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Prairie Turnip Extracts are Potent Immunomodulators that Induce the Expression of Indoleamine Dioxygenase

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Abstract

Native Americans have used the prairie turnip *(Psoralea esculenta)* as a good source of various bioactive compounds that can benefit one's health. For example, prairie turnips found in Iowa are known to reduce inflammation. Inflammation can be caused by many factors, including infection, irritation, or even physical trauma. Inflammation is a basic healing process, but it causes tissue damage. This study was aiming to uncover the mechanism of reducing inflammation in macrophages treated with prairie turnip extracts (PTE). It was hypothesized that the PTE would induce the expression of indoleamine 2, 3-dioxygenase (IDO), which is a marker for anti-inflammation. This enzyme is an important immune response modulator that is present in various cells—including macrophages. It functions by catabolizing tryptophan to help reduce inflammation. AhR (Aryl hydrocarbon receptor) is a regulator of inflammation possibly through regulating IDO transcriptionally. Since inflammation can be primarily mediated by residential macrophages, we treated macrophages derived from THP-1 cells (ATCC, VA) with PTE. To test the hypothesis, the level of mRNAs for IDO and AhR were quantified by performing a series of reverse transcriptase real time polymerase chain reactions. Results showed that the prairie turnip rind extract upregulates the level of IDO mRNAs, which indicates the anti-inflammatory status of cells. Test results also showed that the mechanism of this upregulation may be mediated by AhR. It was concluded that prairie turnip extracts may be potent immunomodulators. Future studies will be aiming to localize IDO and AhR on macrophages in order to confirm this mRNA study.

Keywords: Prairie Turnip Extract, Inflammation, Indoleamine 2,3-Dioxygenase

1. Introduction

1.1 Prairie Turnip

Prairie turnips (*Psoralea esculenta*) are found in the prairies of Iowa and are ethnobotanically, chemically, and pharmacologically important.[1] The genus contains several bioactive compounds such as flavonoids, furanocoumarins, and terpenoids. Native Americans and other indigenous cultures have used the prairie turnip in their medical practices and as a source for its various bioactive capacities.[1] For example, they would crush the roots of the prairie turnip to treat sprains and fractures—in order to help reduce swollen tissue areas, reduce the fever, and/or pain caused by inflammation.[1] The prairie turnip is also used for sore throats, gastroenteritis, and even chest problems.[1] Biological membranes are protected from oxidative stress by the phenolic compounds in *P. esculenta*. These compounds can bind to reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) to neutralize them, thus reducing inflammation.[1] While the *P. esculenta* is one of the lesser studied species of the genus, PTE was found to play an important part in the immune system, specifically affecting the expression of indoleamine 2,3-dioxygenase (IDO).

1.2 Indoleamine 2,3-Dioxygenase

The function of IDO is to suppress the immune system and act as an immune regulator to help control pro-inflammatory signaling.[2] IDO catalyzes the rate-limiting step in the conversion of tryptophan (Trp) to kynurenine (Kyn).[2] The production of Kyn then acts as a natural ligand for the aryl hydrocarbon receptor (AhR).[3] The activation of the immune system is inhibited when IDO acts on multiple Trp substrates.[2] IDO catabolizes Trp to Kyn, which is known to be immunosuppressive since the catabolite is toxic to T helper 1 cells. The inflammation process is driven by the T helper 1 cells, which secrete cytokines and then activate residential macrophages to increase inflammation.[2] The immunologic effects of IDO are not confined to cells that express IDO. Nearby cells are able to sense and respond to secreted Kyn metabolites.[3]

As cells strive to counterbalance inflammation, inflammatory cytokines such as, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) synergistically increase the expression of IDO.[4] The IDO is known to be regulated by various transcription factors that could be stimulated by many different extracellular signals. Various transcription factors regulate the expression of IDO. The IDO promoter contains nucleotide sequences that are important in transcriptional regulation, such as interferon sequence response elements (ISREs), palindromic gamma-activated sequences (GAS) and nuclear factor kappa light chain enhancer of activated B cell (NF-κB) binding sites. Transcription factors, signal transducers and activators of transcription-1 α (STAT-1), interferon regulatory factor-1 (IRF-1), and NF-κB, are binding to ISRE, GAS, and NF-κB binding nucleotide sequences on the IDO promoter respectively. This binding leads to transcription of IDO gene.[4]

1.3 Aryl Hydrocarbon Receptor

AhR can mediate immune suppression and is known to induce IDO expression.[2] It was once identified as a receptor for xenotoxins such as dioxin.[3] The AhR signaling pathway starts with the AhR in the cytoplasm which binds to a ligand.[5] The complex enters the nucleus where transcription occurs.[5] The dioxin response element and other transcriptional regulators are recognized by the AhR/ARNT complex in order to control target gene expression, in this case inducing *IDO*.[5] Recent reports demonstrated that AhR plays a role in producing IDO. One particular report showed that the AhR in a knockout mouse does not produce IDO in dendritic cells. This suggests AhR would be necessary for producing IDO triggered by Toll like receptors 7 and 9, which are endosomal nucleotide sensors involved in the recognition of viruses.[6] The molecular mechanism of AhR induction of IDO is not yet completely understood.

1.4 Background of Quantifying mRNA

The expression of mRNA can be quantified through a variety of methods such as: *in situ* hybridization, RNase protection assays, cDNA arrays, and real-time reverse transcription polymerase chain reaction (RT-PCR).[7] Our research team chose to use quantitative real-time RT-PCR because it is the most widely used method for quantifying gene expression. RT-PCR is used to quantify the mRNA expression of cytokines often expressed at low levels.[7] THP-1 derived macrophages were treated with Prairie turnip extracts (PTE) for a designated time, then the total RNA was extracted and subsequently synthesize complementary DNA (incubation times are indicated in Methodology). Complementary DNA of the THP-1 macrophages were further used for PCR using specific primers for IDO, AhR, and housekeeping gene human 18srRNA. The SsoFast EvaGreen SYBR Green dye (BioRad, CA) binds to the double stranded DNA and emits a fluorescence signal. The signal is then measured by the PCR machine to determine the amount of gene amplification. The specific protocols and further explanations can be found in the Methodology section.[7]

1.5 Hypothesis

Prairie turnips are known to reduce inflammation. It was hypothesized that the prairie turnip extracts (PTE) would induce the gene expression of indoleamine 2, 3-dioxygenase (IDO), which is a marker for anti-inflammation.

2. Methodology

2.1 PTE Preparation and Treatment of Macrophages

THP-1 cells, purchased from ATCC (American Type Cell Collection, VA), were treated with 100 μM of phorbol-12-myristate-13-acetate (PMA, Sigma, MO) and incubated at 37°C with 5% CO_2 for 24 hours. PMA is known to be the most effective at differentiating THP-1 cells (monocytes) into macrophages.[8] The THP-1 cell line was chosen because it has the ability to easily combine a higher growing rate and minimize variability in the cells.[8] After the 24-hour incubation period, the macrophages were then treated with 100 μg of PTE, which was provided by the Briar Cliff University Chemistry Department. To prepare PTE, it must be solubilized in 50% Methanol while heated at 80°C for an hour. Then centrifuge the extracts for 5 minutes at 10,000RPM. After centrifugation, supernatant was collected, and the solvent was evaporated under nitrogen.

2.2 Isolation of RNA and Synthesis of Complementary DNA (cDNA)

RNA was isolated from treated macrophages, which were solubilized by purezol (BioRad, CA). Chloroform was added to the purezol mixture and then centrifuged to inhibit the RNase activity. The mixture separated into three different layers, with the RNA found in the aqueous layer. Isopropyl alcohol was added to the RNA layer to precipitate the RNA, which was then washed in ethanol and resuspended in RNase-free water. The iScript kit (BioRad, CA) was used to synthesize cDNA from the isolated RNA. The kit contains reverse transcriptase that turns isolated RNA to double stranded complementary DNA. The cDNA was synthesized according to the manufacturer's protocol.

2.3 Real Time PCR and quantification

For gene expression studies, Integrated DNA Technologies, Inc (IA) synthesized the following DNA primer sets: human *IDO1*, human *ahR*, and reference gene human *18srRNA* with SsoFast EvaGreen supermix:

human IDO1, 5'-AGA GGA GCA GAC TAC AAG-3'(Forward)
human IDO1, 5'-AGC CAC TTC TTC ATC AA-3'(Reverse)
human AhR, 5'-ACA TCA ACC TAC GCC AGT CGC-3' (Forward)
human AhR, 5'-TCT ATG CCG CTT GGA AGG AT-3' (Reverse)
human 18s rRNA, 5'-CTA CCA CAT CCA AGG AAG CA-3' (Forward)
human 18s rRNA, 5'-TTT TTC GTC ACT AAC TCC CCG-3' (Reverse)

The internal reference gene for the gene-specific amplification was normalized to 18s rRNA. The specific analysis for quantification of each gene is explained in figure legends on results section. The quantified gene is expressed as a cycle of quantification (Cq) by the real time PCR instrument. The amount of IDO and AhR, was normalized to an endogenous housekeeping gene, human 18s rRNA, which is relative to the calibrator MED. The normalized and calibrated Cq numbers were then used to calculate target gene expression. Following the arithmetic formula: $2^{-\Delta\Delta Cq}$, where $\Delta\Delta Cq = \Delta Cq(\text{sample}) - \Delta Cq(\text{Calibrator})$. The ΔCq is the Cq of the target gene subtracted from the Cq of the housekeeping gene. This equation shows the normalized expression of target genes in the PTE treated samples, which are relative to no treatment of PTE.

2.4 Statistical Analysis

A *t* test was used to establish statistical significance in the 95% confidence interval between individual sample groups ($P<0.05$) as indicated in Figures 1 and 2. These sample groups include comparing a positive control group (IFN+TNF on figure 1 and 2) and experimental group (Prairie Turnip Extracts) to a no treatment group (MED in figure 1 and 2). P values found in Figures 1 and 2 were calculated with three combined experiments, each experiment was performed

independently. The individual samples are PTE, IFN+TNF, and medium with no PTE. IFN+TNF acted as the positive control and PTE was compared to the positive control to see how much of an effect it has on the cell.

3. Results

3.1 The Treatment of Macrophages with PTE Increases the Level of IDO mRNA.

The level of IDO mRNA was explored to see how PTE may influence macrophages and increase anti-inflammatory activity. The exploration included treating 100µg (final concentration) of PTE in monocyte (THP-1) derived macrophages (MDM) for 24 hours. Once completed, the amount was evaluated, and levels of mRNA were analyzed relative to untreated cells (Figure 1). Based on this analysis, there was a significant increase in IDO mRNA at almost the same level expression of positive control (Figure 1) in the PTE treated macrophages. Overall, this data shows PTE may increase anti-inflammatory activity of MDM via IDO gene expression. This coincides with the fact that IDO has been associated with anti-inflammatory activity of human macrophages in many different tissues[3].

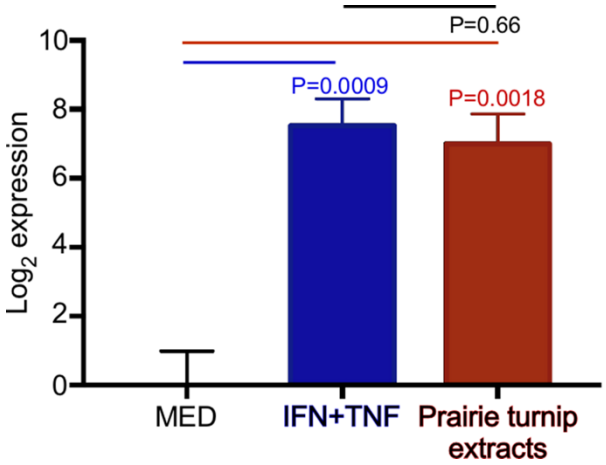

Figure 1. PTE treatment in macrophages increases the IDO mRNA expression. MDM was treated as described in section 3.1. Quantitative analysis of IDO mRNA is presented as the mean $\log_2$ change in gene expression. The P value was calculated with three combined experiments. Each experiment was performed independently. Values were normalized to the mean expression of 18srRNA within a sample group and expressed relative to that of medium (MED) with no PTE or combination of IFN+TNF added.

3.2. The Treatment of Macrophages with PTE Increases the Level of AhR mRNA.

The results show macrophages treated with PTE increase the level of IDO mRNA (Figure 1). This is a strong indicator of anti-inflammatory activity in macrophages. There are several factors that can possibly regulate the increase of IDO mRNA level including extracellular signals and transcription factors. One of the notable anti-inflammatory extracellular signals is IL-27. However, the level of IL-27 mRNA was not increased by PTE treatment (data not shown). Thus, we concluded that IL-27 was not involved in increasing IDO mRNA. The level of AhR mRNA, the possible transcription factor that could increase the level of IDO mRNA, was tested. AhR mRNA was significantly increased over the expression level of the positive control (Figure 2). Overall, this data indicates PTE may mediate the expression of AhR for increasing the expression of IDO.

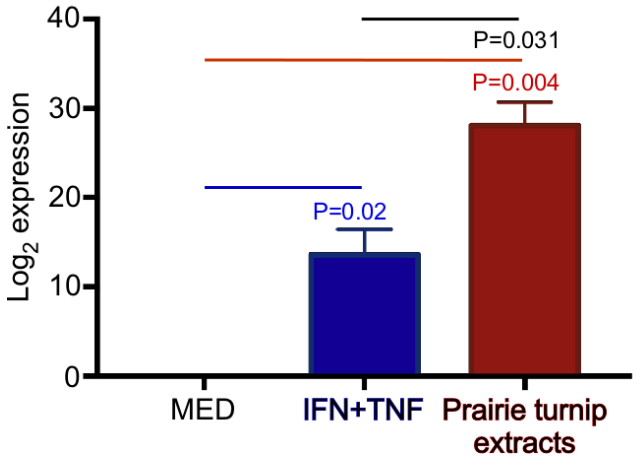

Figure 2. PTE treatment in macrophages increases the AhR mRNA expression. MDM was treated as described in section 3.1. Quantitative analysis of IDO mRNA is presented as the mean $\log_2$ change in gene expression. The P value was calculated with three combined experiments. Each experiment was performed independently. Values were normalized to the mean expression of 18srRNA

4. Discussion and Conclusion

Prairie turnip has been used as a food source in the Siouxland prairies for hundreds of years. Dr. Paul Weber and his research team at Briar Cliff University have studied the chemical properties for a while. His research shows some promising results suggesting prairie turnips possess some antioxidant properties. Recently, the prairie turnip has been investigated for anti-infective research in the Carver Molecular Biology Teaching Lab at Briar Cliff University.

In this study, the exploration of whether PTE would possess anti-inflammatory activity was the focus, since previous studies in the BCU Chemistry department showed antioxidant activity. There are many mammalian cell types that could exhibit anti-inflammatory activity such as lung epithelial cells, M cells on gut epithelial cells, B cells, macrophages, mast cells, and dendritic cells. Macrophages are one of the major cells that mediate and regulate inflammation. THP-1 cells were chosen since they are monocyte driven cells and differentiate well to macrophages with the treatment of PMA.

The process of transitioning inflammatory macrophages to anti-inflammatory macrophages uses many key molecules such as: Interleukin-10 (IL-10), Tumor Growth Factor-beta (TGF-β), and Interleukin-27 (IL-27). These molecules are largely known as cytokines. Along with these molecules, IDO is known to possess immunosuppressive properties. Thus, inflammation could be suppressed by activity of IDO.

The mRNA levels of IDO were studied for two important reasons. First, cytokines such as IL-10 and IL-27 are known to regulate the transcription of immunosuppressive IDO. These cytokines bind to their receptors on cell surfaces and start a specific cellular signaling process to activate distinctive transcription factors such as STAT-3. STAT-3 binds to response elements located within the *IDO* gene. Once STAT-3 binds to the response elements, the IDO gene is transcribed (Figure 3). As shown in Figure 1, IDO mRNA levels were significantly increased upon treatment with PTE. However, previous experiments in this lab found that IL-10 and IL-27 were not detected in PTE treated monocyte driven macrophages using real-time RT-PCR (data not shown). Since there was no increase in IL-27, and IL-10 mRNAs, the hypothesis was formed that an increased IDO gene expression treated by PTE may be due to the increase in transcription factors, such as AhR.

AhR is the transcriptional regulator found in the cell cytoplasm of macrophages. It is involved in cellular activities that include cellular differentiation, anti-inflammatory gene expression, and T helper 2 type immune response. In this study, we explored the involvement of AhR on the increase of IDO gene expression. Interestingly, the mRNA level of AhR was increased following PTE treatment (Figure 2). This data does not suggest AhR is binding with the PTE component such as flavonoids, or phenolic group containing compounds to regulate the IDO gene expression, however it suggests AhR may be involved in the regulation of IDO.

Several experiments are under way including a localization experiment for AhR on cell cytoplasm followed by PTE treatment. Another experiment being studied is an Enzyme Mobility Shift Assay to detect what transcription factors are involved in the expression of IDO. The Briar Cliff University Chemistry department is also working on finding specific components of PTE by using GC-MS (Gas Chromatography – Mass Spectrometry) and HPLC (High Pressure Liquid Chromatography) that are responsible for antioxidant, anti-infective, and anti-inflammatory activities.

From the results, a hypothetical model was proposed that shows how IDO may be upregulated by AhR. AhR follows a ligand binding pathway, and in this case the hypothetical PTE component acts as the ligand. Once the PTE binds to AhR, the complex can enter the nucleus. AhR associates with ARNT (AhR nuclear translocator) to recognize dioxin response elements (DRE) that act as a transcriptional regulator. The DRE may control the gene expression of IDO, which can be transcriptionally regulated due to the promoter binding sites for response elements and transcriptional factors.[2] Therefore, PTE could contain potent immunomodulators that induce the expression of IDO.

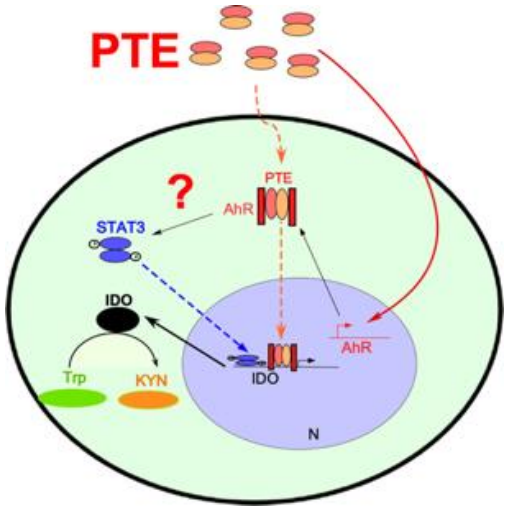

Figure 3. Hypothetical model for IDO induction by PTE. PTE causes AhR to increase, this might be due to PTE acting as a ligand and entering the cell. It binds with the AhR and the AhR/PTE complex can enter the nucleus. It associates with ARNT and recognizes DREs that act as transcriptional regulators to control target gene expression of IDO. IDO then regulates the Trp conversion into KYN. AhR could directly affect STAT3 which could also cause an increase in IDO expression.

Figure 3 explains the possible mechanism of our findings. The components of PTE, such as flavonoids or phenolic compounds may enter the cell either through receptor mediated endocytosis or diffusion. This component binds to AhR localized in the cell cytoplasm to form a transcription factor. The newly formed transcription factor translocates to the nucleus and directly binds to the regulatory elements located on the promoter region of the *IDO* gene. Alternatively, a newly formed transcription factor (AhR + PTE components) may activate a signal transducer and activator of transcription 3 (STAT3, transcription factor). The activated STAT3 translocates to the nucleus to bind the regulatory elements on the *IDO* promoter region. Ultimately, these transcription factors increase the transcription of the *IDO* gene to make mRNA. The IDO mRNA translocates back to the cytoplasm where it can translate into an IDO protein, which would catabolize tryptophan to kynurenine.

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