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Pseudohypericin is necessary for the light-activated inhibition of prostaglandin E2 pathways by a 4 component system mimicking an *Hypericum perforatum* fraction

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ABSTRACT

Hypericum perforatum (Hp) has been used medicinally to treat a variety of conditions including mild-to-moderate depression. Recently, several anti-inflammatory activities of Hp have been reported. An ethanol extract of Hp was fractionated with the guidance of an anti-inflammatory bioassay (lipopolysaccharide (LPS)-induced prostaglandin E_2 production (PGE_2)), and four constituents were identified. When combined together at concentrations detected in the Hp fraction to make a 4 component system, these constituents (0.1 μM chlorogenic acid (compound **1**), 0.08 μM amentoflavone (compound **2**), 0.07 μM quercetin (compound **3**), and 0.03 μM pseudohypericin (compound **4**)) explained the majority of the activity of the fraction when activated by light, but only partially explained the activity of this Hp fraction in dark conditions. One of the constituents, light-activated pseudohypericin, was necessary, but not sufficient to explain the reduction in LPS-induced PGE_2 of the 4 component system. The Hp fraction and the 4 component system inhibited lipoxygenase and cytosolic phospholipase A_2, two enzymes in the PGE_2-mediated inflammatory response. The 4 component system inhibited the production of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α), and the Hp fraction inhibited the anti-inflammatory cytokine interleukin-10 (IL-10). Thus, the Hp fraction and selected constituents from this fraction showed evidence of blocking pro-inflammatory mediators but not enhancing inflammation-suppressing mediators.

1. Introduction

The synthesis of prostaglandins plays a critical role in normal physiological processes as well as acute and chronic inflammatory states (Dubois et al., 1998; Portanova et al., 1996), and the key enzymes involved in prostaglandin biosynthesis are prostaglandin endoperoxide synthases, also known as cyclooxygenases. Cyclooxygenase-1 (COX-1) is responsible for housekeeping functions such as maintenance of gastric mucosa (Smith et al., 1996). Cyclooxygenase-2 (COX-2) is induced by lipopolysaccharide (LPS) to produce prostaglandins, of which prostaglandin E_2 (PGE_2) is one of the main mediators of inflammation (Minghetti et al., 1999; O'Sullivan et al., 1992). Cytosolic phospholipase A_2 ($cPLA_2$) releases arachidonic acid, the substrate for COX and lipoxygenase (LOX) enzymes, from membrane phospholipids.

Cytokines mediate the inflammatory response in a complex manner, during its early, middle, and late stages. Tumor necrosis factor-α (TNF-α), an early pro-inflammatory cytokine, is involved in the pathogenesis of many inflammatory diseases and can regulate the growth, proliferation, and viability of leukocytes (Aggarwal, 2000; Calamia, 2003). Interleukin-10 (IL-10), an anti-inflammatory cytokine predominant in the later phases of inflammation, is a potent inhibitor of macrophage function, and IL-10 can block the synthesis of TNF-α and can inhibit COX-2 induction (Niiro et al., 1995; de Waal-Malefyt et al., 1991). Preparations that can modulate one or many of the mediators of inflammation may be useful for the treatment of inflammatory diseases.

Hypericum perforatum (Hp) contains unusual compounds such as hypericin, pseudohypericin, and hyperforin, as well as compounds present throughout the plant kingdom (Bilia et al., 2002). Raso et al. (2002) found that giving 100 mg/kg of Hp extract by gavage to mice two times daily significantly reduced COX-2 protein

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levels in peritoneal macrophages. Hp extracts and many of the constituents in these extracts (light-activated pseudohypericin, flavonoid compounds, hyperforin) reduced LPS-induced PGE_2 production in RAW 264.7 macrophages (Hammer et al., 2007). Furthermore, Hp extracts exhibited light-independent reductions in LPS-induced PGE_2, but pseudohypericin significantly decreased LPS-induced PGE_2 at 1 and 2 μM only in light-activated conditions and 20 μM hypericin increased PGE_2 with and without LPS only in light-activated conditions. This data established that although individual constituents like pseudohypericin and hypericin needed activation by light to produce an effect on PGE_2, confirming previously reported light-activated bioactivities of hypericin (Bilia et al., 2002; Carpenter and Kraus, 1991), Hp extracts did not differ in light-activated and dark conditions, contrary to previously reported bioactivities (Bilia et al., 2002; Schmitt et al., 2006a,b). Other compounds like the flavonoids and caffeic acid derivatives did not differ in light-activated and dark treatments (Hammer et al., 2007). It is vital to understand the effects of isolated active constituents as well as combinations of active constituents to relate the bioactivity of the constituents to the bioactivity of extracts (Spinella, 2002). Of 10 potentially bioactive constituents tested, only the concentration of pseudohypericin detected in the Hp extracts (0.2–1 μM) was above the level of pure constituent (1 μM) needed to observe a significant reduction in PGE_2 production in RAW 264.7 mouse macrophages (Hammer et al., 2007). However, pseudohypericin's presence in the extracts did not appear to account for the activity of the extracts, suggesting that the interactions of the constituents may be important (Hammer et al., 2007).

Bioactivity-guided fractionation was used to identify constituents present in an Hp ethanol extract that may be responsible for anti-inflammatory activity of the extract. Our hypothesis was that flavonoid compounds were contributing to the anti-inflammatory activity of the Hp extracts, along with other constituents that may interact with the flavonoids. To test this hypothesis, we used a strategy intended to enrich the fractions in flavonoids, and evaluated the fractions for a reduction in LPS-induced PGE_2 production. To compare our anti-inflammatory results to a known compound, we used concentrations of quercetin exceeding the levels found in Hp extracts and that have previously been shown to inhibit inflammatory endpoints of interest as a positive control.

2. Results and discussion

The bioactivities of Hp fractions from four rounds of iterative fractionations are presented in Table 1. The original Hp ethanolic extract significantly inhibited LPS-induced PGE_2 production in RAW 264.7 mouse macrophages at both 10 and 20 μg/ml. There was a significant reduction in cell viability associated with the 20 μg/ml dose of the Hp extract, although the reduction in PGE_2 (46% of PGE_2 control) could not be fully explained by this decreased cell viability data (58% of cell viability control). This original Hp extract was fractionated using ethanol, chloroform, or hexane into three fractions (1A: ethanol, 1B: hexane, and 1C: chloroform). The most active fraction from the first round of fractionation at 10 μg/ml was fraction 1C when compared with other fractions; 36% of the PGE_2 production compared to control and 74% of cell viability compared to control. Subfractionation of fraction 1C by column chromatography with a solvent series of chloroform ($CHCl_3$), acetonitrile (CH_3CN), and methanol (MeOH) led to 4 fractions (2A, 2B, 2C, 2D), of which, 10 μg/ml of fraction 2C most significantly decreased PGE_2 as compared to control (44% of PGE_2 control, 96% of cell viability control) and was the most active of the second round fractions at 10 μg/ml. Fraction 2C was further sub-fractionated using column chromatography with 1:1 CH_3CN:$CHCl_3$ to 1:1 MeOH:CH_3CN (3A, 3B, 3C, 3D, 3E, 3F). Of the third round fractions, fraction 3A significantly decreased PGE_2 (22% of PGE_2 control, 85% of cell viability control) at a concentration as low as 10 μg/ml. Fraction 3A was further sub-fractionated using column chromatography with a step gradient from 10% CH_3CN:$CHCl_3$ to 100% MeOH into 7 fractions (4A, 4B, 4C, 4D, 4E, 4F, 4G). The most active fraction from the last round of fractionation was fraction 4F (58% of PGE_2 control, 101% of cell viability control) at 2 μg/ml; however, the reduction in PGE_2 was not statistically significant.

The concentrations of 10 constituents were quantified in the original Hp extract and the four most active fractions (1C, 2C, 3A, 4F) are shown in Table 2. The most abundant constituents in the original Hp extract were hyperforin (12.5 μM), chlorogenic acid (6.1 μM), rutin (2.7 μM), and hyperoside (1.6 μM) (Table 2). After the first round of fractionation, the concentrations of all the constituents in fraction 1C were at or below 1 μM. It is possible that agents that suppressed the inhibition of PGE_2 production were removed in the earlier stages of fractionation since the concentration of putative active constituents decreased successively from the extract to fraction 1C and then to fraction 2C. In addition, unknowns comprised a larger portion of the later subfractions because the concentration of constituents decreased as the fractionation progressed, although activity remained about the same and was even greater from fraction 2C to 3A. The ratios of the 4 putative bioactive constituents in the fraction seemed to follow the pattern: greatest amount of chlorogenic acid, followed by roughly equal amounts of quercetin and amentoflavone, and the least amount of pseudohypericin (Table 2 figure legend). Ratio analysis of the levels of the four constituents in the extract and active fractions suggested that the greatest activity was obtained when the levels of chlorogenic acid, quercetin, and amentoflavone were approximately the same and that these concentrations were two to three times higher than pseudohypericin, as seen with fraction 3A. Additionally, the lowest activity was seen when only chlorogenic acid and pseudohypericin were detected, as seen with fraction 4F. Although compounds such as hypericin may have non-reversibly adsorbed to the silica gel column, results from the PGE_2 assay confirmed that at least one fraction was active from each round as the fractionation progressed. Additionally, flavonoids were compounds of particular interest in this fractionation and in previous studies, hypericin was shown to increase PGE_2 production in LPS-induced RAW 264.7 mouse macrophages (Hammer et al., 2007).

Since fraction 3A was significantly active in the PGE_2 assay and from the later rounds of fractionation, experiments were conducted to determine if combining its putative bioactive constituents (chlorogenic acid (compound **1**), amentoflavone (compound **2**), quercetin (compound **3**), and pseudohypericin (compound **4**)) into a 4 component system at the amount detected in fraction 3A could explain the reduction in PGE_2 by fraction 3A. These constituents were also studied together as a 4 component system at ten times and one hundred times the amount detected in fraction 3A. None of the four constituents alone reduced PGE_2 in light-activated or dark conditions (Table 3). Combinations of the four constituents revealed that combinations without pseudohypericin (compound **4**) were not effective at reducing PGE_2. Two-way and three-way combinations with pseudohypericin (compound **4**) seemed to explain some of the light-activated activity of the Hp fraction, however; not to as great of an extent as the 4 component system. The combination of all four constituents (34% of PGE_2 control, 101% of cell viability control) was sufficient to explain the anti-inflammatory activity of fraction 3A (12% of PGE_2 control, 85% of cell viability control) in light-activated conditions. Furthermore, this combination of constituents was even more effective at reducing PGE_2 in light-activated than dark conditions. Hyperforin and hypericin were not added to the 4 component system because they were only detected in the fraction and were not able to be