Taurine Chloramine Modulates

Nitric Oxide Production by Activated BV-2 Cells

by

Valeria Serban, MD

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment

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Abstract

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Taurine (2-aminoethanesulfonic acid) is a β-amino acid present in animal cells at high intracellular concentrations, but not incorporated into proteins. Taurine protects against inflammation-induced tissue damage. Although the mechanism through which taurine provides protection is uncertain, the formation of taurine monochloramine (Tau-Cl) and its subsequent actions appears to be important.

Tau-Cl formation requires the presence of a halide-dependent myeloperoxidase (MPO) system, which is associated primarily with neutrophils. Taurine reacts with hypochlorous acid (HOCl), an aggressive oxidant, to form taurine chloramine (Tau-Cl), a more stable and selective oxidant that also functions as a modulator of the inflammatory response by inhibiting the production of inflammatory mediators.

In this work we focused on nitric oxide (NO), a short-lived gas molecule, with roles in neural degeneration, demyelination and inflammatory response. NO is produced from L-arginine by the catalytic enzyme nitric oxide synthase (NOS). Microglia and astrocytes are the major immunoresponsive cells in the CNS and when activated express inducible NOS (iNOS). Although there are reports describing the inhibitory effects of Tau-Cl on inflammatory mediators production by activated astrocytes, Tau-Cl modulation of activated microglia has not yet been examined.

The primary goal of the present investigation was to determine the effects of Tau-Cl on NO production by activated BV-2 cells and the molecular mechanisms through which Tau-Cl exerts its effect. Cultures of BV-2 cells were activated with lipopolysaccharide (LPS), interferon-γ (IFN-γ), and a combination of both LPS and IFN-γ. Tau-Cl dose-dependently inhibited NO production by activated BV-2 cells, regardless of the stimulator. Nitric oxide inhibition was parallel with iNOS protein downregulation by the Tau-Cl treatment. The BV-2 cells responded independently to LPS and IFN-γ, allowing separate investigation of the two signaling pathways: NF-kB for LPS and JAK/Stat for IFN-γ. In addition, Tau-Cl modulated iNOS gene transcription by inhibiting the activity of the transcription factors NF-kB and Stat-1.

This study has clinical relevance, suggesting the role of taurine as a potential therapeutic agent. Intervention with pharmacological doses of taurine in the acute phase of inflammation could, via taurine chloramine formation, downregulate the CNS inflammatory response.

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LIST OF ABBREVIATIONS

AIDS = Acquired Immune Deficiency Syndrome

BV-2 cells = the murine microglia cell line used during experiments

C/EBP = CCAAT/enhancer Binding Protein

COX-2 = Cyclooxygenase -2

CNS = Central Nervous System

EMSA = Electrophoretic Mobility Shift Assay

GABA = Gamma Aminobutyric Acid

GAS = Gamma-IFN Activated Site

HIF-1 = Hypoxia Inducible Factor-1

IFN-γ = Interferon -γ

IkB = a family of inhibitory proteins that prevent NF-kB nuclear translocation

IKK = IkB Kinase

IL-1β = Interleukin -1β

IL-6 = Interleukin -6

IL-12 = Interleukin -12

IL-8 = Interleukin -8

IP-10 (CXCL-10) = an inflammatory chemokine

IRF-1 = Interferon-γ Regulatory Factor

ISRE = IFN-stimulated Response Elements

JAK = Janus Activated Kinase

HOCl = Hypochlorous Acid

LPS = Lipopolysaccharide from the bacterial wall of *E. coli*

MCM = Minichromosome maintenance family of proteins

MCP-1 (CCL-2) = Monocyte Chemoattractant Protein-1

MIP-2 (CXCL-1)= Macrophage Inflammatory Protein-2

MPO = Halide-dependent Myeloperoxidase

NEMO = NF-kB Essential Modulator

NF-kB = Nuclear transcription Factor-kB

NLS = Nuclear Localization Sequence

NO = Nitric Oxide

NO_2^- = Nitrite

NOS = Nitric Oxide Synthase; iNOS inducible, nNOS neuronal, eNOS endothelial

PIAS = Protein Inhibitors of Activated Stat

PGE_2 = Prostaglandin E_2

PMN = Polymophonuclear leukocytes

SBE = Stat Binding Element

SOCS = Suppressor of Cytokine Signaling

Stat = Signal Transducers and Activators of Transcription

Tau-Cl = Taurine Chloramine

TNF-α = Tumor Necrosis Factor -α

BACKGROUND AND SIGNIFICANCE

I. Taurine, Taurine Chloramine and Inflammation

Taurine (2-aminoethanesulfonic acid) is a β-amino acid, which contains a sulfonic acid group instead of a carboxylic acid group, and therefore is not incorporated into proteins (1, 2). Although taurine does not participate in protein synthesis, it is one of the most abundant free amino acids in the cytoplasm of most animal cells, but absent in plants and bacteria (1, 2).

Taurine levels are high in inflammatory cells, electrically excitable tissue, secretory structures, and blood cells, but are relatively low in extracellular fluids. In mammals, the highest concentration of taurine is found in brain, heart and skeletal muscle (1, 2). The highest concentration of taurine is found in the developing brain. During the aging process taurine concentration falls, with levels in the adult being one third of those present in neonates. This pattern has been observed in human, monkey, mouse, rabbit, and rat (1, 2). Taurine is considered essential for brain development and safe for ingestion, and therefore is added to human infant formula. The intracellular concentration of taurine in human leukocytes, monocytes and neutrophils (PMN) ranges from 20 mM to 50 mM, while plasma concentration is 50-100 μM (3). In the CNS, taurine concentration is low extracellularly, around 20-100 μM, and high intracellularly, 6-20 mM (4, 5, 6). Extracellular taurine concentration is reported to increase 5-10 folds during ischemia (7-10). Taurine is actively transported into cells by the taurine transporter protein (TauT) in a Na^+/Cl^- co-dependent process (2).

Physiological actions of taurine include its role as antioxidant against hypochlorous acid (HOCl), through formation of taurine chloramine, and its ability to act as partial GABA agonist at the GABA-A receptor, and also as an agonist for the strychnine-sensitive glycine receptor (2). Taurine stabilizes membranes, regulates calcium fluxes into cells, and modulates intracellular free calcium levels (1, 2). In addition, taurine has an important role in osmoregulation and brain development (1, 2, 12). Taurine interaction with zinc is essential for maintaining retinal rod cell structure and function (1, 2). The liver regulates taurine levels through biosynthesis and the kidney through excretion (2).

Taurine protects against tissue damage in several conditions where inflammation is a common pathogenic feature (11, 13). This has been documented in animal models of oxidant-induced lung injury. Prophylactic administration of taurine protects the lung from damage induced by inhalation exposure to nitrogen dioxide (14), ozone (15), and by intratracheal instillation of bleomycin (16), amiodarone (17), and paraquat (18). All these *in vivo* models of lung damage involve neutrophils recruitment, followed by macrophages, and production of inflammatory mediators.

Although the mechanism through which taurine provides tissue protection is still unknown, the formation of taurine monochloramine (Tau-Cl) and its subsequent actions appears to be significant (13, 19). Recent *in vitro* studies suggest that Tau-Cl may function as a modulator of the inflammatory response because Tau-Cl inhibits production of NO, TNF-α, and other inflammatory mediators generated by activated macrophages and polymophonuclear leukocytes (PMN, neutrophils) (13, 20, 21, 22). Tau-Cl formation

requires the presence of a halide-dependent myeloperoxidase (MPO) system, which is associated primarily with PMN (23-26).

Taurine chemically reacts with hypochlorous acid (HOCl), a highly aggressive oxidant, to form taurine chloramine (Tau-Cl), which is a more stable and selective oxidant than HOCl (23-25, 27). MPO catalyzes the reaction of chloride anion with hydrogen peroxide to form hypochlorous acid, a potent oxidant that causes indiscriminate damage to many essential cellular constituents (23-25).

Activated PMN secrete MPO into the extracellular space. Normally, the extracellular taurine level in the CNS is relatively low, but this level could be increased by exogenous means to subsequently enhance Tau-Cl formation. Most of the Tau-Cl is produced extracellularly and actively transported into the cells through a $Na+/Cl^{-}$ co-dependent system. In addition, small amounts of Tau-Cl may be generated intracellularly, by PMN (26). During an acute CNS inflammatory response, PMN are recruited to the site of inflammation and consequently provide the potential for Tau-Cl to be generated relatively early in the process, at the site of inflammation. Recently, MPO mRNA was isolated from human microglia present in multiple sclerosis lesions and from rodent microglia treated with amyloid-β protein, which demonstrates a potential for Tau-Cl to be generated by microglia, even without PMN recruitment (28, 29).

Although neutrophils and macrophages play an important role during inflammation in general, glial cells are especially involved in mediating inflammatory events in the CNS (30-33). Studies with C6 glioma cells, which retain astrocytic properties, have demonstrated that Tau-Cl inhibits production of NO, PGE_2, MCP-1, and MIP-2 (34-35). The role of Tau-Cl in the modulation of the inflammatory response by

microglia has not been studied yet and represents the subject of the present work. Understanding the effects of Tau-Cl on microglial cells is of critical importance because microglia cells play a key role during CNS inflammatory responses. Microglia not only produce tissue damaging inflammatory mediators, but also stimulate astrocytes to generate even more inflammatory mediators that ultimately lead to neuronal death (35-39).

II. Microglia and Inflammation in the Central Nervous System

Microglia cells are considered brain resident macrophages, and along with astrocytes are the major immunoresponsive cells in the CNS. When stimulated by bacterial products or cytokines, microglia cells produce inflammatory mediators including NO, PGE_2 and TNF-α (36-43). Activated glial cells express iNOS and COX-2, inducible enzymes that generate NO and PGE_2, respectively.

Microglia become activated during CNS inflammatory responses (44, 45), e.g. viral and bacterial infections (46, 47), AIDS dementia complex (48, 49), stroke (50-57), head trauma, multiple sclerosis, and neurodegenerative diseases (Alzheimer and Parkinson Diseases) (58). Increased NO and PGE_2 production is reported to play a role in late stage neuronal death in the penumbra area of an ischemic stroke, hours and days after the injury (57-61). PMN are recruited to the ischemic area within one hour of insult and persist for at least 15 days. In addition to NO and PGE_2, glial cells generate other inflammatory mediators that contribute to neuronal death, including IL-1β, IL-6, IL-12 and TNF-α (32, 33, 62-67).

Glial cells not only produce inflammatory mediators, but also propagate the inflammatory response by producing chemokines (68-70). Chemokines are inducible cytokines of low molecular weight that selectively recruit subpopulations of leukocytes to the site of inflammation. Unlike leukotrienes, bacterial cell wall components, or complement fragments, chemokines are cell-type selective chemoattractants (71). MCP-1, MIP-2 and IP-10 are inflammatory chemokines that play a significant role in various forms of CNS inflammation (72-76).

MCP-1 (CCL-2), a β-chemokine, recruits and activates primarily monocytes to the site of injury in various tissues, including brain (69, 70, 77, 78). Glial cells secrete MCP-1 during inflammatory response in traumatic brain injury (79, 80), ischemic stroke (81-83), experimental immune encephalitis (84, 85) and multiple sclerosis (86).

MIP-2 (CXCL-1), a rat α-chemokine, attracts neutrophils to the site of inflammation (30, 87-89). Neutrophils appear to play a role in causing neuronal damage in models of ischemic stroke (74, 75) and traumatic brain injury (90). MIP-2 is functionally similar to human IL-8. Other studies report IP-10 (CXCL-10) expression in the CNS for several days following ischemia and suggest that IP-10 prolongs the recruitment of leukocytes to the brain (75).

III. Nitric Oxide and Inducible Nitric Oxide Synthase Gene

Nitric oxide (NO), a diffusible gas molecule and short-lived free radical, has important implications in several physiological and pathological conditions. At low concentration NO has a role in neurotransmission, vasodilatation, and inhibition of

platelet aggregation (58). Higher concentrations of NO protect against bacteria and tumor cells invasion. Aberrantly high production of NO is neurotoxic and can cause tissue damage, with role in neural degeneration, demyelination, and inflammation (58). NO is produced during the enzymatic conversion of L-arginine to L-citrulline, by the catalytic enzyme nitric oxide synthase (NOS) (58).

Three separate genes code for the three NOS isoforms: nNOS, eNOS, and iNOS. The iNOS gene is induced by cytokines and expressed mainly by inflammatory and immune cells. The calcium/calmodulin dependent isoforms, nNOS and eNOS, are rapid activated by biological signals that increase intracellular free calcium. Calcium-activated calmodulin binds to nNOS and eNOS and transiently activates the two NOS isoforms. In contrast, iNOS is primarily regulated at the level of gene transcription (58). Calmodulin is tightly bound to iNOS at basal calcium levels, and therefore iNOS is notably distinguished from the constitutive isoforms by its prolonged production of relatively large amounts of NO (58).

Overproduction of NO by iNOS can cause tissue damage that overcomes its potential benefit for host defense. Inducible NOS is implicated in the pathogenesis of many inflammatory responses, e.g., asthma, transplant rejection, inflammatory bowel disease, rheumatoid arthritis, and septic shock (58). In the brain, excessive amounts of NO are derived from iNOS gene activation in glial cells and contribute to demyelination, neuronal degeneration and cell death (58). In addition to iNOS regulation at the transcriptional level, experimental data show involvement of posttranslational mechanisms resulting in rapid inactivation of the iNOS catalytic enzyme in the cytoplasm (58).

Several in vitro studies have documented the induction of the iNOS gene in glial cells treated with proinflammatory cytokines (IFN-γ, TNF-α, IL-1β), viral or bacterial products (LPS), and abnormal protein aggregates (91). There are several transcription factors involved, the most important being nuclear transcription factor-kB (NF-kB), Stat-1, CCAAT/enhancer binding protein (C/EBP), and interferon-γ regulatory factor (IRF-1) (92-95). Transcriptional activation of iNOS gene is NF-kB-dependent (93).

The promoter of the murine iNOS gene has numerous consensus sequences to bind transcription factors (93). They are located at two transcriptional regulatory regions: region-I (from +10 to –300 bp, upstream of the TATA box), and region II (from –1100 to –800 bp). Region-I has been shown to be the principal target for LPS-mediated iNOS gene induction and contains C/EBP and NF-kB binding sites. Region-II is primarily important for IFN-γ-mediated iNOS induction and contains binding sites for IRF-1, Stat-1α and NF-kB (93).

Stat-1-α binds as a homodimer to a region of the iNOS promoter called γ-IFN activated site (GAS), from -942 to -934 bp (96). GAS, also called STAT-binding element (SBE), is present in the promoter region of many other IFN-γ-induced genes, including IRF-1 (97).

IRF-1 is a DNA-binding protein with a basic NH_2-terminal domain, responsible for nuclear DNA binding, and an acidic -COOH terminal region, highly conserved between mice and humans (94). IRF-1 production is induced by cytokines, such as IFN-γ, TNF-α, IL-1 and IL-6. IRF-1 enters into the nucleus rapidly after its synthesis, where it binds to the promoter of IFN-γ-induced genes. In addition, IRF-1 has been shown to facilitate the binding of other transcriptional factors, such as NF-kB, to the iNOS

promoter region (94). IRF-1 initially binds as a monomer to the iNOS promoter, and then a second molecule attaches to generate a dimer and extend the contact of IRF-1 with the DNA. The formation of the IRF-1 dimer is essential for full iNOS gene activation by cytokines (94).

C/EBP is another transcription factor that binds to the promoter of the iNOS gene and has critical importance for iNOS gene transcription after stimulation with IFN-γ or LPS. C/EBP might cooperate with HIF-1 in orchestrating iron-mediated iNOS regulation (98). In addition, C/EBP is important for glucose-mediated iNOS gene expression in rat glial cells stimulated with LPS (95).

Experimental studies proved that iron also participates in the regulation of iNOS gene transcription (98). These data showed the presence of an autoregulatory feedback loop in macrophages that links iron homeostasis with NO formation during host defense against infections and tumor cells. The mechanism through which iron affects iNOS transcriptional regulation is unclear, but activation of hypoxia inducible factor-1 (HIF-1) might be involved (98). The iNOS gene expression is not an all or nothing phenomena. There are graded levels of expression depending on the cooperative nature of the transcription factors. Maximal iNOS expression requires the presence of all factors. Among them, NF-kB appears to be essential, but not sufficient for full iNOS transcription.

IV. Signal Transduction Pathways Regulating the iNOS Gene Expression

Transcription of iNOS is mainly regulated through two signaling pathway: NF-kB and JAK/Stat.

The NF-kB Signaling Pathway

A large number of external stimuli (e.g. bacterial lipopolysaccharide, viral proteins, TNF-α, IL-1β, double stranded DNA, physical and chemical stress) act through the NF-kB (nuclear factor kB) signaling pathway in most cell types, including glia (91, 99-105). NF-kB is required for the activation of most genes implicated in the acute phase of the immune response, including iNOS (101-107).

A conserved DNA binding domain, called the Rel homology domain, characterizes the NF-kB family of proteins. Mammalian cells have five members of the NF-kB family: NF-kB-1 (p50/p105), NF-kB-2 (p52/p100), c-Rel, Rel A (p65) and Rel B. In active DNA-binding form NF-kB is usually a heterodimer composed by various combinations of proteins from the NF-kB/Rel family (99-105). The Rel proteins differ in their abilities to activate transcription. Only p65 (Rel A), Rel B and c-Rel have potent transcriptional-activation domains. Active NF-kB heterodimer in many cell types is composed of p65 (Rel A) and p50 (99-105). Homodimers of p50, NF-kB-1 (p50/p105) and NF-kB-2 (p52/p100) are transcriptionally silent and may function as repressors of gene expression (108).

In the cytoplasm NF-kB is bound to IkB, forming an inactive complex. IkB is a member of a family of inhibitory proteins that prevent NF-kB nuclear translocation. IkB interaction with NF-kB blocks the nuclear localization sequence (NLS) of NF-kB. Among the IkB family of proteins, only -α, -β and -ε contain N-terminal regulatory regions, which are required for stimulus-induced degradation, the key step in NF-kB

activation. IkB-α is a critical component in this pathway and the most studied of the IkB proteins (99-105).

Cell activation leads to rapid phosphorylation of IkB by a large (~800 kD) multisubunit IkB kinase (IKK). IKK is an enzyme with two catalytic subunits, IKK-α and IKK-β, both able to phosphorylate IkB, and one regulatory subunit, IKK-γ or NF-kB essential modulator (NEMO). IKK-α and IKK-β are highly homogenous proteins with 50% sequence identity and contain N-terminal protein kinase domains, as well as leucine zipper and helix-loop-helix motifs (105, 109). IKK-β phosphorylates IkB-α in response to LPS and cytokine activation, and participates in inflammatory gene activation. IKK-α has a major role in dermal and skeletal development (107). NEMO not only stabilizes the multisubunit kinase complex, but is also essential for IKK activation through upstream signals (110, 111). IKK is the point of convergence for most NF-kB-activating stimuli. IKK is also activated by phosphorylation (112-115). However, the complete kinase cascade mediating the effects of LPS and cytokines on IKK-β is yet to be identified (105).

Stimulus-induced phosphorylation of IkB-α (Ser-32 and Ser-36) by IKK-β triggers polyubiquitination by an ubiquitin ligase complex, and subsequently, degradation of IkB by the 26S proteasome complex, which frees NF-kB from the inactive complex (1116-119). Free in the cytoplasm, NF-kB molecule interacts with the nuclear import machinery and translocates into the nucleus (99-101). Once in the nucleus, NF-kB binds, through its NLS, to the regulatory elements (kB site) in the target gene promoter. NF-kB is required for the expression of iNOS, TNF-α, IL-1β, IL-6, IP-10, COX-2, MIP-2 and

MCP-1 genes (106-109). The presence of other transcription factors in the nucleus increases NF-kB transcriptional activity (109).

Termination of NF-kB signaling in the nucleus is very complex and not completely understood. Prior studies demonstrated two negative feedback systems to be involved in regulating nuclear NF-kB transcriptional activity. First, expression of the IkB gene is also NF-kB regulated (99-101). The newly synthesized IkB translocates into the nucleus, binds NF-kB, and the resulting nuclear complex of NF-kB/IkB is transported back to the cytoplasm. Second, the Rel A (p65) subunit of NF-kB can be reversible acetylated and bound to IkB-α, forming a complex that is exported from the nucleus (120).

The IkB-α-dependent nuclear export of NF-kB also replenishes the pool of latent NF-kB in the cytoplasm needed for the next stimulus-induced response. Experimental studies identified a molecular mechanism involved in the negative regulation of NF-kB basal activity, through the phosphorylation of Rel A (p65) at Ser-468. This appears to determine the outcome of NF-kB activation during the inflammatory response (121).

LPS stimulates gene expression predominantly through the activation of the NF-kB signaling pathway (91-96). LPS induces the phosphorylation (Ser-276) of NF-kB p65 by p38 MAP kinase, and increases the transcriptional activity of p65 by strengthening its interaction with constitutive nuclear transcriptional proteins (91). LPS also exerts a rapid effect on the IFN-γ-induced Stat-1 pathway by increasing the activity of a kinase that phosphorylates Stat-1 (Ser-727) and strongly augmenting Stat-1 transcriptional activity (122, 123).

The JAK/Stat Signaling Pathway

Cytokine binding to cell membrane receptors activate, through tyrosine phosphorylation, the Stat family members of transcription factors. Stat proteins are inactive in the cytoplasm and become activated through tyrosine phosphorylation, which typically occurs through cytokine receptor associated tyrosine kinases, i.e., Janus activated kinases (JAKs), or growth factor receptor tyrosine kinase (124-127). Recently, the non-receptor tyrosine kinase (i.e. src) has been found to cause Stat phosphorylation. Phosphorylated Stat forms homo- or hetero- dimers that enter the nucleus and bind to specific DNA promoter sites to increase the transcription of target genes (128-130). Ligand-dependent Stat activation is a transient process, lasting for several minutes to several hours (122). In addition, nuclear Stat dimer may be dephosphorylated by an unidentified protein tyrosine phosphatase to form unphosphorylated Stat monomers in the nucleus (122-127). Stat monomers lack transcriptional activity and translocate back to the cytoplasm, where they can dimerize and get reactivated, completing the activation-inactivation cycle (131).

Seven mammalian Stat proteins (Stat-1, Stat-2, Stat-3, Stat-4, Stat-5a, Stat-5b and Stat-7) have been identified (125). Stat proteins share a number of conserved domains: an amino terminal domain, a coiled-coil domain which allows interaction with other nuclear proteins, such as PIAS (protein inhibitors of activated Stat), a DNA-binding domain, a linker domain, an SH_2 domain, and a tyrosine activation domain (TAD) that can interact with members of the minichromosome maintenance (MCM) family of proteins. While all Stat proteins depend on their carboxyl terminal segment for transcription activation, other

regions of the Stat molecules may also contribute to activation (e.g., amino terminal end) (122).

Stat-1, the founding member of the Stat family is essential for the innate immune response to viral or bacterial infection. Stat-1 is phosphorylated on a single residue (Tyr-701) in response to IFNs, IL-6, and epidermal growth factor stimulation. Tyr-701 phosphorylation is required for Stat-1 dimerization, nuclear translocation, DNA binding, and transcriptional activation (122, 124, 129, 130). In addition, Ser-727 phosphorylation strongly augments Stat-1 transcriptional activity. The kinase responsible for Ser-727 phosphorylation remains to be identified (129, 130). Only IFN-γ activation leads to homodimerisation of tyrosine-phosphorylated Stat-1. In contrast, IFN-α elicits a heterodimer composed of Stat-1 and Stat-2 (128-131).

Several factors have been identified that modulate Stat signaling: PIAS, SOCS, and MCM. PIAS (protein inhibitors of activated Stat) is family of nuclear proteins that downregulates nuclear Stat signals (128, 129, 131).

Five members of PIAS family are known: PIAS-1, PIAS-3, PIAS-xα, PIAS-xβ and PIAS-y (132, 133). PIAS proteins show over 50% homology. They have highly conserved domains, including a zinc binding motif and an acid region. PIAS-1 is specifically associated with Stat-1 homodimer (133). PIAS proteins block Stat-mediated gene activation by inhibiting Stat-DNA binding in the nucleus (125, 134). The PIAS-Stat interaction is dependent on cytokine-induced Stat activation through phosphorylation (Tyr-701) and homodimer formation (133-135). PIAS-1 blocks Stat-1 binding to the DNA promoter gene and inhibits gene activation. PIAS-3 specifically inhibits signaling of active Stat-3 (132, 133).

A family of cytokine-inducible JAK inhibitors, called suppressor of cytokine signaling (SOCS), downregulates Stat signals by inhibiting JAK kinase catalytic activity (135). The SOCS family of proteins has eight members that share a similar structure, with a central SH_2 domain, an N-terminal, and a region of homology at the C-terminus, named the SOCS box. SOCS-1 inhibits signal transduction in response to IFN-γ, IL-6 and growth factors (135). In contrast to SOCS proteins, which are induced in response to cytokines, PIAS proteins are constitutively expressed and function as acute, early response regulators (124, 135).

The MCM (minichromosome maintenance) family of proteins plays a role in the upregulation of Stat-1-mediated gene transcription. Five MCM nuclear proteins are specifically associated with the TAD domain of Stat molecules and upregulate Stat gene transcription. MCM-5 interacts with Stat-1 TAD domain when Ser-727 of TAD domain is phosphorylated (122, 125).

IFNs, the largest subfamily of cytokines, mediate their biologic functions through the JAK/Stat signaling pathway. There are over twenty type-I IFNs (e.g., IFN -α, -β, -ω and –τ) and one type-II IFN (i.e., IFN-γ), each with a specific receptor. IFN-γ binding to its receptor complex leads to rapid phosphorylation and activation of Jak-1 and Jak-2 tyrosine kinases, which in turn phosphorylate Stat-1 that forms homodimer complexes (128-129). Subsequently, Stat-1 homodimers translocate to the nucleus and induce transcription, via binding to the GAS (gamma activation site) of IFN-γ-inducible promoters. IRF-1 is one of the Stat-1 induced genes (92, 128-131, 136).

Although a single Stat-1 homodimer bound to a single GAS element is sufficient to activate transcription, specificity of gene activation can be achieved through co-

operative binding of Stat-1 to several adjacent consensus sites or by forming heterotrimer complex (p84 with Stat-1 homodimer) (93). These complexes bind to the IFN-stimulated response elements (ISRE), a prototype regulatory element for genes activated by IFN-γ. IRF-1 can interact with the ISRE. IRF-1 is constitutively expressed in small amounts in most cell types and is largely inducible by IFN-γ (93). This is important for iNOS gene expression, which has two juxtaposed ISRE elements. The distal ISRE region represents a strong transcriptional activator sequence due to its strong binding to IRF-1, whereas the proximal ISRE site is a weak activator (93, 137).

In BV-2 cells, the ability of IFN-γ alone to induce NO production suggests a cross talk between the NF-kB and JAK/Stat pathways (138-141, 145-147). LPS and IFN-γ induce iNOS gene expression in BV-2 cells through independent, but intricate mechanisms (136, 142-144). Identifying the signaling pathways for iNOS induction by pro-inflammatory cytokines represents an important step towards understanding the molecular mechanisms for neural damage.

Inflammatory response represents a complex process that has synergy and redundancy as important characteristics. Redundancy refers to the requirement of several independent stimuli to act simultaneously in order to generate highly potent substances like cytokines or chemokines. Synergistic action of pro-inflammatory cytokines and bacterial products refers to inflammatory response augmentation by concomitant presence of stimuli, greater than an additive result (136, 145).

SPECIFIC AIMS

Our hypothesis is that Tau-Cl inhibits the production of nitric oxide by activated microglia, similar to its effects on macrophages and C6 glioma cells. The main purpose of this work is to determine the effect of Tau-Cl on NO production by activated BV-2 cells, and to investigate the molecular mechanism of Tau-Cl action by focusing on selected transcription factors that regulate iNOS gene expression.

The specific aims of this research work are as follows:

1) To determine the effects of Tau-Cl on nitric oxide production by activated BV-2 cells. The BV-2 cells were activated with lipopolysaccharide (LPS) alone, interferon-γ (IFN-γ) alone, or with a combination of both LPS and IFN-γ. Tau-Cl was added to the culture media at the time of activation and NO production was evaluated.

2) To delineate the molecular mechanism through which Tau-Cl exerts its effect in activated BV-2 cells. Tau-Cl influence on inducible nitric oxide synthase (iNOS) enzyme expression was evaluated by Western blot analysis. Tau-Cl modulation of iNOS gene expression was determined by Northern blot analysis. Further experiments were conducted to evaluate Tau-Cl effects on cell signaling pathways, focusing on NF-kB and Stat-1 transcription factors. LPS acts mainly through the NF-kB, while IFN-γ exerts its effect primarily through the JAK/Stat pathway. The BV-2 cells respond independently to each of the two activators, LPS and IFN-γ, and this allows us to evaluate the role of each signaling pathway in mediating the effect of Tau-Cl on iNOS gene expression. Accomplishment of these goals would give a better understanding of Tau-Cl function as a potential modulator of the inflammatory response.

METHODS

Cell Culture: The BV-2 cells are immortalized microglial cells that retain morphological and functional characteristics of microglia (148-150). Dr. Michael McKinney (Mayo Clinic, Jacksonville, FL) kindly provided BV-2 microglial cells for use in these studies. The BV-2 cells were grown in DMEM with 10% FCS, 2 mM L-glutamine, and antibiotics at $37^{o}C$ in a humidified 95%/5% mixture of air and CO_2. The BV-2 cells were seeded in 24-well plates (for NO production and Western blot experiments) at a density of 0.5×10^5 cells/well, and Petri dishes of 100 mm diameter (for Northern blot, EMSA and Supershift experiments) at a density of 5×10^5 cells/well and used 2-3 days later when they were 70-90% confluent. Medium was changed when the experiments were initiated. Cells were washed twice with Hanks' balanced salt solution (HBSS), and experiments were conducted in phenol red free DMEM with 2% FCS.

Cell Viability: The BV-2 cells were evaluated morphologically, by MTS (Promega) reduction, and by trypan blue exclusion.

Preparation of Tau-Cl: Equimolar amounts of NaOCl were added dropwise to taurine, with constant vortex, at pH 8.3. Tau-Cl was freshly prepared on the day of use. The identity of Tau-Cl was verified by measuring its UV absorption 190-350 nm (20, 151).

Nitric Oxide Measurement: NO production was evaluated by quantification of nitrite, NO_2^-, a stable metabolic endproduct of NO. NO_2^- accumulation in the culture media was measured using the Greiss reagent. For measurement of NO_2^- accumulation in the media, we sampled the conditioned media (100 μl) and mixed with an equal volume

of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride, and 2.5% phosphoric acid). Samples were incubated 10 minutes at room temperature before determining optical density at 550 nm, using a microplate reader (Cambridge Technology, Inc.) (20). NO_2^- concentrations were determined using a calibration curve with sodium nitrite as standard.

Cell Lysates Preparation and Proteins Measurement: Cell lysates were prepared by washing cells twice with phosphate buffered saline (PBS) before lysing in RIPA buffer (PBS with 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1 % SDS) containing protease inhibitors: PMSF (1 mM), aprotonin (10 µg/ml) leupeptin (1 µg/ml), sodium orthovanadate (1 mM), sodium fluoride (1mM) and pepstatin (1 µg/ml). Cell lysates were collected, incubated on ice for 30 minutes, centrifuged for 15 minutes at 16,000 xg at 4 ^{o}C, and the supernatant was frozen at –70 ^{o}C until used. Concentrations of proteins in the cell lysates were measured using the Micro BCA protein assay regent kit from Pierce Lab (Rockford, IL). Samples of 100 µl of protein were incubated with equal amount of developing reagent, for 2 hours at 37 ^{o}C. Optical density was measured at 570 nm using a microplate reader (Cambridge Technology, Inc.). Protein concentrations were determined using bovine serum albumin (BSA) as standard (Pierce, Rockford, IL).

Western Blotting: Cell lysates were diluted in SDS preparation buffer, subjected to SDS-PAGE, and subsequently transferred to a nitrocellulose filter. Blocking of non-specific binding was done by filter incubation in a solution of PBS with 5% non-fat dry milk and 0.05% Tween 20. Incubation with monoclonal anti-murine iNOS (Transduction Laboratories, Lexington, KY) primary antibody (dilution 1:500) was followed by incubation with goat anti-mouse secondary antibody (dilution 1:1000). Secondary goat

anti-mouse antibody was conjugated to horseradish peroxidase (Jackson ImmunoResearch) (34, 35). Western blots were developed using the ECL chemiluminescence detection system (Amersham, Arligton Heights, IL).

Northern Blot Analysis: Total RNA was extracted from cells with Tri-Reagent (Molecular Research Center, Inc.) before being size-fractionated by electrophoresis in 1% agarose-formaldehyde gel and transferred overnight to Nytran membrane. RNA was cross-linked to membranes by UV irradiation. Blots were prehybridized in hybridization solution (ExpressHyb Hybridization Solution, Clontech, Palo Alto, CA) for 1 hour at 68°C followed by hybridization with [^{32}P] dCTP random prime-labeled cDNA at 68°C for 16-18 hours. Plasmids containing cDNA probe for iNOS (murine) and GAPDH (rat) were available in the lab, graciously provided by Dr. Carl Nathan (Cornell University, New York, NY) and Dr. Rong Dong (London University, London, UK), respectively. Blots were washed three times at room temperature in 2xSSC containing 0.5% SDS for 10 min each, followed by two washes at 50°C in 0.1xSSC containing 0.1% SDS. RNA hybridized with cDNA probe was visualized after autoradiography, using Kodak XAR-5 film exposed at –70°C (34, 152).

Nuclear protein extract: Cells were washed twice in ice-cold PBS, suspended in 1 ml PBS in a microfuge tube, pelleted (12,000 xg for 15 sec), resuspended in 400 μl of ice-cold nuclear extraction buffer A (10 mM HEPES, pH 7.9 containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF), and allowed to swell on ice for 15 minutes. Nonidet NP-40 (25 μl of 10% solution) was added and the tube was vigorously vortexed for 10 seconds. The nuclei were collected by centrifugation (12,000 xg for 30 seconds), suspended in 100 μl of ice-cold nuclear extraction buffer (20 mM

HEPES, pH 7.9 containing 2.5% glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF), and vigorously rocked at 4°C for 15 minutes on a shaking platform. Nuclear extracts were clarified by centrifugation (16,000 xg for 20 minutes, at 4°C) and the supernatant was stored in aliquots frozen at –70°C until used (152, 153).

Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assays: DNA-protein binding interactions were performed using 3-5 μg of nuclear protein extract incubated for 15 minutes at 22°C with 1 ng of [^{32}P] labeled double stranded oligonucleotide probe (custom synthesized by Bioserve Biotechnologics, Laurel, MD) in 20 mM Tris-HCl, pH 7.9, containing 50 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.1% Nonidet NP-40, 1 mM DTT, 0.5% BSA, and 2 μg poly dI-dC in a final volume of 20 μl (152).

Gel Supershift: Supershift analyses were performed using the indicated polyclonal antibodies (Santa Cruz). The antibody was included in above reaction mixture and incubated at 4°C for 90 minutes before adding the [^{32}P] labeled oligonucleotide probe, followed by incubation at 22°C for 15 minutes. The DNA-protein complexes were fractionated in 6% native polyacrylamide gels run in 0.5xTBE buffer. Gels were dried under vacuum and bands were visualized by autoradiography.

The double-stranded oligonucleotide probe was labeled using the Klenow fragment of DNA polymerase and [^{32}P] dCTP. The probe used in the experiments has the consensus NF-κB sequence 5'-AGTTGAGG<u>G</u>GACTTTCCCAGGC - 3', and the mutant probe sequence has one G base change to C (underlined) (152). The GAS sequence for binding Stat-1 is 5'-GATGTATTTCCCAGAA<u>AA</u>GGAA C-3' and the underlined AA bases were changed to GC in the mutant probe (152).

RESULTS OF THE EXPERIMENTAL STUDIES

Microglia cells are key players during inflammatory events in the CNS. Taurine, via taurine chloramine formation is suggested to protect against tissue damage. Although there are reports describing the inhibitory effects of Tau-Cl on the production of inflammatory mediators by activated astrocytes and other immunoresponsive cells, Tau-Cl modulation of activated microglia has not yet been examined and represents the subject of the presented work.

I) Tau-Cl Inhibition of NO Production by Activated BV-2 Cells

a) NO Production by BV-2 Cells in Response to IFN-γ and LPS Activation

Rationale: Activation of the iNOS gene and, consequently, NO production are involved in early inflammatory response. Experiments were first conducted to evaluate NO production by BV-2 cells. Optimal conditions for cell responsiveness to different activators were determined to allow further studies with Tau-Cl.

Procedure: The BV-2 cells were grown in 24-well plates and activated with either LPS (0.01, 0.1, 1, and 10 μg/ml), or IFN-γ (10, 100, 200, 500, and 1000 U/ml), or a combination of both (0.1 μg/ml LPS with 10 U/ml IFN-γ). Unactivated BV-2 cells were used as controls. NO production was evaluated 24 hours later by quantification of nitrite, NO_2^-, a metabolic end product of NO, using the Griess reagent.

Results: Unactivated BV-2 cells did not produce detectable amounts of NO_2^- over the course of the experiments. Cells activated with LPS produced NO_2^- in a concentration dependent manner. A robust and consistent response (70 µM NO_2^-) was produced in response to 1 µg/ml LPS (Fig.1-A), and 1 µg/ml LPS was selected for use in subsequent studies.

The BV-2 cells activated with IFN-γ alone produced NO_2^- in the culture media in a similar fashion to LPS stimulation. Unactivated BV-2 cells did not generate NO_2^-. Physiological levels of IFN-γ, 100-200 U/ml, were sufficient to produce a robust response, 30-50 µM NO_2^- (Fig.1-B), and 100 U/ml IFN-γ was used for further studies.

Concentrations of IFN-γ and LPS were selected to synergistically activate the BV-2 cells. When used alone, IFN-γ (10 U/ml) elicited 5 µM NO_2^-, and LPS (0.1 µg/ml) elicited 7 µM NO_2^-, but the combination of both was strongly synergistic, eliciting 53 µM NO_2^- (Fig.1-C).

Similar results were obtained in five independent experiments and the concentration of activators selected for further use was standardized at 1 µg/ml LPS as single activator, 100 U/ml IFN-γ as single activator, and 10 U/ml IFN-γ + 0.1 µg/ml LPS for the synergistic combination.

b) Tau-Cl Dose-dependently Inhibits NO Production by Activated BV-2 Cells

Rationale: Experiments were conducted to determine the optimal concentration of Tau-Cl that inhibits NO production by the activated BV-2 cells.

Procedure: The BV-2 cells were 85-90% confluent in 24-well plates when the experiments were initiated. Increasing concentrations of Tau-Cl (0.1 mM, 0.15 mM, 0.2 mM, 0.25 mM, and 0.5 mM) were added at the time of activation in three different settings: IFN-γ (100 U/ml) alone, LPS (1 μg/ml) alone, and a synergistic combination of IFN-γ (10 U/ml) + LPS (0.1 μg/ml). NO_2^- accumulation in the media was measured 24 hours later using the Griess reagent. Cell viability was assessed and no toxicity was observed within the range of Tau-Cl concentrations used.

Results: Approximately 90% inhibition of NO production by 0.25 mM Tau-Cl was observed in BV-2 cells activated with LPS or IFN-γ as single activators. When the BV-2 cells were activated with the synergistic combination of LPS and IFN-γ, Tau-Cl inhibited NO production 75-80 % at 0.25 mM, and 90% at 0.5 mM. Tau-Cl at 0.15 mM and 0.2 mM had different degrees of inhibition depending on activator, but 0.1 mM had no inhibitory effect (Fig.1-D).

Similar results were obtained in five independent experiments. These results demonstrate the effectiveness of a physiological concentration of Tau-Cl on NO production by the BV-2 cells.

In addition, Tau-Cl was effective regardless of the type of activator used. This was important for studying the Tau-Cl effect on the molecular mechanisms involved, because LPS triggers NF-kB, while IFN-γ works through the Stat-1 signaling pathway. In this way, we can analyze in future experiments Tau-Cl effect on NF-kB and, separately, on Stat-1 transcription factors.

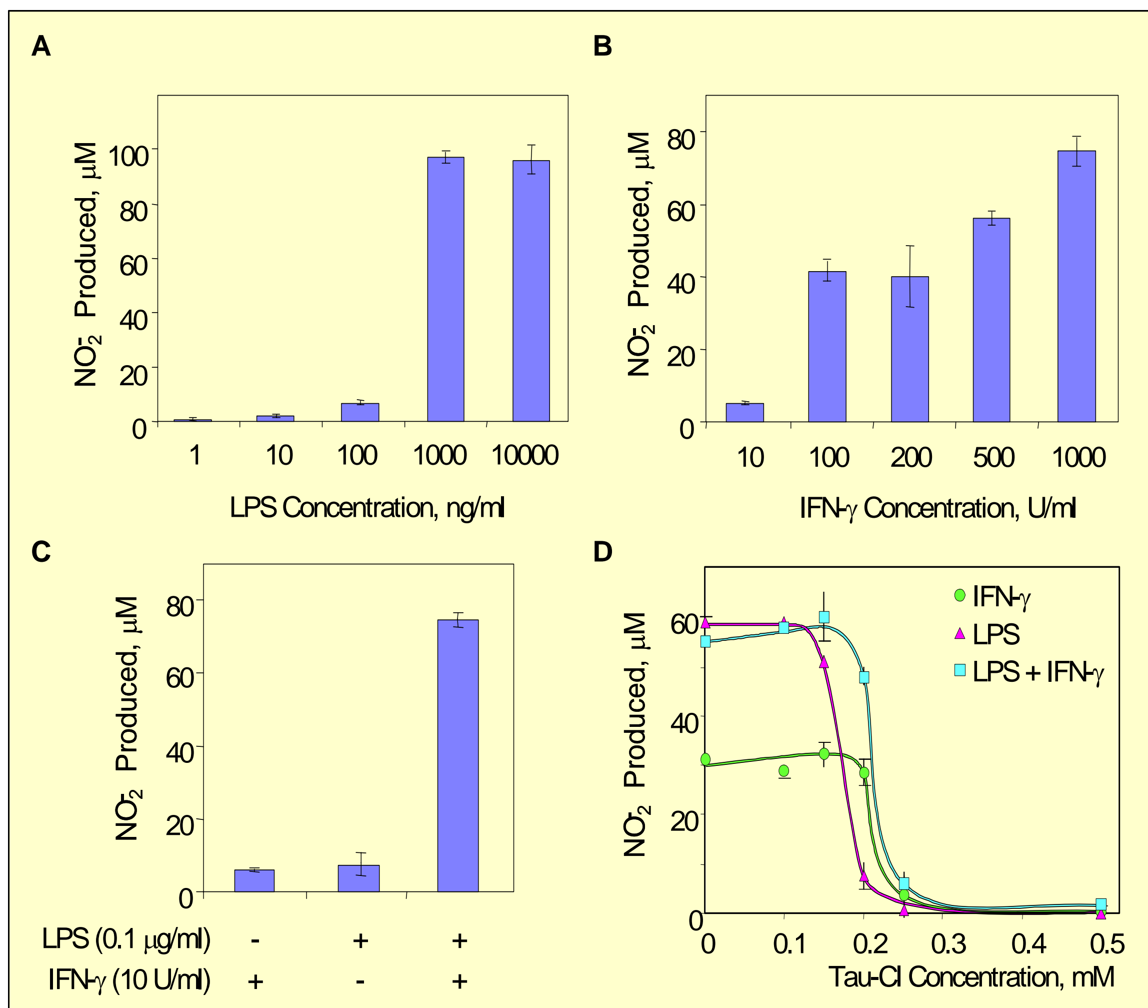

Figure 1

Activated BV-2 Cells Produce NO and Tau-Cl Inhibits NO Production

(A), (B), (C) The BV-2 cells were activated as indicated and NO production was measured 24 hrs later. (D) The BV-2 cells were activated with LPS (1 μg/ml), IFN-γ (100 U/ml), and the synergistic combination of both, LPS (0.1 μg/ml) + IFN-γ (10 U/ml). Tau-Cl was added at the time of activation and NO was measured 24 hours later. Values represent x ± SD for triplicate samples. Similar results were obtained in five independent experiments.

c) The Kinetics of NO Production by Activated BV-2 Cells

Rationale: We conducted experiments to determine NO production by the BV-2 cells at different time points following activation. Our goal was to delineate when NO production would be detectable, maximal, and its kinetics. These results would lead subsequent work in evaluating the effects of Tau-Cl on iNOS protein and mRNA iNOS expression.

Procedure: The BV-2 cells were grown in 24-well plates and activated with IFN-γ (100 U/ml) or LPS (1 μg/ml) when 80-90% confluent. Tau-Cl (0.5 mM) was added at the time of activation. NO_2^- accumulation in the media was measured 4, 8, 16 and 24 hours following activation using the Griess reagent. Separate wells were used for each time point.

Results: NO production over time in different activation settings is shown in (Fig. 2). Nitric oxide was not detectable 4 hours following stimulation, regardless of the activator used. At 8 hours of activation, BV-2 cells produced small amounts of NO (10 μM NO_2^-) when activated with LPS, but were unresponsive to IFN-γ. After 16 hours of activation, LPS induced near maximum amounts of NO (70 μM NO_2^-) while IFN-γ induced a little more than half-maximum amounts of NO (35 μM NO_2^-). After 24 hours of activation, NO_2^- accumulation in the conditioned media of cells activated with LPS had a plateau, increasing from 70 to 80 μM at 16 and 24 hours, respectively. Cells activated with IFN-γ increased dramatically their NO production, from 35 μM at 16 hours to about 60 μM at 24 hours. Tau-Cl inhibited the amount of NO_2^- accumulated in the conditioned media at every time point examined, regardless of the activator.

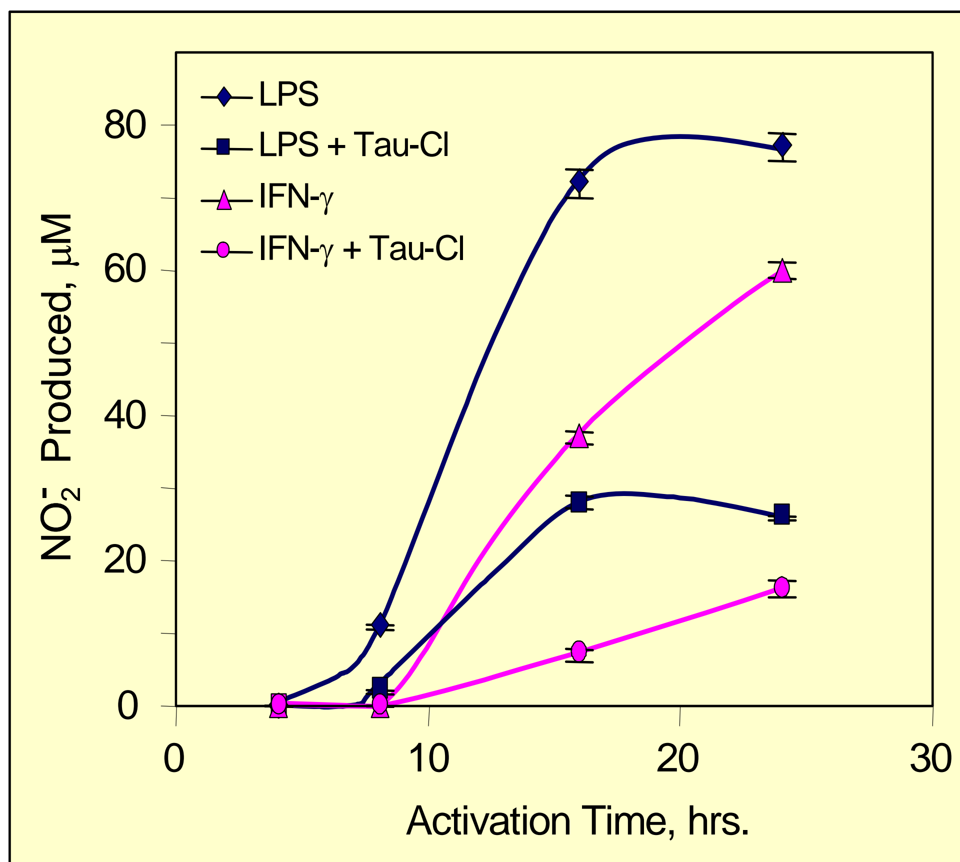

Figure 2

Tau-Cl Dose-dependently Inhibits NO Production by Activated BV-2 Cells

The BV-2 cells were activated with LPS (1 μg/ml) or IFN-γ (100 U/ml) in the absence or presence of Tau-Cl (0.5 mM), and NO_2^- was measured at the times indicated. Values represent x ± SD for duplicate samples. Similar results were obtained in additional four independent experiments.

It is interesting that the shape of the curve in the kinetics of NO production, although different for LPS and IFN-γ, was not altered by Tau-Cl treatment.

We concluded from the kinetics experiments that LPS is a more potent activator than IFN-γ, inducing NO production earlier and faster than IFN-γ. LPS elicited NO production 8 hours following activation which increased 7-fold 8 hours later (16 hours after stimulation). IFN-γ induced NO by 16 hours of activation, and reached maximum at 24 hours, having a more linear curve. Extrapolation from the curve obtained with IFN-γ suggest that 10 μM NO_2^- would have been attained 10 hours following activation and increased approximately 4-fold 8 hours later (18 hours after stimulation). Tau-Cl had strong inhibitory effects (~70%) on NO production by activated BV-2 cells, regardless of the activator. Similar results were obtained in additional four independent experiments.

d) Tau-Cl Dose-dependently Inhibits iNOS Protein Expression in Activated BV-2 Cells

Rationale: Experiments were conducted to determine the effects of Tau-Cl on iNOS protein expression in the activated BV-2 cells.

Procedure: The BV-2 cells were grown in 24-well plates and activated with 1μg/ml LPS in the presence of different concentrations of Tau-Cl (0.15 mM, 0.25 mM, and 0.5 mM). NO_2^- accumulation in culture media was measured 24 hours later using the Griess reagent and selected protein lysates were prepared for Western blot analysis of iNOS protein expression.

Results: Tau-Cl dose-dependently inhibited accumulation of NO_2^- in the conditioned media of BV-2 cells activated with LPS (Fig. 3). Based on these results, culture wells were selected for preparing cell lysates for subsequent Western blot analysis of iNOS. Western blot analysis of cell lysates showed a dose-dependent inhibition of iNOS protein expression by Tau-Cl when BV-2 cells were activated with LPS (Fig. 3). Unactivated cells did not express detectable levels of iNOS enzyme. LPS activated cells expressed iNOS protein and this was dose-dependently inhibited by Tau-Cl. In addition, Tau-Cl produced a parallel inhibition of iNOS protein expression and NO production. Similar results were obtained in two additional independent experiments.

Our data demonstrated maximum NO production when cells were activated with LPS, and this was the reason for using only LPS as stimulator in the Western blot analysis. Since iNOS activity is regulated primarily at the level of gene transcription, subsequent experiments were conducted to determine Tau-Cl effects on iNOS mRNA expression.

II) Tau-Cl Modulates iNOS mRNA Expression in Activated BV-2 Cells

Rationale: The second aim of this work was to determine the effects of Tau-Cl on iNOS mRNA expression. Tau-Cl inhibits NO production and iNOS protein expression by the activated BV-2 cells. Subsequent experiments examined Tau-Cl effects on iNOS mRNA expression by Northern blot. Initially, experiments were conducted to select time points for comparing the effects of different activators on iNOS mRNA expression.

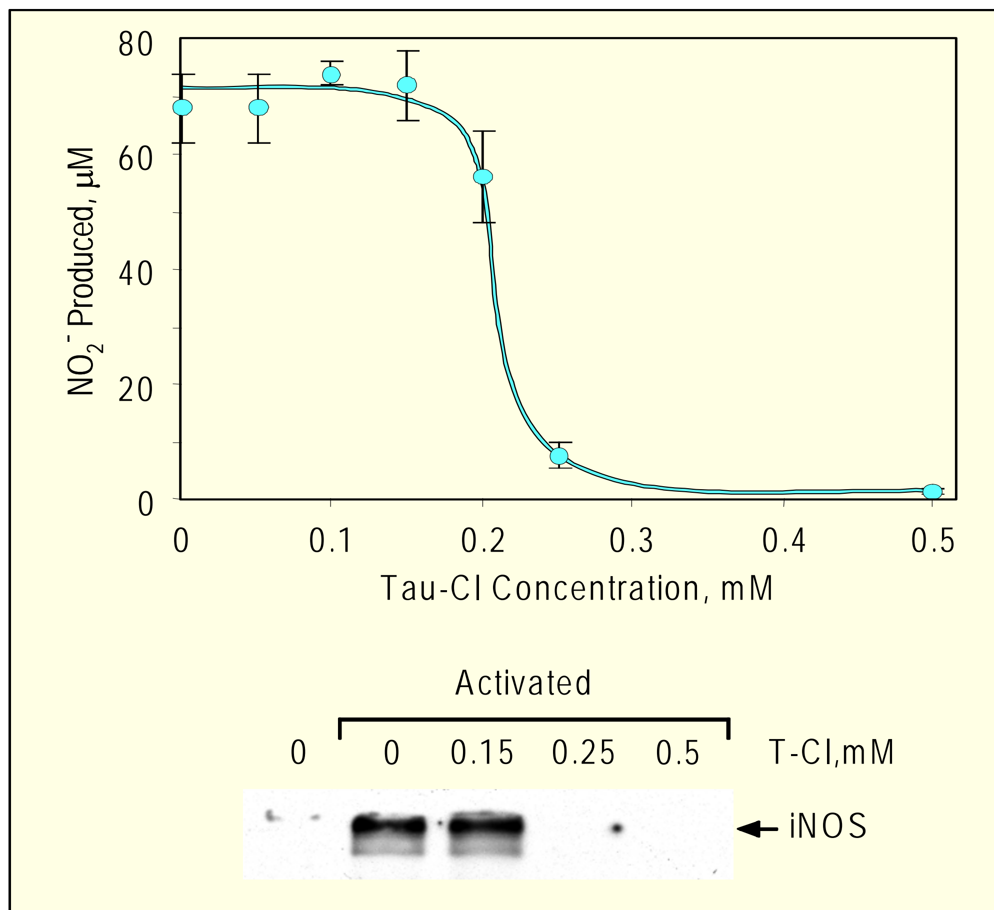

Figure 3

Tau-Cl Dose-dependently Inhibits iNOS Protein Expression

The BV-2 cells were activated with LPS (1µg/ml) and Tau-Cl (0.15 mM, 0.25 mM and 0.5 mM) was added at the time of activation. NO production was measured 24 hours later and iNOS protein expression in the cell lysate was evaluated by Western blot. Similar results were obtained in two additional independent experiments.

Procedure: The BV-2 cells were grown in 100 mm diameter plastic tissue culture dishes to prepare adequate amounts of material for total mRNA extraction. The BV-2 cells were examined in three different settings: unactivated, activated, and activated in the presence of Tau-Cl. The BV-2 cells were activated with LPS (1 μg/ml) alone, IFN-γ (100 U/ml) alone, or with the synergistic combination of LPS and IFN-γ. Tau-Cl (0.5 mM) was added at the time of activation. Parallel dishes for all conditions were incubated for 24 hours, and NO_2^- accumulation in the conditioned media was measured using the Griess reagent. Initial studies of the temporal pattern of iNOS mRNA expression indicated 4-hour and 16-hour time points to be most informative (data not shown) and were selected for further studies.

Total RNA was extracted from cells using Tri-Reagent at 4 and 16 hours following activation. RNA was measured and equal amounts of RNA were loaded in lanes for separation on the gel by electrophoresis. The iNOS mRNA expression was determined by Northern blot. The same blot was subsequently striped and re-probed for GAPDH mRNA.

Results: At 4 hours following activation (Fig. 4), the synergistic combination of LPS and IFN-γ elicited stronger iNOS mRNA expression than LPS alone. IFN-γ activation did not produce detectable amount of iNOS mRNA at 4 hours. Tau-Cl inhibited iNOS mRNA production at 4 hours in cells activated with either LPS or the synergistic combination. Unactivated BV-2 cells did not express detectable amounts of iNOS mRNA. As control, we used GAPDH mRNA. Similar results were obtained in four independent experiments.

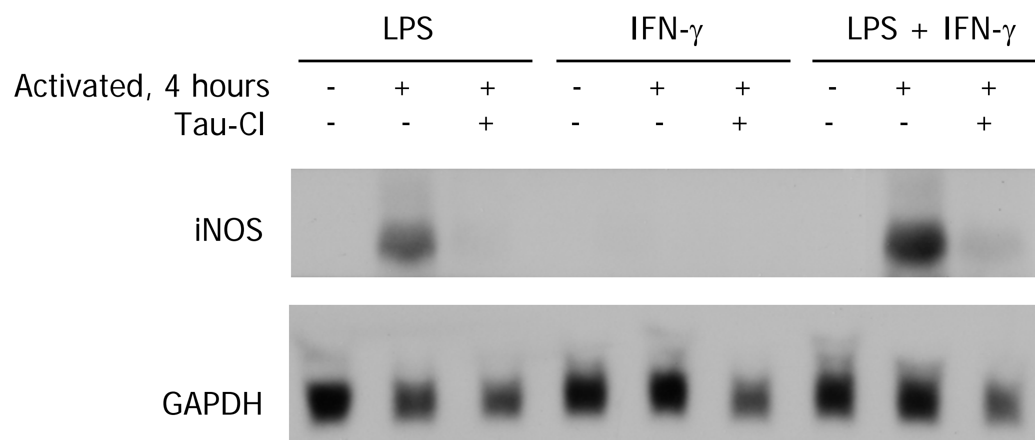

Figure 4

The iNOS mRNA Expression 4 hours Following Activation

The BV-2 cells were activated with LPS (1 μg/ml), IFN-γ (100 U/ml), and the synergistic combination of both, LPS (0.1 μg/ml) with IFN-γ (10 U/ml). Tau-Cl (0.5 mM) was added at the time of activation. Total RNA was extracted from the cells 4 hours following activation and analyzed by Northern blot for iNOS mRNA and GAPDH mRNA expression. Similar results were obtained in four independent experiments.

At 16 hours following stimulation (Fig. 5), IFN-γ produced the strongest iNOS mRNA signal. Activation with LPS alone and the synergistic combination of both activators induced iNOS mRNA that persisted for 16 hours, but the signal was weaker than with IFN-γ alone. The GAPDH mRNA was used as a control. Unactivated BV-2 cells did not express detectable amounts of iNOS mRNA. Tau-Cl inhibited iNOS mRNA expression 16 hours following IFN-γ-stimulation. These results demonstrate activator-dependent kinetics of iNOS mRNA expression and inhibition by Tau-Cl, regardless of the activator. Similar results were obtained in four independent experiments.

Among the three activators used, the synergistic combination of LPS with IFN-γ was consistently the most potent and persistent inducer of iNOS gene expression. The iNOS mRNA had similar expression when the BV-2 cells were stimulated with LPS or the synergistic combination of activators, and demonstrated the iNOS mRNA induction as early as 4 hours that persisted for 16 hours. LPS in conjunction with IFN-γ was a stronger activator than LPS alone. The iNOS mRNA expression for IFN-γ-activation showed later induction of the iNOS gene transcription, at 16 hours.

The temporal expression of iNOS mRNA varied with the mode of activation and may relate to activator-dependent signaling pathways. Tau-Cl inhibited iNOS mRNA expression at both time points for all three activator conditions. Further experiments were conducted to analyze the influence of Tau-Cl on the signaling pathways that regulate the iNOS gene expression, focusing on NF-kB and Stat-1 transcription factors.

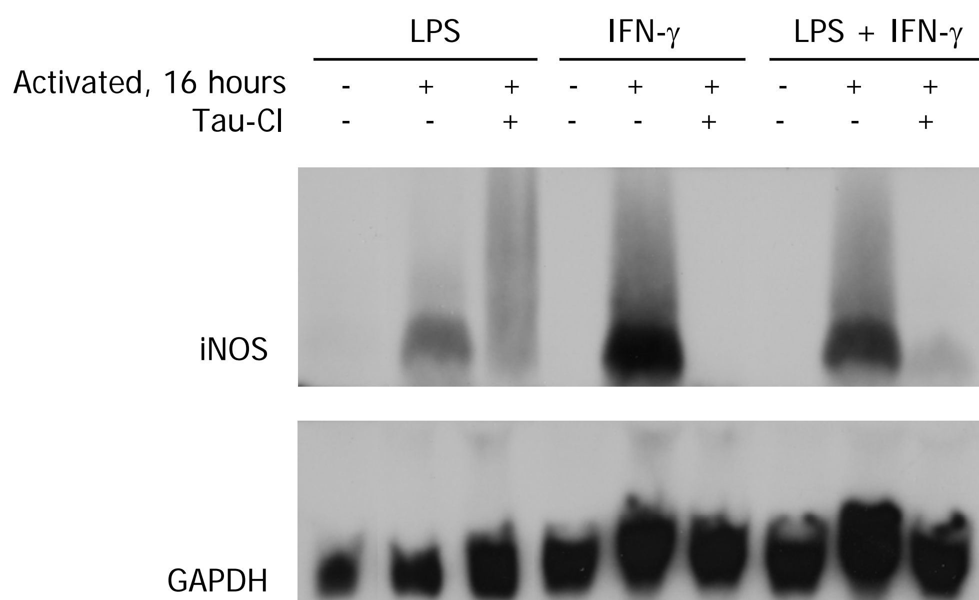

Figure 5

The iNOS mRNA Expression 16 hours Following Activation

The BV-2 cells were activated with LPS (1 μg/ml), IFN-γ (100 U/ml), and the synergistic combination of both, LPS (0.1 μg/ml) with IFN-γ (10 U/ml). Tau-Cl (0.5 mM) was added at the time of activation. Total RNA was extracted from the cells 16 hours following activation and analyzed by Northern blot for iNOS mRNA and GAPDH mRNA expression. Similar results were obtained in four independent experiments.

III) Tau-Cl and the NF-kB Signaling Pathway

Rationale: NF-kB is involved in regulating transcription of most inflammatory genes and the influence of Tau-Cl on this pathway was determined. NF-kB in unactivated cells is bound to the IkB, as a complex in the cytoplasm. After activation, NF-kB translocates to the nucleus as a dimer, composed most frequently of two subunits: p50 and p65. Possible sites for Tau-Cl action include: active NF-kB transcription factor binding to its consensus sequence in the promoter region of NF-kB-regulated genes (in the nucleus), IkB protein (in the cytoplasm) that binds NF-kB preventing its nuclear translocation, and IKK that phosphorylates IkB and the kinases that activated IKK (in the cytoplasm). We focused our attention on Tau-Cl effects on NF-kB binding to its consensus sequence.

Nuclear NF-kB binding activity was analyzed using the electrophoretic mobility gel shift assay (EMSA). In addition, supershift assays were performed to analyze the composition of the NF-kB transcription factor. Most cells have p50 and p65 subunits, with a p50/p65 heterodimer as active transcription component, and p50/p50 homodimer as a silent or repressive constituent (105).

Procedure: The BV-2 cells were grown in 100 mm diameter plastic tissue culture dishes and the nuclear protein extract was prepared following the procedure described in the methods section. The BV-2 cells were examined in three different settings: unactivated, activated, and activated in the presence of Tau-Cl. The BV-2 cells were activated with LPS (1 μg/ml), IFN-γ (100 U/ml), and the synergistic combination LPS (0.1 μg/ml) with IFN-γ (10 U/ml). Tau-Cl (0.5 mM) was added at the time of activation.

Parallel dishes for all conditions were incubated for 24 hours, and NO_2^- accumulation in the conditioned media was measured using the Griess reagent. Nuclear protein extracts were prepared one hour following activation (152). Nuclear protein extracts were examined by EMSA for the ability of nuclear NF-kB to bind to its cognate recognition site in the double-stranded oligonucleotide probe. NF-kB subunit composition was analyzed by supershift assay using antibodies (Santa Cruz) to p65, p50, and c-Rel.

Results: When the BV-2 cells were stimulated with LPS (Fig. 6), NF-kB was recovered in the nucleus one hour later. Two bands were identified in the EMSA containing the NF-kB transcription factor. Unactivated BV-2 cells constitutively expressed small amounts of both NF-kB bands, although this was not consistently observed. The presence of NF-kB in the nucleus of unstimulated cells is probably related to its role in cell growth and development, as well as apoptosis. The activated BV-2 cells expressed more of both NF-kB bands than unactivated cells. Tau-Cl inhibited the nuclear NF-kB signal in the BV-2 cells activated with LPS. Similar results were obtained in five independent experiments.

The subunit composition of the two bands of the NF-kB complex was determined in the BV-2 cells stimulated with LPS using supershift assays (Fig. 7). In addition, the composition of the NF-kB complex was determined when the BV-2 cells were activated with LPS in the presence of Tau-Cl. Antibodies to p50, p65, and c-Rel NF-kB subunits were pre-incubated with nuclear protein extracts before measuring binding to the labeled double-stranded oligonucleotide probe. The NF-kB bands containing subunits that were reactive with the correspondent antibodies were shifted upward in the gel lane.

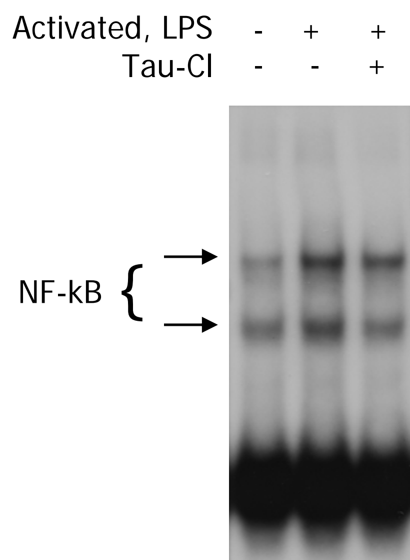

Figure 6

The NF-kB Activity in the LPS-stimulated BV-2 Cells

The BV-2 cells were activated with LPS (1 μg/ml) and NF-kB activity was measured by EMSA in the nuclear protein extracts one hour following activation. Tau-Cl (0.5 mM) was added at the time of activation. The NF-kB complex consisted of two bands (arrows). Similar results were obtained in five independent experiments.

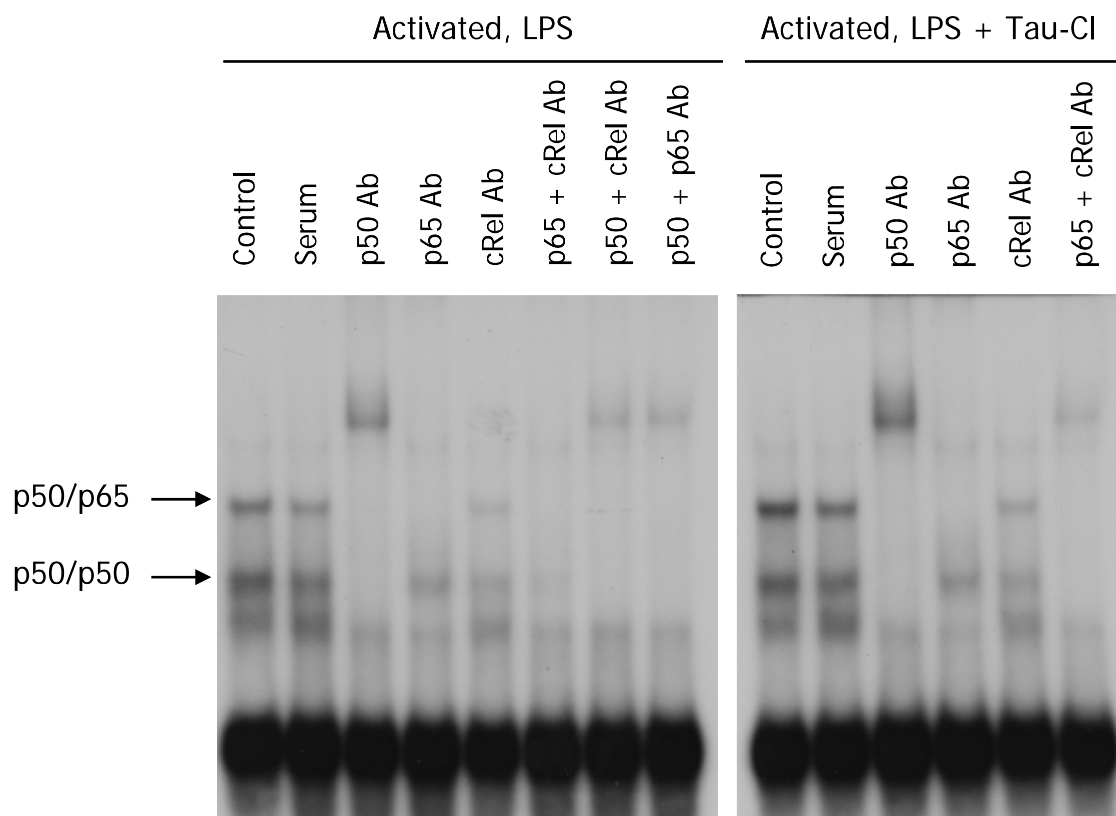

Figure 7

Supershift Assay of the Nuclear NF-kB in the LPS-activated BV-2 Cells

The BV-2 cells were activated with LPS (1 µg/ml) in the absence (left panel) and presence (right panel) of Tau-Cl (0.5 mM) and nuclear protein extracts were prepared one hour following activation. Gel shift assays were conducted using antibodies to the NF-kB subunits p50, p65, and c-Rel. Supershift assays identified the composition of the two bands of the NF-kB complex. Similar results were obtained in three independent experiments.

Nuclear protein extracts from cells activated with LPS exhibited two NF-kB bands that were supershifted by p50 antibody (Fig. 7 - left panel). In addition, p65 antibody shifted the upper band only. The c-Rel antibody did not shift any band when used alone, or in combination with either p65 or p50 antibody. These results are consistent with the upper band being a p50/p65 heterodimer and the lower band being a p50/p50 homodimer. The combinations of antibodies used in the supershift assays support this finding: p65 antibody shifted only the upper band, p50 antibody shifted both bands, and p50 with p65 antibody shifted the same two bands.

The BV-2 cells activated with LPS in the presence of Tau-Cl (Fig. 7 - right panel) exhibited NF-kB complex composition similar to that obtained with LPS activation in the supershift assays. Antibody against p50 subunit shifted both bands, and p65 antibody shifted only the upper band. In addition, c-Rel antibody did not retard the migration of any band. These results demonstrated that the composition of NF-kB is not altered by the Tau-Cl treatment: the upper band is a heterodimer p50/p65 and the lower band is a homodimer p50/p50. Similar results were obtained in three independent experiments.

Nuclear protein extracts from BV-2 cells activated with IFN-γ yielded results that were in contrast to that obtained with LPS activation, in regards to nuclear NF-kB activity (Fig. 8). Although the two bands of NF-kB complex were sometimes present in nuclear protein extracts of unactivated BV-2 cells, no increase was observed after one hour of activation with IFN-γ. Similar results were obtained in six independent experiments.

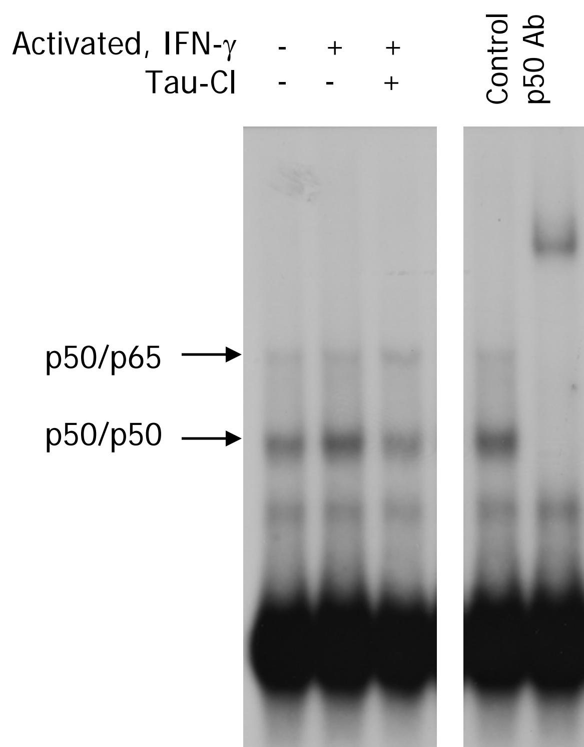

Figure 8

NF-kB Activity in the IFN-γ-stimulated BV-2 Cells

The BV-2 cells were activated with IFN-γ (100 U/ml) and NF-kB activity was measured by EMSA in the nuclear protein extract one hour following activation. Tau-Cl (0.5 mM) was added at the time of activation. The NF-kB complex consisted of two bands (arrows) that were supershifted by the p50 antibody. Similar results were obtained in six independent experiments.

The presence of the two bands for the nuclear NF-kB complex one hour following IFN-γ stimulation was observed in all three conditions: unactivated, activated, and activated in the presence of Tau-Cl. Of particular interest was the activated lane, where no increase in the heterodimer signal (upper band) was detected one hour following stimulation (Fig. 8). The upper band is the transcriptionally active form of the NF-kB complex. These results are consistent with IFN-γ acting mainly through Stat-1 signaling pathways, and not primarily though NF-kB. It is likely that the BV-2 cells might have an autocrine secretion of cytokines, which could act through NF-kB later, but was not detected one hour following activation.

In the supershift assays (Fig. 9) antibodies against p50 and p65 subunits were used to characterize the composition of the NF-kB complex when the BV-2 cells were activated with IFN-γ. Results were similar to those obtained with LPS experiments. The NF-kB complex was identified in two bands; the upper band was shifted with antibodies against p50 and p65 subunits, but the lower band shifted only with the antibody against p50. The NF-kB supershift results are consistent with the upper band being a heterodimer p50/p65 and the lower band being a homodimer p50/p50. Similar results were obtained in three independent experiments.

Experiments were conducted with BV-2 cells stimulated with the synergistic combination of LPS and IFN-γ, and NF-kB activity in the nuclear protein extract was analyzed by EMSA (Fig. 10). NF-kB signal was not consistently detected in nuclear protein extracts from unactivated cells. The synergistic stimulation of the BV-2 cells with both LPS with IFN-γ elicited a strong NF-kB response, with two bands being identified (Fig. 10). The bands were similar to those obtain when LPS was used as single activator.

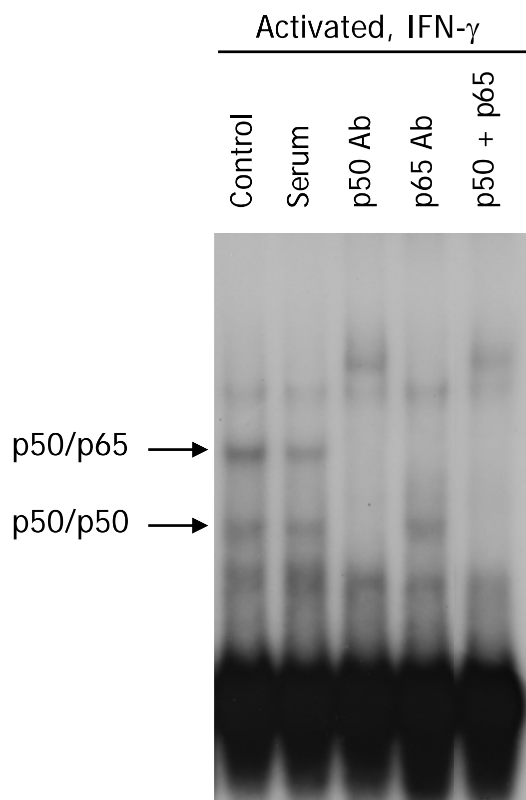

Figure 9

Supershift Assay of the Nuclear NF-kB in the IFN-γ-activated BV-2 Cells

The BV-2 cells were activated with IFN-γ (100 U/ml) and Tau-Cl (0.5 mM) was added at the time of activation. Nuclear protein extracts were prepared one hour following activation. Gel shifts assays were conducted using antibodies to NF-kB subunits p50, p65, and c-Rel. Supershift assays identified the composition of the two bands of the NF-kB complex. Similar results were obtained in three independent experiments.

Tau-Cl had strong inhibitory effects on the NF-kB signal, especially on the upper band, which represents the active heterodimer that drives expression of NF-kB targeted genes. Similar results were obtained in five independent experiments.

The supershift assay identified the components of active nuclear NF-kB when the BV-2 cells were stimulated with the synergistic combination of LPS and IFN-γ (Fig. 11). Antibodies against different components of NF-kB were used: p50, p65, and c-Rel. The upper band shifted with both p50 and p65 antibodies, but not with c-Rel. The lower band shifted with p50 antibody only, but not with p65 and c-Rel antibodies. We concluded that the upper band is a heterodimer p50/p65 and the lower band is a p50/p50 homodimer. Similar results were obtained in three independent experiments. The supershift assay yielded similar results with the LPS and IFN-γ experiments.

In summary, NF-kB was present in the nucleus of BV-2 cells one hour following stimulation, regardless of the activator used. There were constantly two band identified for the NF-kB complex. The composition of the bands was the same, regardless of the activator used and the composition of the band was not altered by Tau-Cl treatment. The upper band consisted of a heterodimer p50/p65 and the lower band was identified as a homodimer p50/p50.

Tau-Cl decreased the amount of NF-kB recovered in the nuclear protein extract of BV-2 cells activated with LPS or with the synergistic combination, LPS and IFN-γ. When BV-2 cells were stimulated only with IFN-γ, no detectable activation of the NF-kB pathway was detected one hour following activation.

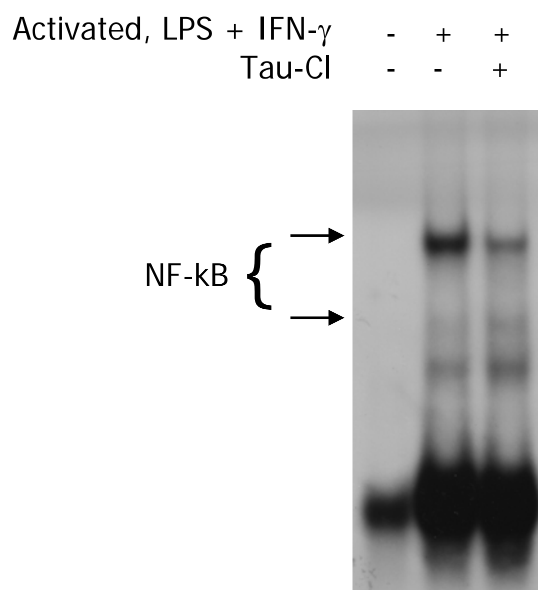

Figure 10

NF-kB Activity in the LPS with IFN-γ-stimulated BV-2 Cells

The BV-2 cells were activated with LPS (0.1 μg/ml) and IFN-γ (10 U/ml). Tau-Cl (0.5 mM) was added at the time of activation. NF-kB activity was measured by EMSA one hour following activation in the nuclear protein extracts. The NF-kB complex consisted of two bands (arrows). Similar results were obtained in five independent experiments.

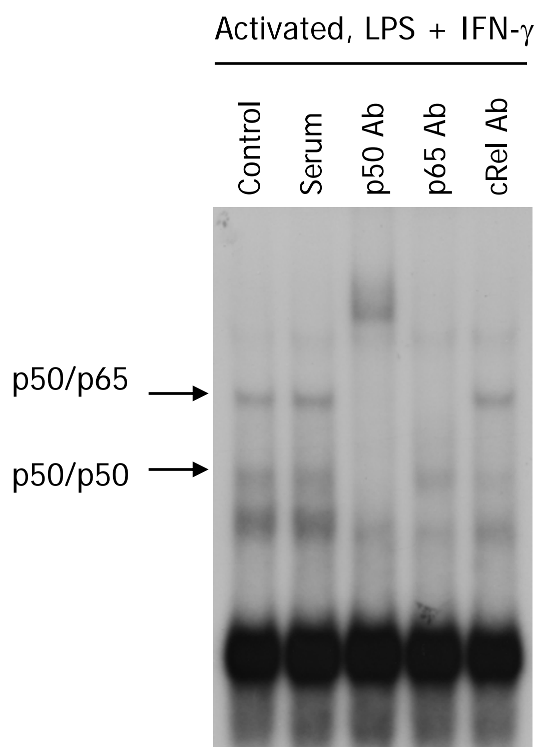

Figure 11

Supershift Assay of the Nuclear NF-kB in the LPS with IFN-γ-activated BV-2 Cells

The BV-2 cells were activated with LPS (0.1 μg/ml) and IFN-γ (10 U/ml). Tau-Cl (0.5 mM) was added at the time of activation and nuclear protein extracts was prepared one hour following activation. Gel shift assays were performed using antibodies to the NF-kB subunits p50, p65, and c-Rel. Supershift assays identified the composition of the two bands of the NF-kB complex. Similar results were obtained in three independent experiments.

IV) Tau-Cl and the Stat-1 Signaling Pathway

Rationale: Cell activation with IFN-γ triggers the Stat-1 signaling pathway. IFN-γ stimulation activates JAKs kinases, which in turn phosphorylate Stat-1 at Tyr-701. Two molecules of phospho-Stat-1 form an active homodimer, which translocates to the nucleus and activates transcription of target genes. The BV-2 cells can be activated by IFN-γ alone, allowing the Stat-1 pathway to be studied independent of NF-kB. The iNOS gene expression is in part regulated by Stat-1. Experiments were conducted to evaluate Tau-Cl effect on nuclear phospho-Stat-1 activity and its composition was analyzed through the supershift assays.

Procedure: The BV-2 cells were grown in 100 mm tissue culture dishes and activated with LPS (1 μg/ml) alone, IFN-γ (100 U/ml) alone, and the synergistic combination of both LPS (0.1 μg/ml) with IFN-γ (10 U/ml), in the absence and presence of Tau-Cl (0.5 mM). Unactivated BV-2 cells were used as control. Nuclear proteins were extracted one hour following activation and analyzed through EMSA to determine the activity of nuclear phospho-Stat-1.

Results: When the BV-2 cells were stimulated with LPS alone there was no band detected for active nuclear GAS/Stat-1 (data not shown) in nuclear protein extracts, indicating that LPS did not activate GAS/Stat-1 pathway one hour following activation. Similar results were obtained in five independent experiments.

However, when IFN-γ was used as single stimulator two bands were recovered from the nuclear protein extract (Fig. 12). These bands were confirmed to contain GAS/Stat-1 and were absent from the nuclear protein extracts of unactivated cells. In

addition, cells activated with IFN-γ in the presence of Tau-Cl had greatly reduced amounts of both bands relative to BV-2 cells activated in the absence of Tau-Cl (Fig. 12 - left panel). Similar results were obtained in five independent experiments.

To characterize the composition of the two bands containing Stat-1 supershift assays were performed (Fig. 12 - right panel). Antibody specific for p91 and a second antibody that reacts with both p84 and p91 were used. Antibody to only p84 was not available. Both bands were shifted with p91 antibody, indicating that both bands had p91 as a component. It is possible that the lower band was a p91/p84 heterodimer and the upper band a p91/p91 homodimer, but this was not examined further. Stat-1-α represents p91 and Stat-1-β represents p84. The composition of the band was not altered by Tau-Cl (data not shown). Similar results were obtained in three independent experiments.

Experiments were conducted with BV-2 cells stimulated with the synergistic combination of LPS and IFN-γ and nuclear GAS/Stat-1 was analyzed by EMSA (Fig. 13 - left panel). Unactivated BV-2 cells had no detectable activity for GAS/Stat-1. Activated cells expressed two bands as part of the GAS/Stat-1 complex. Tau-Cl inhibited both bands. Similar results were obtained in five independent experiments.

The supershift assay was performed to further characterize the composition of GAS/Stat-1 (Fig. 13 - right panel). Antibody to p91 and antibody to p91 and p84 were used. Both bands were shifted with p91 antibody, similar to IFN-γ activation. We concluded that both bands had p91 as a subunit, and their composition was likely upper band p91/p91 homodimer, and lower band p84/p91 heterodimer. Tau-Cl inhibited both bands, but had no influence on the composition of GAS/Stat-1 complex (data not shown). Similar results were obtained in three independent experiments.

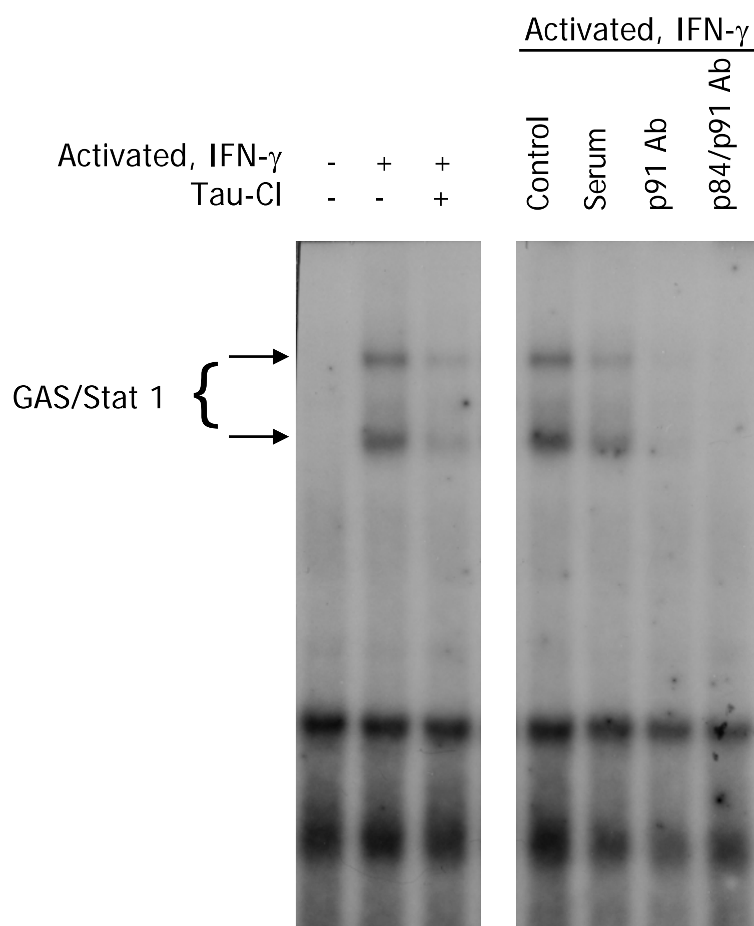

Figure 12

GAS/Stat-1 Activity in the IFN-γ-stimulated BV-2 Cells and the Supershift Assay

The BV-2 cells were activated with IFN-γ (100 U/ml). Tau-Cl (0.5 mM) was added at the time of activation and nuclear protein extracts was prepared one hour following activation. EMSA was performed and two bands of GAS/Stat-1 complex were detected (arrows) (left panel). Supershift assays for GAS/Stat-1 were performed using antibodies to p91, and p84 with p91 subunits (right panel). Similar results were obtained in five independent experiments.

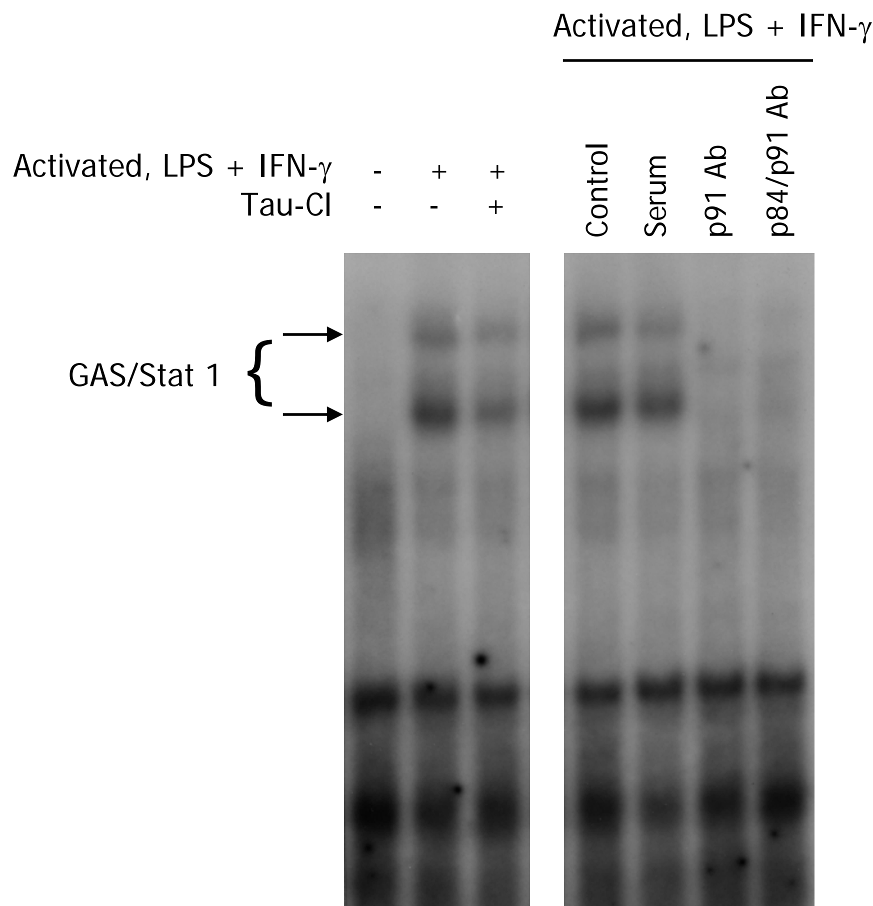

Figure 13

GAS/Stat-1 Activity in the LPS with IFN-γ-stimulated BV-2 Cells and the Supershift Assay

The BV-2 cells were activated with LPS (0.1 μg/ml) and IFN-γ (10 U/ml). Tau-Cl (0.5 mM) was added at the time of activation and nuclear protein extracts was prepared one hour following activation. EMSA was performed and two bands of GAS/Stat-1 complex were detected (arrows) (left panel). Supershift assays for GAS/Stat-1 were performed using antibodies to p91, and p84 with p91 subunits (right panel). Similar results were obtained in five independent experiments.

DISCUSSION

The BV-2 cells are a good model for studying signaling pathways because they respond independently to LPS and IFN-γ. Cells activated with LPS or with LPS together with IFN-γ produced more NO at a faster rate than cells activated with IFN-γ alone. Our work demonstrates that Tau-Cl inhibits, in a dose-dependent manner, NO production by the activated BV-2 cells, regardless of the activator used. There was a parallel inhibition of iNOS mRNA expression with NO production by cells activated in the presence of Tau-Cl. Activation of the BV-2 cells with the synergistic combination of LPS and IFN-γ or with LPS alone induced iNOS mRNA expression 4 hours later. However, activation with IFN-γ alone required more than 4 hours before iNOS transcripts were detected.

Results obtained in our studies are consistent with the idea that LPS acts through the NF-kB signaling pathway with no effect on Stat, while IFN-γ acts through the JAK/Stat signaling pathway. No effect of IFN-γ stimulation was observed on NF-kB signaling one hour after activation, even though iNOS mRNA was eventually elicited and iNOS expression is thought to be NF-kB dependent. Activation of cells with LPS and IFN-γ together, acts through both NF-kB and JAK/Stat signaling pathways.

Our experiments showed that IFN-γ activates the BV-2 cells to produce NO, but the amount of NO produced were relatively low compared with LPS stimulation and required longer activation time. Slower iNOS gene induction with IFN-γ could be explained by the production of autocrine factors in response to IFN-γ that would later activate the NF-kB pathway. LPS activated BV-2 cells secrete IL-1β (140) and TNF-α (154). In addition, IRF-1 is an important transcription factor for iNOS gene expression

(93), and IRF-1 is an IFN-γ-regulated gene (93, 145). The degree of iNOS gene expression depends on the coordinate presence of multiple transcription factors (93). LPS triggers the classic NF-kB pathway, where the heterodimer p50/p65 is activated through action of IKK on IkB (105). However, an alternative NF-kB pathway triggered by TNF family member proteins function through a mechanism not involving IkB and results in active NF-kB that has a different subunit composition: p52 and Rel B (155, 156, 157). It seems possible that IFN-γ may activate the NF-kB signaling pathway via autocrine factors that were secreted, e.g., TNF-α. Although TNF-α mRNA does not appear to be upregulated by IFN-γ stimulation in most cell types (158), IFN-γ is reported to stimulate TNF-α production by microglia (159). Also, IFN-γ activation of Stat-1 combined with IFN-γ induced expression of IRF-1 and C/EBP-β may be sufficient to induce low levels of iNOS mRNA expression, especially when coordinated with autocrine actions of TNF-α stimulating the NF-kB signaling pathway. These speculations warrant further studies with BV-2 cells.

Our experiments demonstrated that Tau-Cl dose-dependently inhibited NO production, iNOS enzyme protein and iNOS mRNA expression, regardless of the activator used in these studies. The ability of Tau-Cl to function as an inhibitor of NO production was first reported in the RAW 264.7 cells, a murine derived cell line, activated with LPS and IFN-γ (160). Subsequently, several studies confirmed that Tau-Cl inhibits the production of NO and extended the effects of Tau-Cl to other inflammatory mediators in a variety of cell types, including astrocytes (34, 35), alveolar macrophages, peritoneal macrophages, neutrophils, human peripheral blood monocytes, human synoviocytes, and cell lines of various tissue origins (161-181).

The mechanism through which Tau-Cl exerts its effect appears to be at the level of cell signaling that regulates iNOS gene expression. Our studies show that Tau-Cl has an inhibitory effect on both NF-kB and GAS/Stat-1 signaling in the activated BV-2 cells. Tau-Cl inhibited NF-kB activity when the cells were activated with either LPS or the synergistic combination of LPS with IFN-γ. Conversely, when the cells were activated with IFN-γ as single activator or with LPS and IFN-γ together, Tau-Cl inhibited JAK/Stat signaling. The effects of Tau-Cl on attenuating the NF-kB pathway have been previously reported in the NR8383 cells (152, 165), astrocytes (164), and the Jurkat cells (146, 182). The inhibitory effects of Tau-Cl on Stat signaling has been reported only in the NR8383 cells (166).

Further studies are required to determine the molecular sites of Tau-Cl intervention on the NF-kB signaling pathway in the activated BV-2 cells. Previous studies demonstrate that phosphorylation of IkB is inhibited in activated cells in the presence of Tau-Cl (146, 152), but different mechanisms were proposed. In the NR8383 cells activated with LPS plus IFN-γ, Tau-Cl is suggested to inhibit activation of the multiprotein complex, IKK, by upstream kinases that phosphorylate IKK (152). In this regard, Tau-Cl was recently reported in inhibit both p38 and ERK mitogen-activated protein kinases (MAPKs) in the RAW 264.7 cells activated with LPS (183). In the Jurkat cells activated with TNF-α, Tau-Cl was demonstrated to oxidize Met-45 of IkB in such a way as to inhibit its further degradation by the proteasome (146, 182). It seems likely that both mechanisms may be involved in mediating the effects of Tau-Cl. Inhibitory effects of Tau-Cl on Stat signaling also demonstrate more than one mechanism of Tau-Cl action is operative in these cells.

In other studies with the BV-2 cells (138, 139, 145), MAPKs were reported to be involved in mediating LPS and IFN-γ effects on iNOS gene transcription. MAPKs are composed of three major cascades: p38, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK) (138, 139, 145). LPS was reported to act mainly though p38 in murine microglia, but not through ERK (138-140, 145). IFN-γ induced ERK only, which occurred 6 hours after activation (138, 145). However, some controversy exists because IFN-γ is also reported to stimulate both p38 and ERK MAPKs (184). In addition, since the effect of IFN-γ on MAPKs is observed 6 hours after activation, it seems likely that this may be an indirect effect of IFN-γ acting via some autocrine factor, e.g., TNF-α. Both LPS and IFN-γ induce early, within 2 hours from activation, expression of IRF-1 mRNA in the BV-2 cells, which represents an important transcription factor for iNOS gene expression in microglia (138). IFN-γ stimulates c/EBP-β-dependent gene expression, including iNOS, via the ERK pathway (184).

Similar to our results, others report that LPS and IFN-γ differentially regulate iNOS expression in the BV-2 cells (138, 145). LPS is reported to rapidly induce iNOS mRNA expression, within 2 hours of activation. In contrast, IFN-γ induces iNOS mRNA expression between 5 and 8 hours after stimulation (138). In our hands, LPS induced iNOS mRNA 4 hours following activation, but IFN-γ required up to 16 hours. The differences between these findings are trivial, but could relate to the use of RT-PCR for quantifying mRNA because RT-PCR detects smaller amounts of mRNA than Northern blot analysis does. In addition, the experiments had different conditions: number of cells, volume, and degree of cell confluency are also important factors in responses of the cultured BV-2 cells.

To further investigate Tau-Cl downregulation of NO production by IFN-γ-activated BV-2 cells, we suggest future work to evaluate the amount of Stat-1 present in the nucleus. In the NR8383 cells activated with LPS and IFN-γ, Tau-Cl inhibited induction of IRF-1 and decreased Stat-1 binding to GAS (166); however, activated Stat-1 migration into the nucleus appeared unimpaired. Perhaps an amino acid required for binding to GAS was oxidized in a way that did not affect formation of Stat-1 dimer and migration into the nucleus. Similar mechanisms of Tau-Cl action may be involved in the BV-2 cells, but this remains to be determined.

It is worth noting that NO production by microglia activated with IFN-γ may require autocrine effects of TNF-α (159), and Tau-Cl inhibits production of TNF-α by post-transcriptional mechanisms (167). In this regard, the density of the BV-2 cells, i.e., the degree of confluency, is an important variable that one must control for in these experiments. In general, the amount of NO produced is directly proportional to the number of cells in culture when stimulated with LPS combined with IFN-γ, as would be expected. However, we determined that the density of cells or degree of confluency has a greater influence on the amount of NO produced when cells are stimulated with single activators, especially with IFN-γ. As a consequence of cell number being too low, NO is produced by the BV-2 cells activated with the combination of LPS and IFN-γ, but not with IFN-γ alone, at least not at levels detectable with the Griess reagent. For this reason, it was important to standardize the seeding cell densities and the degree of confuency in our studies. We interpret these observations to be consistent with the idea that a putative autocrine factor, i.e., TNF-α, is required for IFN-γ to be an effective activator of the BV-

2 cells and if the density of the cells is too low, insufficient levels of autocrine factors is achieved to be effective.

The promoter region of the murine iNOS gene has binding sites for NF-kB, IRF-1, Stat-1, C/EBP, and AP-1 (93). In addition, NF-kB nuclear translocation and DNA-binding was reported for BV-2 cells treated with LPS only, but not with IFN-γ (143, 144). Our results are consistent with these findings, demonstrating active NF-kB in the nucleus one hour following LPS activation, but not with IFN-γ (143, 144). Further studies are required to evaluate NF-kB signaling 6-10 hours following IFN-γ stimulation and to determine the role, if any, of TNF-α and of other potential autocrine factors.

The molecular mechanism of synergistic induction of inflammatory genes represents an important topic for research. It has been shown that IFN-γ activation of Stat-1 transcription factor triggered production of IRF-1. Stat-1 and IRF-1 independently activated transcription of IFN-γ-induced genes. In addition to their independent action, recent studies have shown a certain degree of cooperation: IFN-γ and LPS each enhance the action of IRF-1 and contribute to synergistic gene induction (93, 136).

The most important mechanism for synergistic iNOS induction appears to result from Stat-1/IRF-1 interaction with NF-kB. The majority of promoters activated synergistically by IFN-γ and LPS have binding sites for Stat-1 or IRF-1 and NF-kB (136, 143, 144). NF-kB binding to its recognition site strongly enhances gene transcription if the promoter has already been activated by Stat-1 or IRF-1 binding to GAS and ISRE, respectively. NF-kB and IRF-1 interaction occurs after binding to their recognition sites on the iNOS promoter. In addition, it has been demonstrated that LPS augments IFN-γ-induced Stat-1 transcription, independent of NF-kB activation. Phosporylation of Ser-727

of Stat-1 enhances its trans-activation function and is thought to represent the mechanism of enhanced Stat-1 activity by LPS (136, 140, 146, 147).

Recent experimental studies implicated Tau-Cl in inducing apoptosis in unactivated cells (185, 186). It was shown that Tau-Cl causes apoptosis through direct damage to the mitochondria by opening of the permeability transition pore and mitochondrial swelling (185). In, addition, it has been demonstrated that Tau-Cl induces cell cycle-independent proliferative arrest followed by activation of a cell death pathway involving BCL-2 family and caspase activation (186). Thus Tau-Cl may help resolve inflammatory response by downregulating production of inflammatory mediators and inducing apoptosis in other cells.

Clinical studies with Tau-Cl demonstrate its role as modulator of the inflammatory response in periodontal inflammatory disease (187), epidemic keratoconjunctivitis (188), and acute otitis externa (189). In addition, taurine, via taurine chloramine formation may stabilize platelets and atherosclerotic plaques with therapeutic application to coronary artery disease (190). Research studies report taurolidine, a taurine derivate, has bactericidal properties, enhancing immune activity of macrophages and neutrophils and protect in sepsis (191).

The present studies potentially have clinical relevance by opening new possibilities for neuroprotection, suggesting that taurine, via taurine chloramine, could act as a neuroprotective agent by downregulating nitric oxide production by microglia that are activated during the CNS inflammatory response.

CONCLUSION

Taurine has been reported to protect against inflammation-induced tissue damage in several animal models (11-18). An important mechanism of taurine protection relates to formation of taurine chloramine and its subsequent actions (11, 13). Neutrophil-associated MPO produces HOCl, which chemically reacts with taurine to form Tau-Cl, at the site of inflammation. Further, it has been shown that Tau-Cl inhibits production of inflammatory mediators by activated astrocytes (34, 35, 164), macrophages, alveolar macrophages, peritoneal macrophages, neutrophils, human peripheral blood monocytes, human synoviocytes, and cell lines of various tissue origins (161-181). The present studies demonstrate that Tau-Cl inhibits production of NO by the activated BV-2 cells, which retain microglia cells characteristics. We focused on the molecular mechanism through which Tau-Cl regulates iNOS gene expression, in particular, on the transcription factors NF-kB and Stat-1. Tau-Cl inhibits NO production in a dose-dependent manner that parallels iNOS protein expression. Tau-Cl exerts its effect primarily through inhibiting iNOS gene transcription, as demonstrated by decreased expression of iNOS mRNA on Northern blot analysis. Our results are consistent with published data (34, 35, 161-181) regarding Tau-Cl inhibition of several inflammatory mediators, e.g., NO, TNF-α, COX-2, MCP-1, and MIP-2, by activated immunoresponsive cells, e.g., macrophages and astrocytes.

LPS, IFN-γ, and the combination of both, LPS with IFN-γ, were used to activate the BV-2 cells. In this way we were able to explore separately both signaling pathways: NF-kB known to mediate signaling for LPS activation, and JAK/Stat for IFN-γ. Our

results show that LPS acts through the NF-kB pathway while no signal was detected for Stat-1. On the other hand, IFN-γ activation induces signaling viar Stat-1, but not through NF-kB when measured one hour following stimulation. The activated BV-2 cells produce autocrine factors/cytokines (e.g., TNF-α, IL-1β) when stimulated with IFN-γ. These cytokines may subsequently activate the NF-kB pathway for a full expression of iNOS gene. This would account for the signaling process taking longer with IFN-γ activation to express iNOS mRNA. For this reason, it might be helpful to examine nuclear protein extracts at later time points, to evaluate nuclear NF-kB and Stat-1 when the BV-2 cells are activated with IFN-γ alone. Tau-Cl does not change the composition of the transcription factor dimers, regardless of the activator used.

These studies have clinical relevance, suggesting that taurine, via Tau-Cl formation at the site of inflammation; have the potential to be a protective agent against inflammation-induced brain damage, and gives hope for cure. Intervention with pharmacological doses of taurine in acute phase of inflammation could, via taurine chloramine formation, downregulate the CNS inflammatory response and warrants further investigations in future.

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