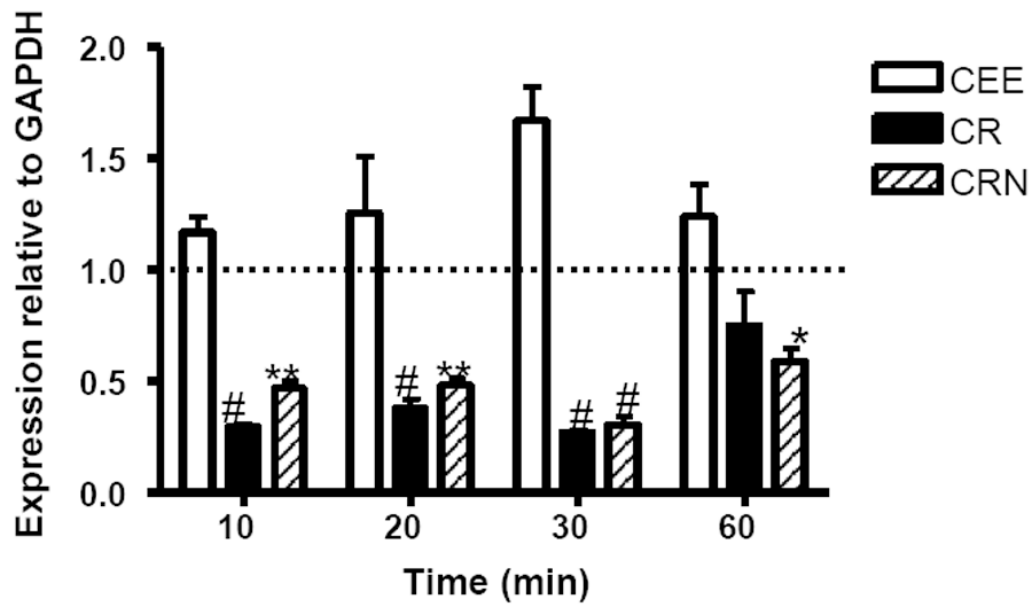


Figure 1.

CR and CRN suppress TLR-2 transcript levels in RAW 264.7 cells. RAW 264.7 cells were cocultured with 0.1 mM of CEE (open bars), CR (filled bars), or CRN (striped bars) for 10 to 60 minutes. TLR-2 mRNA levels were measured by qRT-PCR. Values represent the mean transcript level \pm the SEM, relative to GAPDH transcript levels. Statistical analysis was performed using a two-way ANOVA, with untreated cells as the control. * < 0.05 ; ** < 0.01 ; # < 0.001 . Data are representative of three experiments.

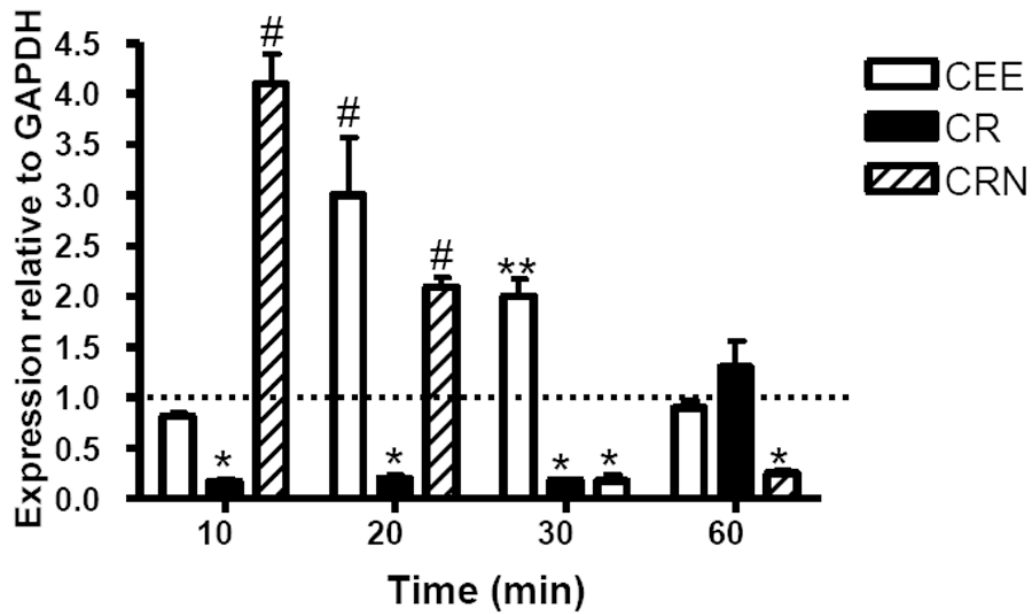


Figure 2.

CR suppresses TLR-3 transcript levels in RAW 264.7 cells while CEE and CRN upregulate TLR-3 mRNA levels. RAW 264.7 cells were cocultured with 0.1 mM of CEE (open bars), CR (filled bars), or CRN (striped bars) for 10 to 60 minutes. TLR-3 mRNA levels were measured by qRT-PCR. Values represent the mean transcript level \pm the SEM, relative to GAPDH transcript levels. Statistical analysis was performed using a two-way ANOVA, with untreated cells as the control. * < 0.05 ; ** < 0.01 ; # < 0.001 . Data are representative of three experiments.

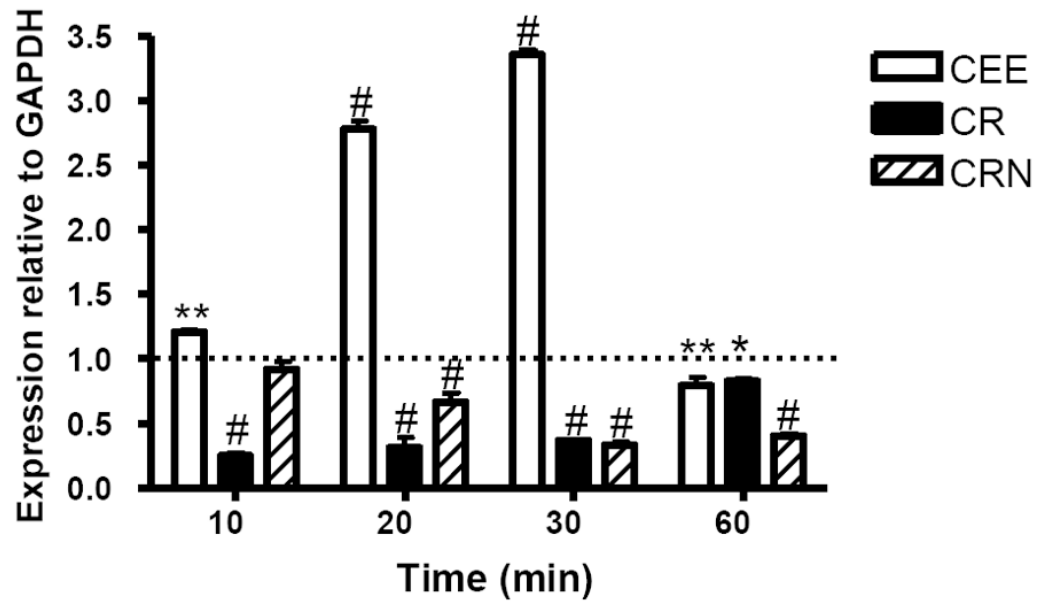


Figure 3. TLR-4 mRNA levels are repressed in RAW 264.7 cells by CR and CRN, while CEE enhances TLR-4 expression. RAW 264.7 cells were cocultured with 0.1 mM of CEE (open bars), CR (filled bars), or CRN (striped bars) for 10 to 60 minutes. TLR-4 mRNA levels were measured by qRT-PCR. Values represent the mean transcript level \pm the SEM, relative to GAPDH transcript levels. Statistical analysis was performed using a two-way ANOVA, with untreated cells as the control. * < 0.05 ; ** < 0.01 ; # < 0.001 . Data are representative of three experiments.

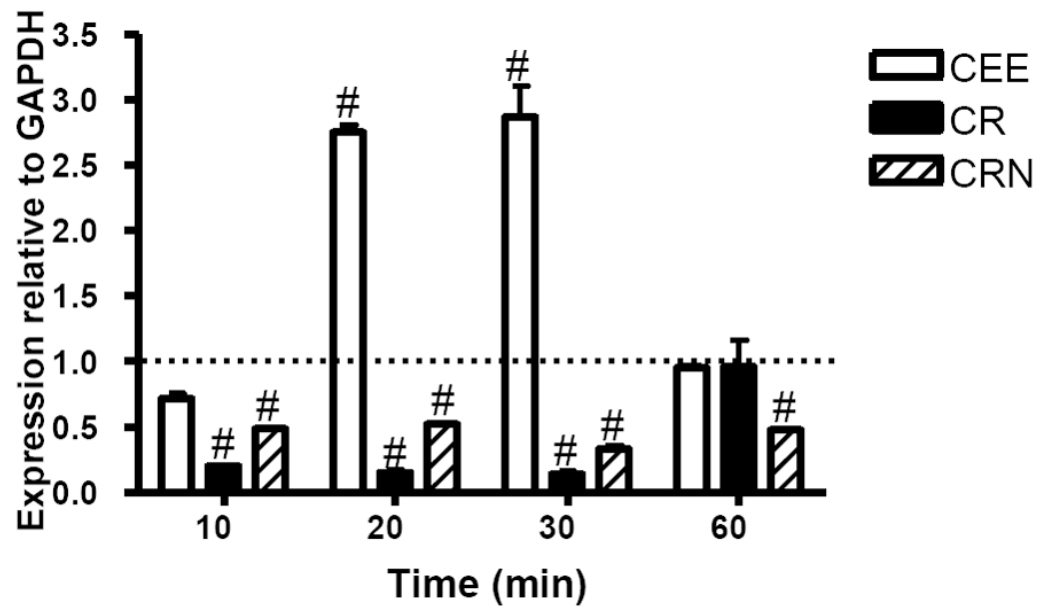


Figure 4.

CR and CRN suppress TLR-7 transcript levels in RAW 264.7 cells while CEE upregulates TLR-7. RAW 264.7 cells were cocultured with 0.1 mM of CEE (open bars), CR (filled bars), or CRN (striped bars) for 10 to 60 minutes. TLR-7 mRNA levels were measured by qRT-PCR. Values represent the mean transcript level \pm the SEM, relative to GAPDH transcript levels. Statistical analysis was performed using a two-way ANOVA, with untreated cells as the control. * < 0.05; ** < 0.01; # < 0.001. Data are representative of three experiments.

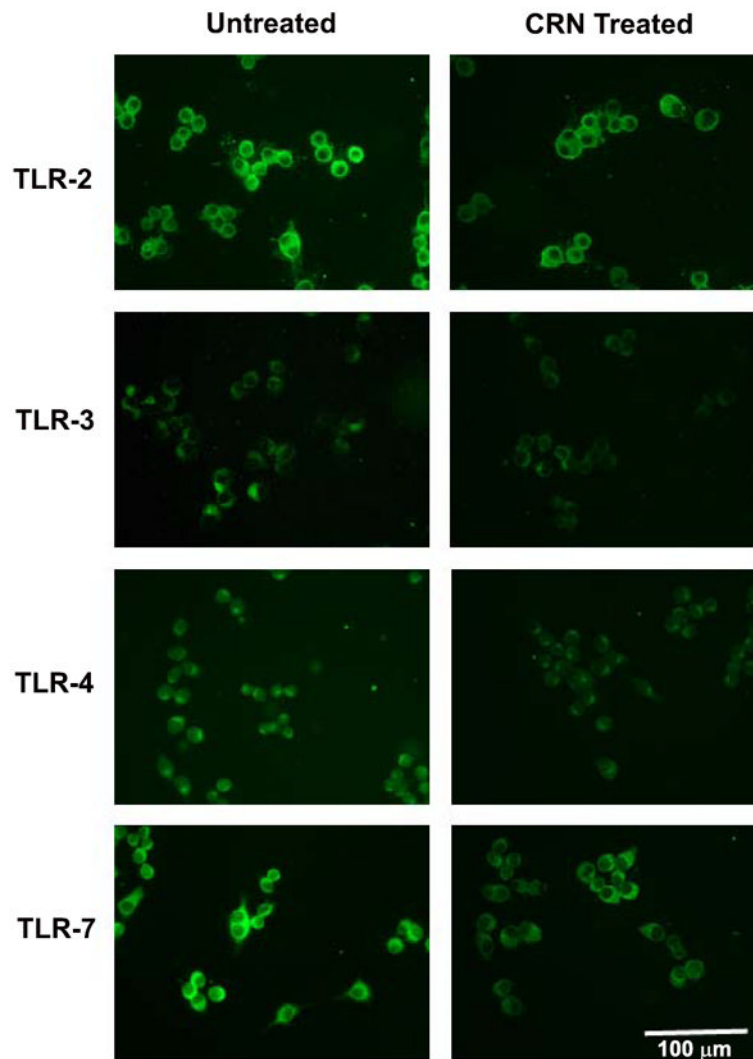


Figure 5. Altered expression of TLR-2, -3, -4 and -7 following exposure of RAW 264.7 cells to 0.1 mM of CRN as determined by immunofluorescent staining. Immunohistochemical staining revealing that exposure to CRN results in a decrease of TLR-2, TLR-3, TLR-4, and TLR-7 relative to the levels observed in the control-treated cells.

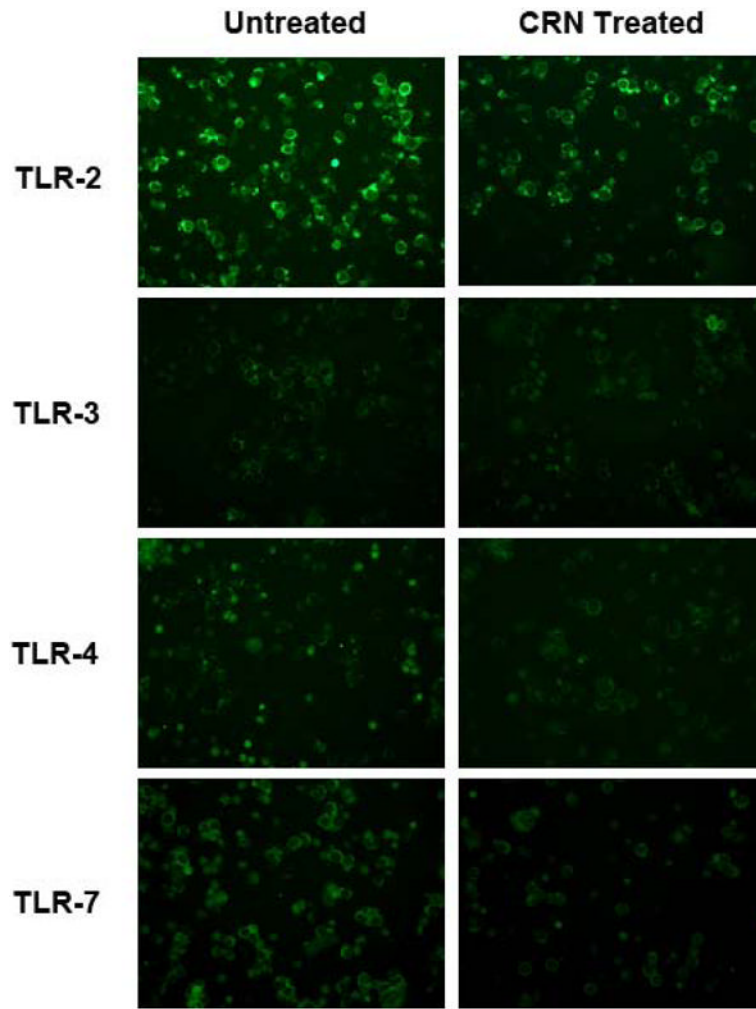


Figure 6. Reduced expression of TLR-2, -3, -4 and -7 following exposure of mouse splenocytes to 0.1 mM of CRN as determined by immunofluorescent staining. Immunohistochemical staining of cytopins prepared from splenocytes treated with CRN demonstrates that CRN results downregulates TLR-2, TLR-3, TLR-4, and TLR-7 relative to the levels observed in the control-treated cells.

TABLE 1

Primers used for realtime PCR studies

Primer Name	Forward Primer 5'→3'	Reverse Primer 5'→3'	Accession #
mTLR-2	TGTTCCCTGTGTGCTGGT	ACAAAGTGGTTGTCGCCTGCT	NM_011905
mTLR-3	CCTTGCGTTGCGAAGTGAAG	CAATTGTCTGGAAACACCCCG	NM_126166
mTLR-4	AGAAATTCCTGCAGTGGGTCA	TCTCTACAGGTGTTGCACATGTCA	NM_021297
mTLR-7	CGTGGA CTGCACAGACAAG	GTTCAGCCTACGGAAGGAATC	NM_133211
mGAPDH	GTGGCAAAGTGGAGATTGTTG	CATTCTCGGCCTGACTGTG	NM_008084