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Role of JAK in the Induction of iNOS in Macrophages using Pharmacological Inhibitors

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ABSTRACT

Inducible nitric oxide synthase (iNOS) is induced in different cell types by cytokines and lipopolysaccharide (LPS). Cytokine signal transduction is believed to be mediated primarily through the JAK/STAT pathway. We, therefore, examined the effects of JAKs (Janus Kinase) in the induction of iNOS in macrophages using pharmacological inhibitors by using cell culture, Griess assay, protein assay, and western blotting techniques and also to compare the responses of JAK1and JAK2 inhibitor at different concentrations. The result of this study concludes that JAK inhibitors, i.e., JAK 1 and AG-490 down-regulates multiple signaling pathways such as STAT1-activation, iNOS gene expression and NO production in LPS treated macrophages. JAK2 proved to be an active ingredient and has shown anti-inflammatory effects. Activated JAK2 regulates the phosphorylation of JAK through the activation of PI3K thus plays a pivotal role in LPS-induced iNOS expression.

Keywords: Pharmacological inhibitors, JAK, Nitric oxide, Macrophages

INTRODUCTION

Nitric oxide (NO) is an extremely unstable signaling molecule with a half-life of few seconds. It diffuses out of the cell and acts locally affecting nearby cells leading to local effects. NO is a gaseous lipophilic free radical cellular messenger and is very unstable. It plays an important role in the protection of endothelium against cardiovascular diseases. The cardioprotective roles of NO include blood pressure regulation and vascular tone, inhibition of platelet aggregation and leukocyte adhesion and prevention of smooth muscle cell proliferation. Lower bioavailability of NO is the potent factor leading to cardiovascular diseases [1]. Three isoforms of the nitric oxide synthase (NOS) have been known i.e., a neuronal type called nNOS, an epithelial type called eNOS and an inducible form called as iNOS.

Macrophages are phagocytic cells that recognize a wide range of signaling molecules such as immune stimulatory lipopolysaccharides (LPS) from the gram-negative bacteria cell wall and cytokines like IFN-γ and TNF-α produced from other immune cells. LPS and TNF-α activate the MAPK pathway which thereby activates nuclear factor kappa-B (NF-κB) and activator protein-1 (AP1) that are the key pro-inflammatory transcription factors [2,3]. The activated macrophages produce NO that mediates cytotoxic and cytostatic effects against pathogenic microbes and tumor cells [4-7]. The enzyme that is responsible for NO production by macrophages is the inducible isoform of nitric oxide synthase (iNOS). The iNOS catalyzes the oxidation of one of the equivalent guanidine nitrogen of arginine to form NO and citrulline [8]. The iNOS generate high concentrations of NO as compared to the neuronal and endothelial isoform of NOS and account for the cytotoxic and cytostatic effects of NO on the target cells [9].

The 2 major pathways mainly involved in the iNOS gene expression within macrophages are the mitogen-activated protein kinase (MAPK) pathway, the Janus kinase (JAK) and Signal Transducer and Activator of Transcription (STAT) pathway collectively known as the JAK/STAT pathway. Janus kinases (JK) are large proteins activated

by phosphorylation of tyrosine. They are mainly intracellular tyrosine kinase ranging from 120-140 kDa and are involved in the signaling cascade of cytokines in association with the cytokine receptors such as interferon. There are 4 types of Janus kinase enzymes namely JAK1, JAK2, JAK3, and TYK2. The members of the JAK family act as an important protein tyrosine kinases [10]. All of these enzymes are activated by phosphorylation of tyrosine-the phosphorylation results in the activation of STAT molecules. The activation of STATs leads to dissociation from the receptor and thus allows there movement as homo or heterodimers into the nucleus and binds to specific regions of the promoter gene [11]. It has been reported from both *in vitro* and *in vivo* studies with tyrosine kinase inhibitors that activation of tyrosine kinase is necessary for an array of biological responses to LPS, including activation of JNK [12]. The tyrosine kinase 2 (Tyk2) is important for LPS-induced endotoxin shock [13]. Tyk2 mainly belongs to the JAK family and the best-known substrate for these factors is the family of STAT proteins [14]. JAK2 is involved in the LPS-induced expression of iNOS in skin-derived dendritic cells [15]. However, there is little information about the molecular mechanisms through which JAK2 transduces LPS-induced signals to downstream molecules in order to activate pro-inflammatory genes. On the basis of sequence similarities between the JAKs, 7 JAK homology (JH) regions have been identified [16]. The JH1 domain is present at the C terminus and is a classical tyrosine kinase domain surrounded by a pseudokinase domain (JH2) that may have a regulatory role [17]. The N-terminal of the JAKs contains domains JH3-JH7 and is comprised of Src homology 2 domain and a FERM domain (four-point-one, ezrin, radixin, moesin) involved in binding of cytokine receptor [18,19]. The JAK/STAT pathway is one of the major mechanisms by which cytokine receptors transduce intracellular signals [20,21]. This system is monitored at various levels like JAK activation, nuclear trafficking of STAT factors and negative feedback loops [20,22-24].

In the present study, we are going to investigate the role of JAKs (Janus Kinase) in the induction of iNOS in macrophages using pharmacological inhibitors by using cell culture, Griess assay, protein assay, and western blotting techniques and also to compare the responses of JAK1and JAK2 inhibitor at different concentrations.

MATERIALS AND METHODS

Cell Culture

J774 macrophages cells were grown in DMEM culture medium until it becomes very confluent by changing the medium after every 48 hours. Then the culture medium was transferred into 10 petri dishes. In this, DMEM culture medium, Bovine serum albumin provides supplements to grow the cells, and antibiotic Penicillin was added to avoid the contamination. The petri dishes were kept in an incubator for 24 hours at the room temperature.

JAK1 Inhibitor in Different Concentrations

Ten plates were incubated with 2 ml of complete DMEM to become confluent. The plate's numbers 1 to 5 were used as a control and from 6 to 10 as activated. In plate number 1 only DMEM was added and in plate number 2 100 µl of JAK1 inhibitor from 1 µM was added. The plate number 3 was treated with 100 µl from 10 µM of JAK1 inhibitor and plate number 4 with 100 µl from 10 mM of JAK1 inhibitor. Plate number 5 was treated with 10 µl from 10 mM of JAK1 inhibitor, and final volumes of the plates were made up to 1 ml with DMEM. Plate number 6 was activated with 10 µl of 10 mM LPS. Plates number 7 to 10 were also treated with JAK1 inhibitor as in 2 to 5, but after 30 minutes they were activated with LPS and final volume was made up to 1 ml with DMEM (Table 1).

Table 1 Showing petri dishes numbers, LPS volume and macrophage activator (LPS) volume, JAK1 inhibitor in different concentrations, a volume of DMEM culture medium and the total volume of the petri plates

Plate No.	LPS Vol. (µl)	JAK1 Inhibitor (µl)	DMEM (µl)	Total Vol. (µl)
1	-	-	1000	1000
2	-	100 µl from 1 µM	900	1000
3	-	100 µl from 10 µM	900	1000
4	-	100 µl from 100 µM	900	1000
5	-	10 µl from 10 mM	990	1000
With Macrophage Activator (LPS)				
6	10	-	990	1000
7	10	100 µl from 1 µM	890	1000
8	10	100 µl from 10 µM	890	1000
9	10	100 µl from 100 µM	890	1000
10	10	10 µl from 10 mM	980	1000

JAK2 Inhibitor (AG490) in Different Concentrations

Ten plates were incubated with 2 ml of complete DMEM to become confluent. The plates from 1 to 5 were used as a

control and from 6 to 10 as activated. In plate number 1 only DMEM was added and in plate number 2 100 µl of JAK2 inhibitor (AG490) from 1 µM was added. Plate number 3 was treated with 100 µl from 10 µM of JAK2 inhibitor. Plate number 4 was treated with 100 µl from 10 mM of JAK2 inhibitor and plate number 5 was treated with 10 µl from 10 mM of JAK2 inhibitor and final volume of the plates was made up to 1 ml with DMEM. Plate number 6 was activated with 10 µl of 10 mM LPS. The plates from 7 to 10 were also treated with JAK2 inhibitor as in 2 to 5, but after 30 minutes they were activated with LPS and final volume was made up to 1 ml with DMEM.

The Standard Nitrite Griess Assay

For standard graph preparation stock solution of sodium nitrite was prepared freshly by weighing 0.345 g of sodium nitrite and was dissolved in 5 ml of complete DMEM (i.e., DMEM with penicillin and 10% Bovine serum albumin). About 100 mM stock solution was diluted to get 1 mM working solution. A standard curve was prepared by making 5 ml of each concentration. About 100 µl each standard solution to the outer wells and media from cells to the inner wells were added, in triplicates on the non-sterile 96-well plate. The working Griess reagent solution was prepared by mixing equal volumes of Griess reagent I and Griess reagent II. About 100 µl of the working solution was added to each well on the 96-well plate. Plates were incubated for 10 min at room temperature. Absorbance was read at 540 nm on place reader.

The Protein Assay

For protein assay, Bovine serum albumin (BSA) standards were prepared freshly. For Bovine serum albumin (BSA) standards preparation, 10 mg/ml BSA stock was prepared by dissolving 0.05 gm of BSA in 5 ml of distilled water, and it was diluted with 9 ml of distilled water to get 1 mg/ml concentrated bovine serum albumin. About 50 µl of each standard was added in triplicate to the outer wells and 5 µl of cell lysates to the inner walls of a non-sterile 96-well plate. About 5 µl of lysis buffer was added to the standards. And 50 µl of DDW was added to the lysates to bring the volume up to 55 µl. Around 200 µl of the working Bio-Rad solution was added to all the wells. Plates were incubated at room temperature for 30 min. Absorbance was read at 540 nm on a plate reader.

Western Blotting (Protein Analysis)

The cells were activated according to the condition of the experiment for a specified time. The activation is confirmed with the Griess assay. After confirming activation, cells were lysed to extract the protein. The extracted proteins were transferred from a Sodium dodecyl sulfate (SDS) polyacrylamide gel to an adsorbent i.e., Nitrocellulose membrane. The proteins were then detected using suitable primary and secondary antibody and were visualized by detection reagent.

RESULTS

JAK2 Inhibitor in Different Concentrations

The standard nitrite Griess assay: The cells were treated with the LPS in the absence or presence of increasing concentrations of JAK inhibitors, JAK1 inhibitor, and AG-490. The nitric oxide production was detected as nitrite accumulation in the culture medium. LPS strongly induced the nitric oxide production in J774 macrophages and it was inhibited in a concentration-dependent manner by JAK1 inhibitor and AG-490. Increasing concentration of the sodium nitrite gives dose-dependent absorbance response. Figure 1 represents the concentration-dependent response produced by sodium nitrite.

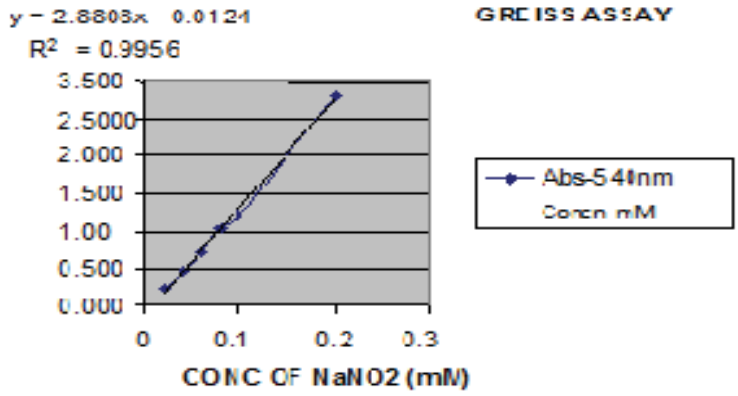

Figure 1 Standard nitrite curve

The concentration of the nitrite samples of C1, JAK1 inhibitor (AG490), LPS, JAK1 with LPS, JAK2 with LPS were obtained (Figure 2). It has been observed that there is less concentration of nitrite in macrophages cells when cells were treated with a JAK1 inhibitor, but the cells treated with AG490 (JAK2 inhibitor) showed more production of nitric oxide.

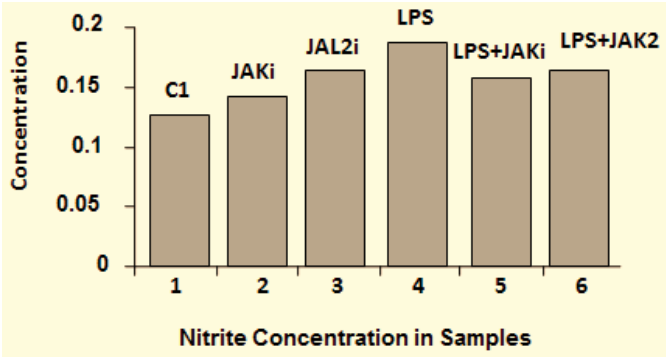

Figure 2 Nitrite concentration in samples

The effect of JAK2 inhibitor, i.e. AG-490 on iNOS protein expression was investigated by western blot analysis (Figure 3). The LPS induced iNOS protein expression in J774 macrophages was reduced in a concentration-dependent manner by AG-490. Initially, cells were treated with LPS and then AG-490 was added for the duration of 30 min. Then the medium was also replaced with the fresh medium drug for 30 minutes duration. Nuclear proteins were extracted from the cells by cell lysis process. The protein concentrations of the samples were measured. In this method, various dilutions (µg/ml) were used such as 0, 0.2, 0.4, 0.6,1 and 2 in the absence or presence of the LPS.

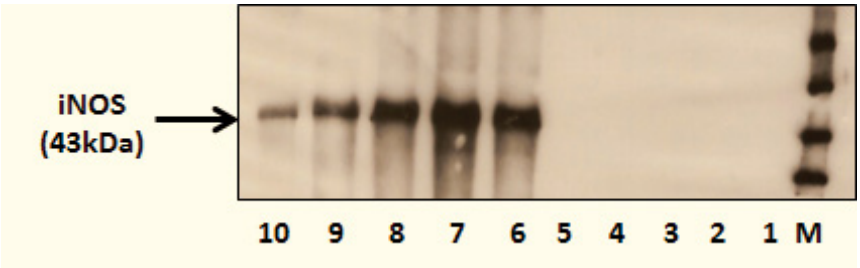

Figure 3 Western blot analysis (JAK2 inhibitor bands)

Five bands were detected when the cells were treated with JAK1 inhibitor as well as JAK2 inhibitor (Figure 4). The intensity of iNOS bands detected in JAK2 inhibitors was more. Less intensity of bands was obtained in the cells treated with JAK1 inhibitor. The intensity of bands increases with the increasing dose of drugs.

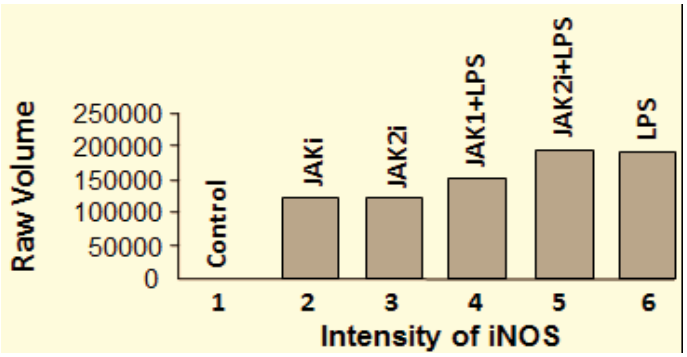

Figure 4 Intensity of the iNOS bands

JAK1 Inhibitor in Different Concentrations

The effect of JAK1 inhibitor on iNOS protein expression was investigated by western blot analysis (Figure 5). LPS

induced iNOS protein expression in J774 macrophages was reduced in a concentration-dependent manner by JAK1 inhibitor. Initially, cells were treated with LPS and then JAK1 inhibitor was added for 30 min. Then the medium was also replaced with the fresh medium drug for 30 minutes duration. Nuclear proteins were extracted from the cells by cell lysis process. The protein concentrations of the samples were measured. In this method, various dilutions (µg/ml) were used such as 0, 0.2, 0.4, 0.6, 1 and 2 in the absence or presence of the LPS.

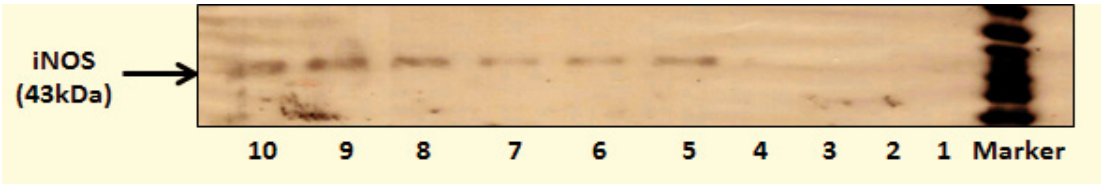

Figure 5 Western blots analysis (JAK1 bands)

After treating the cells with 2 different pharmacological agents such as JAK1 inhibitor and JAK2 inhibitor they were lysed, and the protein concentration was measured. There is less production of protein content in cells treated with JAK1 inhibitor and there is increasing production of proteins in the cells treated with JAK2 inhibitor (Tyrphostatin AG490). From the above results, it has been confirmed that the samples treated with JAK1 inhibitor have less intensity (low raw volume) indicating there is less production of protein (Figure 6).

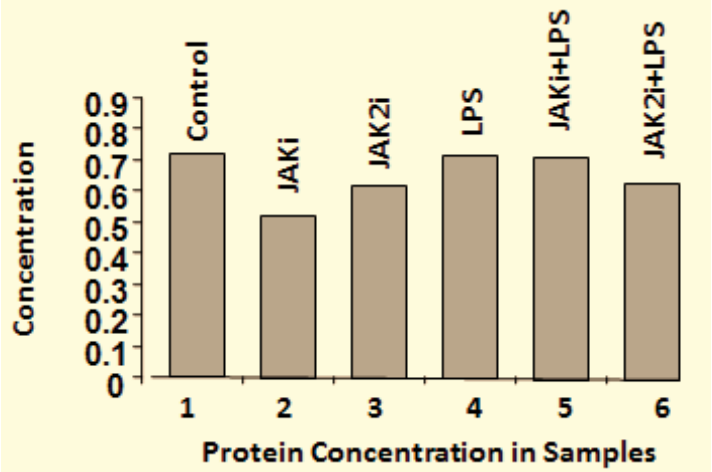

Figure 6 Protein concentration in samples

DISCUSSION

In the present study, the effects if two JAK inhibitors, AG-490 and JAK1 inhibitor on the activation of the JAK-STAT signaling pathway, iNOS expression and NO production in LPS treated macrophages was carried out. These two inhibitors suppressed the LPS induced iNOS expression and NO production along with inhibition of STAT pathway activation. Here the stimulation of cytokine involves the ligation of 2 different receptor subunits and these results in the formation of JAK heterodimers and their subsequent autophosphorylation.

Lipopolysaccharide-induced iNOS expression and NO production in J774 macrophages were inhibited by JAK inhibitors such as JAK1 and AG-490 based on dose-dependent manner along with their inhibitory action on STAT1 activation. When the JAK inhibitors were added to the cell culture 24 h after LPS, there was no effect on nitric oxide production. It has been suggested that the compounds do not inhibit iNOS activity. The results revealed that AG-490 inhibited the LPS induced iNOS expression in macrophages. It has been found that STAT1 is essential for the optimal induction of LPS-stimulated iNOS gene expression. Here LPS stimulated the type 1 interferon IFN production by monocytes and macrophages. It suggests that interferon plays a critical role for LPS induced iNOS gene expression by the activation of STAT1. STAT1 and NF-kB have shown transcriptional activation of many inflammatory genes. These contain cognate binding sites in their promoters. Here LPS was acted as a strong inducer of NF-kB which may activate transcription of iNOS gene expression, but this signal was not enough for the optimal induction of genes.

JAK-STAT and MAPK multiple signaling pathways were activated in macrophages treated with lipopolysaccharide. In this study, a JAK2 inhibitor causes a dose-dependent inhibition of phosphorylation of JAK2. It has been shown that JAK2 was a specific inhibitor that can suppress LPS-induced iNOS expression. This AG490 strongly suppressed the JAK2 phosphorylation. Thus JAK2 regulates iNOS expression mediated by AP-1 pathway. Tyrphostin inhibits LPS-induced iNOS expression at cellular signaling levels while this expression activates JAK2 mediated JNK signaling cascade with the attendant to AP-1 activation. AG490 blocked LPS induced iNOS expression through the blockage of JAK2 signaling. This finding provides the first molecular basis for the anti-inflammatory and anti-carcinogenic action of AG 490. The regulation of iNOS expression was also controlled at the level of mRNA stability due to the transcriptional regulation. In the present study JAK inhibitors, AG490 and JAK1 inhibitor did not affect the rate of degradation of iNOS mRNA in cells. It has been suggested that AG-490 and JAK1 inhibitor inhibits iNOS expression at the transcriptional level and they do not regulate mechanisms involved in the iNOS mRNA stabilization.

AG-490 causes a dose-dependent inhibition of the phosphorylation of JAK2. The concentration of the nitrite samples evaluated by performing nitrite assay revealed that there is less production of nitrite in macrophages cells when treated with JAK1 inhibitor as compared to cells treated with JAK2 inhibitor that showed high production of nitric oxide. It suppressed iNOS expression by blocking JNK phosphorylation. Activator protein-1 is involved in the regulation of LPS induced iNOS. In the present study JAK2 inhibitor, AG490 markedly suppressed LPS-induced phosphorylation. Thus AG-490 inhibited the iNOS expression by blocking JNK mediated AP-1 activation. In the present study, iNOS was chosen for the target molecule to investigate the molecular mechanism behind the anti-inflammatory activity. The iNOS is one of the potent mediators of pro-inflammation and carcinogenesis. Nitric oxide which was generated from iNOS was involved in the promotional stage of tumor genesis. Hence, inhibitors such as JAK1 and JAK2 interfered in iNOS expression and have the ability of cancer chemoprevention. JAK2 proved to be an active ingredient and has shown anti-inflammatory effects. Hence, it can be concluded that JAK2 is mainly involved in the regulation of iNOS expression as compared with JAK1. Activated JAK2 regulates the phosphorylation of JNK through the activation of PI3K, thus JAK2 plays a pivotal role in LPS-induced iNOS expression.

CONCLUSION

The result of this study concludes that JAK inhibitors, i.e., JAK 1 and AG-490 down-regulates multiple signaling pathways such as STAT1-activation, iNOS gene expression and NO production in LPS treated macrophages. A better knowledge of the mechanisms regulating iNOS expression and NO production in inflammation could facilitate the development of novel anti-inflammatory drugs acting through iNOS pathway. JAK inhibitors-JAK1 and AG-490 can regulate iNOS gene expression in macrophages which can be stimulated with the LPS to mimic a state of infection and inflammation. AG-490 suppressed LPS-induced iNOS expression through the inhibition of JAK2 mediated JAK pathway with the attendant to AP-1 activation. This finding provides the first molecular basis for the anti-inflammatory and anti-carcinogenic actions of AG-490. Hence, inhibitors such as JAK1 and JAK2 interfered in iNOS expression and have the ability of cancer chemoprevention. JAK2 proved to be an active ingredient and has shown anti-inflammatory effects. Activated JAK2 regulates the phosphorylation of JAK through the activation of PI3K thus plays a pivotal role in LPS-induced iNOS expression.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

[1] Naseem, Khalid M. "The role of nitric oxide in cardiovascular diseases." *Molecular Aspects of Medicine,* Vol. 26, 2005, pp. 33-65.

[2] Whiteside, Simon T., and Alain Israël. "IκB proteins: structure, function, and regulation." *Seminars in Cancer Biology*, Vol. 8, No. 2, 1997, pp. 75-82.

[3] Sharp, Gemma C., et al. "A computational model of lipopolysaccharide-induced nuclear factor kappa B activation: a key signalling pathway in infection-induced preterm labour." *PloS One,* Vol. 8 No. 7, 2013.

[4] Hibbs Jr, John B., et al. "Nitric oxide: a cytotoxic activated macrophage effector molecule." *Biochemical and Biophysical Research Communications,* Vol. 157, 1988, pp. 87-94.

[5] Marletta, Michael A., et al. "Macrophage oxidation of l-arginine to nitrite and nitrate: nitric oxide is an intermediate." *Biochemistry*, Vol. 27, 1988, pp. 8706-11.

[6] Stuehr, D. J., and C. F. Nathan. "Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells." *Journal of Experimental Medicine,* Vol. 169, 1989, pp 1011-20.

[7] Stuehr, Dennis J. "Purification, and properties of nitric oxide synthases." *Methods in Enzymology,* Vol. 268, 1996, pp. 324-33.

[8] Xie, Qiao-wen, and Carl Nathan. "The high-output nitric oxide pathway: role and regulation." *Journal of Leukocyte Biology,* Vol. 56, 1994, pp. 576-82.

[9] O'Shea, John J., Massimo Gadina, and Robert D. Schreiber. "Cytokine signaling in 2002: new surprises in the Jak/Stat pathway." *Cell*, Vol. 109**,** 2002, pp. 121-31.

[10] Hambleton, Julie, et al. "Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharide-stimulated macrophages." *Proceedings of the National Academy of Sciences,* Vol. 93, 1996, pp. 2774-78.

[11] Dell'Albani, P., et al. "Role of the JAK/STAT signal transduction pathway in the regulation of gene expression in CNS." *Neurochemical Research*, Vol. 28, No. 1, 2003, pp. 53-64.

[12] Kamezaki, Kenjirou, et al. "The role of Tyk2, Stat1 and Stat4 in LPS-induced endotoxin signals." *International Immunology,* Vol. 16, 2004, pp. 1173-79.

[13] Rane, Sushil G., and E. Premkumar Reddy. "Janus kinases: components of multiple signaling pathways." *Oncogene*, Vol. 19, 2000, pp. 5662-79.

[14] Cruz, M. T., et al. "LPS induction of I kappa B-alpha degradation and iNOS expression in a skin dendritic cell line is prevented by the janus kinase 2 inhibitor, Tyrphostin b42." *Nitric Oxide,* Vol. 5, 2001, pp. 53-61.

[15] Wilks, A. F., et al. "Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase." *Molecular and Cellular Biology,* Vol. 11, 1991, pp. 2057-65.

[16] Yeh, Tammie C., et al. "A dual role for the kinase-like domain of the tyrosine kinase Tyk2 in interferon-α signaling." *Proceedings of the National Academy of Sciences,* Vol. 97, 2000, pp. 8991-96.

[17] Kampa, Dione, and Joan Burnside. "Computational and functional analysis of the putative SH2 domain in Janus Kinases." *Biochemical and Biophysical Research Communications,* Vol. 278, 2000, pp. 175-*82.*

[18] Haan, Claude, et al. "Mapping of a region within the N terminus of Jak1 involved in cytokine receptor interaction." *Journal of Biological Chemistry,* Vol. 276, 2001, pp. 37451-58.

[19] Hilkens, Catharien MU, et al. "A region encompassing the FERM domain of Jak1 is necessary for binding to the cytokine receptor gp130." *FEBS letters,* Vol. 505*,* 2001, pp 87-*91.*

[20] Ihle, James N. "The Janus protein tyrosine kinase family and its role in cytokine signaling." *Advances in Immunology,* Vol. 60, 1995, pp. 1-35.

[21] Ivashkiv, Lionel B. "Cytokines and STATs: how can signals achieve specificity?." *Immunity,* Vol. 3, 1995, pp. 1-4.

[22] Leonard, Warren J., and John J. O'Shea. "Jaks and STATs: biological implications." *Annual Review of Immunology,* Vol. 16, 1998, pp. 293-322.

[23] Johnson, Howard M., et al. "Hypothesis: ligand/receptor-assisted nuclear translocation of STATs." *Proceedings of the Society for Experimental Biology and Medicine,* Vol. 218, 1998, pp. 149-55.

[24] Endo, Takaho A., et al. "A new protein containing an SH2 domain that inhibits JAK kinases." *Nature,* Vol. 387, 1997, pp. 921-24.