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Piperine blocks interleukin-2-driven cell cycle progression in CTLL-2 T lymphocytes by inhibiting multiple signal transduction pathways

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HIGHLIGHTS

- Piperine is an alkaloid found in black pepper.
- Piperine inhibits IL-2-driven T lymphocyte proliferation without causing cell death.
- Piperine inhibits activation of STAT3, STAT5, ERK1/2 and Akt.
- Piperine causes G_0/G_1 and G_2/M phase cell cycle arrest.

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ABSTRACT

Piperine, a pungent alkaloid found in the fruits of black pepper plants, has diverse physiological effects, including the ability to inhibit immune cell-mediated inflammation. Since the cytokine interleukin-2 (IL-2) is essential for the clonal expansion and differentiation of T lymphocytes, we investigated the effect of piperine on IL-2 signaling in IL-2-dependent mouse CTLL-2 T lymphocytes. Tritiated-thymidine incorporation assays and flow cytometric analysis of Oregon Green 488-stained cells showed that piperine inhibited IL-2-driven T lymphocyte proliferation; however, piperine did not cause T lymphocytes to die or decrease their expression of the high affinity IL-2 receptor, as determined by flow cytometry. Western blot analysis showed that piperine blocked the IL-2-induced phosphorylation of signal transducer and activator of transcription (STAT) 3 and STAT5 without affecting the upstream phosphorylation of Janus kinase (JAK) 1 and JAK3. In addition, piperine inhibited the IL-2-induced phosphorylation of extracellular signal-regulated kinase 1/2 and Akt, which are signaling molecules that regulate cell cycle progression. Piperine also suppressed the expression of cyclin-dependent kinase (Cdk) 1, Cdk4, Cdk6, cyclin B, cyclin D2, and Cdc25c protein phosphatase by IL-2-stimulated T lymphocytes, indicating G_0/G_1 and G_2/M cell cycle arrest. Piperine-mediated inhibition of IL-2 signaling and cell cycle progression in CTLL-2 T lymphocytes suggests that piperine should be further investigated in animal models as a possible natural source treatment for T lymphocyte-mediated transplant rejection and autoimmune disease.

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1. Introduction

The cytokine interleukin 2 (IL-2), originally named T cell growth factor, is important for T lymphocyte proliferation and the development of effector cells, including $CD8^+$ cytotoxic T lymphocytes (Malek and Castro, 2010; Pipkin et al., 2010). In the absence of IL-2 signaling, $CD8^+$ T lymphocytes receive improper programming during a primary immune response, resulting in the inability of $CD8^+$ memory T lymphocytes to generate a robust recall response and expand in response to rechallenge or persistent infection (Bachmann et al., 2007; Mitchell et al., 2010; Williams et al., 2006). Additionally, T lymphocytes have a greater dependence on IL-2 signaling in non-lymphoid tissues compared to lymphoid tissues, possibly due to the higher cytokine levels and the promotion of cell–cell interactions within the structural organization of lymphoid tissues, which is lacking in non-lymphoid tissues (D'Souza and Lefrancois, 2003; D'Souza et al., 2002). Because IL-2 plays such important roles in shaping T lymphocyte responses, it is an excellent therapeutic target for suppressing inappropriate T lymphocyte activation that results in

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transplant rejection (Halloran, 2010), autoimmune diseases such as multiple sclerosis (Venken et al., 2010) and rheumatoid arthritis (Cope et al., 2007), and chronic inflammatory conditions such as inflammatory bowel disease (Zenewicz et al., 2009). IL-2 has been successfully targeted in vivo using daclizumab and basiliximab, which are monoclonal antibodies (Ab) that inhibit IL-2 signaling by binding to and blocking the α chain (CD25) of the high affinity IL-2 receptor (IL-2R) (Waldmann, 2007). Daclizumab and basiliximab are currently used to prevent transplant rejection (Mottershead and Neuberger, 2007; Ramirez and Marino, 2007) and daclizumab has shown promise in the treatment of multiple sclerosis (Wynn et al., 2010). However, these monoclonal Ab are costly, have to be administered by injection, and there is a risk that repeated administration will induce neutralizing Ab or cause anaphylaxis (Baudouin et al., 2003; Buttmann and Rieckmann, 2008). The search for new agents that block IL-2 signaling is therefore an active area of research.

IL-2-induced heterotrimerization of the IL-2R results in the phosphorylation and activation of Janus kinase 1 (JAK1) and JAK3, which are constitutively associated with the β-chain and the γ-chain of the IL-2R, respectively, to induce intracellular signaling (Miyazaki et al., 1994). The subsequent phosphorylation of multiple tyrosine residues on the IL-2Rβ chain by JAK1 and JAK3 creates docking sites for a variety of signaling molecules, including the adapter protein Shc (Ellery and Nicholls, 2002) and signal transducer and activator of transcription (STAT) 3 and STAT5 (Malek and Castro, 2010). Phosphorylation of STAT5 and STAT3 by JAK1 and JAK3 causes the STAT molecules to homodimerize or heterodimerize and translocate to the nucleus (Johnston et al., 1995). Although both STAT5 and STAT3 are activated by IL-2R signaling, STAT3 is activated to a lesser degree than STAT5 (Delespine-Carmagnat et al., 2000). STAT5 activation promotes T lymphocyte proliferation and transcription of various genes involved in cell cycle progression (e.g., *c-myc*,*cyclin D2*) and cell survival (e.g., *bcl-2* and *bcl-x*) (Lord et al., 2000). STAT5 activation is regulated by phosphatases such as Src homology 2 domain-containing tyrosine phosphatase 2 (Chen et al., 2003; Yu et al., 2000). Activation of STAT5 and STAT3 is also regulated by suppressor of cytokine signaling proteins, which are induced by IL-2R signaling in T lymphocytes as part of a negative feedback loop (Aman et al., 1999; Cohney et al., 1999; Sporri et al., 2001). IL-2-induced phosphorylation and activation of Shc results in early activation of the extracellular signal-regulated kinase (ERK) 1/2 and Akt pathways through recruitment of adapter proteins Grb2-Sos and Grb2-Gab2, respectively (Gu et al., 2000).

Natural products such as phytochemicals are a promising source of new immunomodulatory drugs (Forward et al., 2011; Hellinger et al., 2014; Pae et al., 2010; Wu et al., 2009). The fruits of black pepper plants contain piperine, which is an alkaloid responsible for the pungent flavor of black pepper spice (Srinivasan, 2007). Importantly, many of the diverse physiological effects associated with the use of black pepper in traditional Indian and Chinese medicines have been attributed to piperine (Szallasi, 2005). Recent studies have revealed that piperine exerts potent anti-inflammatory activity in mice experiencing endotoxin shock (Bae et al., 2010) and in rat models of arthritis (Bang et al., 2009; Kim and Lee, 2009; Umar et al., 2013). The ability of piperine to inhibit inflammation has been attributed to a reduction in inflammatory mediator expression by macrophages in the presence of piperine (Kim et al., 2012; Ying et al., 2013). However, piperine also inhibits lipopolysaccharide-induced maturation of dendritic cells, suggesting a decreased capacity to present antigen to T lymphocytes (Bae et al., 2012). In addition, piperine suppresses mouse T lymphocyte activation by mitogens (Dogra et al., 2004) and inhibits synthesis of Th2 cytokines in a mouse model of asthma (Kim and Lee, 2009). However, the effect of piperine on signaling pathways that control T lymphocyte activation has not yet been studied.

In view of the pivotal role that IL-2 plays in regulating T lymphocyte responses (Malek and Castro, 2010), we used IL-2-dependent mouse CTLL-2 T lymphocytes to determine the effect of piperine on IL-2 signaling. CTLL-2 cells are a clone of cytotoxic T lymphocytes that depend on IL-2 rather than T cell receptor stimulation for proliferation (Gillis and Smith, 1977), and are therefore frequently used to study IL-2R signaling in isolation from signal transduction events associated with T cell receptor stimulation (Forward et al., 2011; Kaltenberg et al., 2010; Moon and Nelson, 2001). Our analysis of the effect of piperine on the IL-2-stimulated growth of CTLL-2 T lymphocytes shows that piperine inhibited proximal and distal signaling events associated with IL-2 binding to IL-2R.

2. Material and methods

2.1. Cell culture

IL-2-dependent mouse CTLL-2 $CD8^+$ T lymphocytes (Gillis and Smith, 1977) were obtained from the American Tissue Culture Collection (Manassas, VA) and maintained at 37 °C in a humidified incubator with 5% CO_2. Culture medium consisted of RPMI 1640 medium (Sigma–Aldrich Canada, Oakville, ON) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin–streptomycin, 2 mM L-glutamine, and 5 mM HEPES (Invitrogen Canada Inc., Burlington, ON), and 50 U/ml recombinant mouse IL-2 (PeproTech Inc., Rocky Hill, NJ).

2.2. Reagents

Phosphate buffered saline (PBS), rapamycin, and piperine were purchased from Sigma–Aldrich (Oakville, ON). A 100 mM stock solution of piperine was prepared in dimethyl sulfoxide (DMSO; Sigma–Aldrich) and stored at −80 °C. LY-294002 was purchased from Enzo Life Sciences (Plymouth Meeting, PA). PD 98059 and *N′*-((4-oxo-4H-chromen-3-yl) methylene) nicotinohydrazide (CNH) were purchased from EMD Chemicals, Inc. (Gibbstown, NJ). Anti-phospho-JAK1 (Tyr 1022/1023), anti-phospho-STAT5 (Tyr 694), anti-phospho-STAT3 (Tyr 705), anti-STAT3, anti-phospho-Akt (Ser 473), anti-Akt, anti-cyclin D2, anti-cyclin-dependent kinase 4 (Cdk4), and anti-Cdk6 Ab were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-STAT5a, anti-Cdk1, anti-Cdc25c, and anti-cyclin B Ab were purchased from Millipore (Billerica, MA). Anti-β-actin, anti-phospho-JAK3 (Tyr 980), anti-phospho-ERK1/2, and anti-ERK1/2 Ab, as well as goat anti-mouse IgG Ab coupled to horse radish peroxidase (HRP), bovine anti-goat IgG Ab coupled to HRP, donkey anti-rabbit IgG Ab coupled to HRP, and Stattic were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Phycoerythrin (PE)-labeled anti-CD25 Ab and rat IgG2b were purchased from BD Biosciences (Mississauga, ON) and eBioscience, Inc. (San Diego, CA), respectively. Paraformaldehyde was purchased from Bio-Shop Canada Inc. (Burlington, ON). All cell culture plastics were purchased from Sarstedt Inc. (Montreal, QC) unless otherwise noted.

2.3. Cell proliferation assays

To determine rounds of cell division, CTLL-2 T lymphocytes were stained with 2 μM Oregon Green 488 dye (Invitrogen) in warm PBS for 10 min at room temperature. Serial halving of the fluorescent vital dye occurs with each round of cell division (Lyons, 2000). Excess dye was inactivated by FCS and cells were incubated for 30 min at 37 °C in warm fully supplemented RPMI 1640 medium. Cells were then plated at 1×10^5 cells/well in 24-well

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