Immunopharmacology and inflammation

Evaluation of the anti-inflammatory, anti-catabolic and pro-anabolic effects of E-caryophyllene, myrcene and limonene in a cell model of osteoarthritis

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ABSTRACT

Osteoarthritis is a progressive joint disease and a major cause of disability for which no curative therapies are yet available. To identify compounds with potential anti-osteoarthritic properties, in this study, we screened one sesquiterpene, E-caryophyllene, and two monoterpenes, myrcene and limonene, hydrocarbon compounds for anti-inflammatory, anti-catabolic and pro-anabolic activities in human chondrocytes. At non-cytotoxic concentrations, myrcene and limonene inhibited IL-1β-induced nitric oxide production (IC50 = 37.3 µg/ml and 85.3 µg/ml, respectively), but E-caryophyllene was inactive. Myrcene, and limonene to a lesser extent, also decreased IL-1β-induced NF-κB, JNK and p38 activation and the expression of inflammatory (iNOS) and catabolic (MMP-1 and MMP-13) genes, while increasing the expression of anti-catabolic genes (TIMP-1 and -3 by myrcene and TIMP-1 by limonene). Limonene increased ERK1/2 activation by 30%, while myrcene decreased it by 26%, relative to IL-1β-treated cells. None of the compounds tested was able to increase the expression of cartilage matrix-specific genes (collagen II and aggrecan), but both compounds prevented the increased expression of the non-cartilage and anti-catabolic effects in human chondrocytes and, thus, its ability to halt or, at least, slow down cartilage destruction and osteoarthritis progression warrants further investigation.

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1. Introduction

Osteoarthritis (OA) is a multifactorial degenerative joint disease characterized by inflammation and progressive loss of the articular cartilage, associated with changes in the subchondral bone and other joint tissues. It affects 10–15% of the world population and is a major cause of disability, not only in the elderly, as well as in the workforce population (Zhang and Jordan, 2010). Existing therapeutic approaches are mainly symptomatic, thus novel drugs with disease-modifying and chondroprotective properties, the so-called disease-modifying osteoarthritic drugs, are required to halt disease progression and decrease its huge socio-economic impact (Goldring and Goldring, 2007; Kaplan et al., 2013).

Plant-derived compounds show important biological properties that can be explored in the context of OA for identification of compounds with potential anti-osteoarthritic activity (Calixto et al., 2004; Khalife and Zafarullah, 2011). Among compounds of plant origin, those found in essential oils present favorable pharmacokinetic properties, namely lipophilicity and low molecular weight (Miguel, 2010). Our previous studies have been focused in identifying essential oils with anti-inflammatory and anti-catabolic properties in human chondrocytes to be used as sources of compounds with potential anti-osteoarthritic activity (Neves et al., 2009; Rufino et al., 2014a). In this context, we recently identified the essential oils of Eryngium duriaeii subsp. juresium and Lavandula luisteri as possessing anti-inflammatory properties...
in human chondrocytes (Rufino et al., 2014a). The current study aims at identifying active compounds of these essential oils and further characterizing their pharmacological properties in human chondrocytes. For this, mechanisms relevant as pharmacological targets for the development of anti-osteoarthritic drugs need to be addressed. Although OA etiology is not yet completely understood, pro-inflammatory cytokines, like interleukin-1β (IL-1β), play a central role in disease development and progression by inducing the expression of cartilage matrix-degrading enzymes and impairing anabolic and anti-catabolic responses in chondrocytes (Goldring et al., 2008). The consequent upregulated degradative process, together with impaired reparative responses, results in progressive cartilage loss, the hallmark of OA.

Matrix metalloproteinases (MMPs) and aggrecanases are the main enzymes responsible for hydrolyzing the major articular cartilage-specific matrix components, collagen II and aggrecan. This is accompanied by impaired reparative responses involving downregulation of the natural MMP inhibitors, the tissue inhibitor of metalloproteinases (TIMP) family, decreased synthesis of collagen II and aggrecan and increased expression of non-articular cartilage matrix components, like collagen I (Troeberg and Nagase, 2012). Moreover, increased production of pro-inflammatory and pro-catabolic mediators, like nitric oxide (NO), amplifies and perpetuates cartilage destruction (Boileau et al., 2002; Rosa et al., 2008; Sasaki et al., 1998). The transcription factor, Nuclear Factor-kB (NF-kB), and the family of mitogen-activated protein kinases (MAPK) play a central role in modulating the expression of those catabolic and inflammatory mediators (Goldring and Otero, 2011) and, thus, agents that suppress their activity have the potential to effectively decrease cartilage destruction and, therefore, OA progression (Berenbaum, 2004). Furthermore, compounds that can also restore anabolic and anti-catabolic gene expression have the potential to promote cartilage repair.

Therefore, we used primary human chondrocyte cultures stimulated with IL-1β as an in vitro cartilage degradation model that emulates the damage seen in OA. Using this model, we evaluated the inhibition of IL-1β-induced NO production as a simple screening assay. The essential oils of E. duriae subsp. juresianum and L. luisieri were separated into several fractions that were then screened using the assay mentioned above. The chemical composition of the fractions tested was elucidated and compounds present in the active fractions and commercially available were selected for screening using the same assay. The compounds selected were obtained with a high degree of purity from commercial sources and those found to be capable of inhibiting IL-1β-induced NO production were selected for further assessment of anti-osteoarthritic potential. For this, we evaluated their ability to modulate IL-1β-induced signaling pathways involved in the expression of inflammatory and catabolic genes, namely activation of NF-kB and the MAPK family members, Jun terminal Kinase (JNK), p38 and Extracellular signal-Regulated Kinase 1/2 (ERK1/2). Then, we evaluated the ability of those compounds to counteract the effects of IL-1β on the expression of inflammatory (iNOS), catabolic (MMP-1 and -13), anti-catabolic (TIMP-1 and -3) and extracellular matrix (collagen I, collagen II and aggrecan) genes in human articular chondrocytes.

2. Material and methods

2.1. Essential oil fractionation and chemical analysis

The essential oils of E. duriae subsp. juresianum and L. luisieri were fractionated by flash chromatography on silica gel 63–200 μm (Merck) using a 2 cm × 40 cm Omnifit (Sigma-Aldrich) glass columns. Elution was made in step gradients from 100% of n-pentane, n-pentane /ethyl ether mixtures, till a final concentration of 100% ethyl ether. Collected fractions were analyzed by gas chromatography–mass spectroscopy (GC/MS) and combined if having similar composition. Analysis was performed using a Hewlett-Packard 6890 gas chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 μm), interfaced with an Hewlett-Packard mass selective detector 5973 (Agilent Technologies) operated by HP Enhanced ChemStation software, version A.03.00. GC–MS parameters: oven temperature program: 70–220°C (3°C min⁻¹), 220°C (15 min); injector temperature: 250°C; carrier gas: helium, adjusted to a linear velocity of 30 cm s⁻¹; splitting ratio 1:40; interface temperature: 250°C; MS source temperature: 230°C; MS quadrupole temperature: 150°C; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35–350 units; scans s⁻¹: 4.51. Compounds were identified through their GC retention and mass spectra. Retention indices, calculated by linear interpolation relative to retention times of C₆–C₂₃ of n-alkanes, were compared with those of authentic products included in laboratory database (CEF/Faculty of Pharmacy, University of Coimbra) and/or the literature data. Acquired mass spectra were compared with reference spectra from the laboratory database, Wiley/NIST library (McLafferty, 2009) and literature data (Adams, 1995; Cavaleiro et al., 2011; Joulain, 1998; Videira et al., 2013). Relative amounts of individual components were calculated based on the total ion chromatogram peak areas.

2.2. Cartilage samples and chondrocyte isolation

Human chondrocytes were isolated by enzymatic digestion (Rosa et al., 2008) of knee cartilage from the distal femoral condyles of multi-organ donors (20–70 years old, mean = 52.9, n = 31) and patients (58–73 years old, mean = 65, n = 5) undergoing total knee arthroplasty at the Orthopaedic Department and Bone Bank of the University and Hospital Center of Coimbra (CHUC). The cartilage samples presented variable degrees of degradation, ranging from intact to severely damaged. All procedures were approved by the Ethics Committee of CHUC (protocol approval numbers 8654/DC and HUC-13-05).

2.3. Cell cultures and treatments

Primary non-proliferating chondrocyte cultures were established from non-pooled cartilage samples. The human chondrocytic cell line, C28/I2, kindly provided by Prof. Mary Goldring (currently at the Hospital for Special Surgery, New York) and Harvard University, was used to evaluate NF-kB–DNA binding activity. The cell cultures were serum-starved for at least 8 h and maintained thereafter in serum-free culture medium. The test compounds, diluted in DMSO (Sigma-Aldrich, St Louis, MO, USA) to achieve the concentrations indicated in figures and their legends, were added to the chondrocyte cultures 30 min before the pro-inflammatory stimulus (IL-1β, 10 ng/ml) and maintained for the experimental period indicated in figure legends. The final concentration of DMSO did not exceed 0.1% (v/v). E-caryophyllene (purity > 98.5%) and myrcene (purity ≥ 95.0%) were from Sigma-Aldrich (St Louis, MO, USA) and limonene (purity ~ 90%) was from Fluka.

2.4. Nitric oxide production

Nitric oxide production was evaluated as the amount of nitrite accumulated in primary chondrocyte culture supernatants after 24 h treatment with IL-1β, following pre-treatment with non-cytotoxic concentrations of the fractions of the essential oils or the test compounds. Nitrite concentration was measured using the
spectrophotometric method based on the Griess reaction (Green et al., 1982).

2.5. Western blot analysis

Total and cytoplasmic extracts were prepared as described before (Goldring and Otero, 2011). Proteins were separated by SDS-PAGE under reducing conditions and electrotransferred onto PVDF membranes. These were probed overnight with the following primary antibodies: mouse monoclonal anti-human iNOS (R&D Systems, Minneapolis, MN), mouse polyclonal anti-human phospho-β-Tub 1a-γ or rabbit polyclonal anti-human β-Tub α, anti-human phospho-JNK, anti-human phospho-p38 or anti-human phospho-ERK1/2 (Cell Signaling Technology, Inc., Danvers, MA) and then with anti-rabbit or anti-mouse alkaline phosphatase-conjugated secondary antibodies (GE Healthcare, UK). Mouse anti-human β-Tubulin monoclonal antibody was used to detect β-Tubulin as a loading control. Enhanced Chemiluminescence reagent (GE Healthcare) was used to detect immune complexes. Image analysis was performed with ImageQuant TL software (GE Healthcare). The results presented are the normalized ratio between the intensities of the bands corresponding to the protein of interest and to the protein used as loading control.

2.6. NF-κB transcription factor assay

A colorimetric ELISA-based assay (NoShift Transcription Factor Assay Kit, Novagen, La Jolla, CA) was used to evaluate the presence of active NF-κB dimers, capable of binding to the cognate consensus oligonucleotide sequence. For this purpose, nuclear extracts from C-28/I2 cells were incubated with a biotinylated consensus NF-κB oligonucleotide (NoShift NF-κB Reagents; Novagen) and the assay performed according to the manufacturer's instructions. The absorbance intensity in each sample is directly proportional to the amount of NF-κB–DNA complexes formed and, thus, to the amount of active NF-κB dimers present in each sample. In parallel, the specificity of the reaction was confirmed in competition assays where addition of a 10-fold molar excess of non-biotinylated wild-type or mutant oligonucleotides (NoShift NF-κB Reagents; Novagen) to binding reactions containing nuclear extracts from IL-1β treated cells abrogated or did not affect, respectively, the formation of NF-κB–DNA complexes.

2.7. Total RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from human condrocytes using TRIzol® Reagent (Invitrogen, Life Technologies, Co; Paisley, UK) and quantified using a NanoDrop ND-1000 spectrophotometer at 260 nm. Purity and integrity of RNA were assessed as the 240/260 nm. The cDNA was reverse-transcribed using the iScript Select cDNA Synthesis Kit (Bio-Rad), beginning with 1 μg of RNA. Specific sets of primers for iNOS, MMP-1, MMP-13, TIMP-1, TIMP-3, collagen II, collagen I, aggrecan and HPRT-1 (Table 1) were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, CA). PCR reactions were performed using 25 μg/ml of transcribed cDNA in a final volume of 20 μl.

2.8. Statistical analysis

Results are presented as mean ± S. E. M. Each subject contributed only once to the statistical analysis which was performed using GraphPad Prism (version 5.00). The Kolmogorov–Smirnov test was used to assess normality for the observations themselves or for the observed differences. As this test showed that in all experiments the results were normally distributed, the statistical analysis was performed using the paired t-test for comparison of each condition with its respective control and one-way ANOVA for comparison of all conditions. Results were considered statistically significant at \( P < 0.05 \).

3. Results

3.1. Selection of inhibitors of IL-1-induced iNOS expression and NO production

Various fractions of the essential oils of E. duriae subsp. jureisanum and L. luisieri were separated in amounts sufficient for pharmacological screening. The composition of these fractions was fully elucidated and is reported in Table 2.

The fractions obtained were then tested at non-cytotoxic concentrations ranging from 10 to 50 μg/ml (Supplementary data). The results (Fig. 1 panel A) show that the hydrocarbon-containing fractions (F1, F3, F5) and both essential oils decreased IL-1β-induced NO production in a concentration-dependent manner, the highest concentrations achieving an inhibition of 55% and 75%, respectively, relative to cells treated with IL-1β alone. The other three fractions of the essential oil of L. luisieri also showed some inhibitory activity which, nonetheless, did not exceed 35% relative to IL-1β-treated cells. Fractions F2.4 and F2.5 of the essential oil of E. duriae subsp. jureisanum were only tested at a concentration of 10 μg/ml, as higher concentrations were found to be cytotoxic (Supplementary data). These fractions, as well as fraction F2.7, at

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Oligonucleotide primer pairs used for qRT-PCR.</th>
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<tr>
<td>Gene name</td>
<td>Genbank accession number</td>
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<tr>
<td>iNOS</td>
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<td>MMP-1</td>
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<td>MMP-13</td>
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a concentration of 50 μg/ml, also significantly inhibited IL-1β-induced NO production, although to a lesser extent than found with F₁. No significant effects were obtained with F₂,3 at any of the concentrations tested. Therefore, the hydrocarbon-containing fractions (F₁ and F₂) of both essential oils were considered the most promising for selection of compounds for further studies.

As shown in Table 2, these fractions (Fₐ and F₁) are composed of monoterpenic and sesquiterpene hydrocarbons, mainly α-pinene and 3,5-dimethyl-1,4,4-trimethylcyclopentene, and E-caryophyllene, α-neocallitropsene, germacrene D, β-selinene and bicyclogermacrene, respectively. Of these compounds, only α-pinene and E-caryophyllene are readily available from commercial sources.
and we have recently reported the differential activity of $\alpha$-pinene enantiomers as inhibitors of pro-inflammatory and catabolic pathways in human chondrocytes (Rufino et al., 2014b). Nonetheless, both essential oils have other minor components in common, namely the monoterpene hydrocarbons, myrcene and limonene, which were thus selected for pharmacological evaluation. Thus, high purity $E$-caryophyllene, myrcene and limonene (structural formulas are depicted in Fig. 2), obtained from commercial sources indicated in Section 2, were screened for their ability to inhibit IL-1$\beta$-induced NO production.

The obtained results (Fig. 1 panel B) show that, at non-cytotoxic concentrations (Supplementary data), myrcene and limonene effectively inhibited IL-1$\beta$-induced NO production, while $E$-caryophyllene had no significant effect at any of the concentrations tested.

Since myrcene and limonene showed inhibitory activity towards IL-1$\beta$-induced NO production, various non-cytotoxic concentrations were then tested to determine the respective concentration required to inhibit NO production by 50% (IC$_{50}$) and thus, to compare their relative potencies. The IC$_{50}$ values obtained are 37.3 ± 1.1 $\mu$g/ml for myrcene and 85.3 ± 1.2 $\mu$g/ml for limonene.

To determine whether the observed inhibition of NO production by myrcene and limonene is due to inhibition of iNOS expression, its mRNA (Fig. 3 panel B) and protein (Fig. 3 panel A) levels were evaluated. Treatment with myrcene, 50 $\mu$g/ml, significantly diminished IL-1$\beta$-induced iNOS mRNA and protein levels by 78% and 69%, respectively, while inhibition by limonene, even at a concentration four fold higher, did not exceed 39% and 60%, respectively.

3.2. Inhibition of IL-1$\beta$-induced NF-$\kappa$B activation by myrcene and limonene

To further elucidate the mechanisms by which the two compounds, myrcene and limonene, inhibit iNOS expression and to further evaluate their potential as anti-osteoarthritic drugs, their ability to inhibit IL-1$\beta$-induced NF-$\kappa$B activation was determined. NF-$\kappa$B activation requires the phosphorylation, ubiquitination and proteasomal degradation of its natural inhibitor, NF-$\kappa$B Inhibitor-$\alpha$ (I$\kappa$B-$\alpha$), which, in unstimulated cells, retains NF-$\kappa$B dimers in the cytoplasm. Once I$\kappa$B-$\alpha$ is degraded, the freed NF-$\kappa$B dimers translocate to the nucleus and bind to specific sequences in the promoter region of target genes promoting their transcription (Hayden and Ghosh, 2008). Thus, we evaluated the protein levels of phosphorylated and total I$\kappa$B-$\alpha$ by western blot and the binding of the freed NF-$\kappa$B dimers to a specific DNA sequence by ELISA.

Since human cartilage samples are scarce and a large number of cells is required, the ability of the test compounds to inhibit...
IL-1β-induced NF-κB-DNA binding was evaluated in the human chondrocytic cell line, C-28/I2, while the levels of phosphorylated and total IκB-α were evaluated in primary human chondrocytes. The results show that treatment with IL-1β dramatically increased IκB-α phosphorylation (Fig. 4 panel A) and decreased total IκB-α levels, reflecting its almost complete degradation (Fig. 4 panel B), followed by increased NF-κB-DNA binding (Fig. 4 panel C). Treatment with Bay 11-7082 (Bay), a specific NF-κB inhibitor that selectively prevents IκB-α phosphorylation, or with the test compounds markedly reduced IL-1β-induced IκB-α phosphorylation (Fig. 4 panel A) and degradation (Fig. 4 panel B), as well as NF-κB-DNA binding (Fig. 4 panel C). Interestingly, although the degree of inhibition of IκB-α phosphorylation and of NF-κB-DNA binding achieved with Bay was significantly higher than that obtained with myrcene and limonene, IκB-α degradation was similarly decreased by the three compounds.

### 3.3. Effects of myrcene and limonene on IL-1β-induced MAPK activation

Together with NF-κB activation, signaling pathways involving activation of members of the MAPK family also play an important role in the proteolytic cartilage degradation process, namely in the expression of MMPs (Mengshol et al., 2000). Thus, the ability of myrcene and limonene to inhibit IL-1β-induced MAPK activation was assessed by evaluating their phosphorylation levels.

Myrcene and limonene showed quite distinct effects on IL-1β-induced JNK (Fig. 5 panel A), p38 (Fig. 5 panel B) and ERK1/2 (Fig. 5 panel C) phosphorylation. Myrcene significantly reduced IL-1β-induced phosphorylation of the three MAPKs, while limonene was effective in reducing p38 phosphorylation by near 39%, but increased phosphorylated ERK1/2 by 30% and had no significant effect on JNK.

### 3.4. Modulation of inflammatory, catabolic, anti-catabolic and extracellular matrix gene expression by myrcene and limonene

Then, we evaluated the ability of the compounds tested to counteract the effects of IL-1β on the expression of catabolic, anti-catabolic and extracellular matrix genes, which, at least in part, are mediated by NF-κB and the MAPKs (Goldring and Otero, 2011). As expected, treatment of human chondrocytes with IL-1β (10 ng/ml) increased MMP-1 and -13 mRNA levels by nearly 9- and 5-fold, respectively (Fig. 6 panel A), while decreasing TIMP-1 and -3 expressions (Fig. 6 panel B). Myrcene decreased the mRNA levels of both MMPs by nearly 60%, relative to IL-1β-treated cells; limonene reduced MMP-1 and -13 levels by 51% and 39%, respectively. Nevertheless, the reduction of MMP-13 levels elicited by limonene did not reach statistical significance.

On the other hand, limonene did not significantly change the inhibitory effect of IL-1β on TIMP-1 and -3 mRNA levels, even though it showed a tendency to increase TIMP-1 levels that did not
Fig. 5. Effects of myrcene and limonene on IL-1β-induced activation of JNK, p38 and ERK1/2 in human chondrocytes. Phosphorylated levels of JNK (panel A), p38 (panel B) and ERK (1/2) (panel C) were analyzed in total cell extracts of human chondrocytes left untreated (Control, Ctrl) or treated for 5 min with IL-1β, 10 ng/ml, following a pre-treatment for 30 min with the indicated concentrations of myrcene, limonene or a specific inhibitor of the activation of each MAPK. Each column represents the mean ± S. E. M. of, at least, 4 independent experiments. Representative images are shown. **P < 0.01, ***P < 0.001 relative to IL-1β-treated cells and §§§P < 0.001 relative to control cells. iJNK: JNK inhibitor, SP600125 (20 μM); ip38: p38 inhibitor, SB203580 (20 μM); iERK: ERK1/2 inhibitor, U0126 (10 μM).

Fig. 6. Effects of myrcene and limonene on IL-1β-induced changes in the expression of catabolic and anti-catabolic genes. mRNA levels of MMP-1 and MMP-13 (panel A) and TIMP-1 and TIMP-3 (panel B) were evaluated by qRT-PCR. Each bar represents the mean ± S. E. M. of, at least, 4 independent experiments in which human chondrocytes were left untreated (Control, Ctrl) or treated for 12 h (panel A) or 24 h (panel B) with IL-1β, 10 ng/ml, in the presence or absence of the indicated compounds added to the cell cultures 30 min before IL-1β. *P < 0.05, **P < 0.01, ***P < 0.001 relative to IL-1β-treated cells and §§§P < 0.001 relative to control cells.
reach statistical significance. On the contrary, myrcene not only completely reversed the inhibitory effect exerted by IL-1β, as it effectively increased TIMP-1 and -3 levels approximately 2- and 1.3-fold above those in untreated control cells, respectively, which correspond to even larger increases if compared to TIMP-1 and -3 mRNA levels in cells treated with IL-1β alone.

To assess the potential ability of the test compounds to inhibit the deleterious effects of IL-1β in anabolic responses that are essential for repair of damaged articular cartilage, the expression of collagen II and aggrecan was evaluated. Furthermore, the ability of the test compounds to decrease the expression of the non-cartilage-specific collagen I gene, induced by IL-1β, was also evaluated. Chondrocyte treatment with 10 ng/ml IL-1β, for 24 h, significantly increased collagen I mRNA levels, while decreasing those of collagen II and aggrecan, relative to untreated control cells (Fig. 7). Treatment of human chondrocytes with myrcene or limonene caused no significant changes on collagen II (Fig. 7 panel A) and aggrecan (Fig. 7 panel B) mRNA levels compared to those in cells treated with IL-1β alone. Nonetheless, both treatments were able to completely abolish or even reverse the increase in collagen I mRNA levels induced by IL-1β.

4. Discussion

The results obtained in this study identify two monoterpene hydrocarbons, myrcene and limonene, as capable of inhibiting IL-1β-induced NO production in human chondrocytes. The specific activities (activity/µg) relative to inhibition of IL-1-induced NO production, of myrcene and limonene are 1.33%/µg and 0.57%/µg, respectively, while for the active fractions (F₁ and F₈) of the essential oils of E. duriae subsp. juresianum and L. luisieri they are 1%/µg and 3%/µg, respectively. Since myrcene and limonene are only minor components of those fractions, it is likely that other constituents are also active and contribute to the effects observed. Moreover, since both fractions contain several distinct compounds, none of which is present in sufficiently high amounts to justify the effects observed, either the active compound in those fractions is significantly more potent than myrcene and limonene or various active compounds, including these two, act synergistically, or at least, additively, to achieve a similar or even higher degree of inhibition of IL-1-induced NO production. Unfortunately, as mentioned in Section 3.1, the major components of those fractions are either not readily available from commercial sources or have been previously studied, as is the case for (+)-ç-pinene that we showed to have anti-inflammatorly and anti-catabolic activities in human chondrocytes (Ruffino et al., 2014b). Thus, identification of the other active compounds in those fractions is, at present, impracticable.

On the other hand, the sesquiterpene hydrocarbon, E-caryophyllene, which is a major component of the active fraction of the essential oil of E. duriae subsp. juresianum, is completely inactive. This finding is somewhat unexpected as E-caryophyllene has been reported to exert anti-inflammatory effects by activating cannabinoid CB2 receptors (Bento et al., 2011; Medeiros et al., 2007) and endogenous and synthetic cannabinoids have been reported to decrease inflammation in animal models of arthritis (Sumariwalla et al., 2004) and to inhibit IL-1-1-induced NO production in bovine chondrocytes (Mbvundula et al., 2005).

The two active compounds, myrcene and limonene, show clear qualitative and quantitative differences in terms of ability to inhibit IL-1β-induced responses. Myrcene was the most potent in inhibiting NO production, as indicated by an IC₅₀ value less than half of that found for limonene. Myrcene was also more effective than limonene in preventing other inflammatory and catabolic responses in human chondrocytes, namely expression of iNOS, MMP-1 and MMP-13 induced by IL-1β, likely reflecting, at least in part, the stronger inhibition of NF-kB and the ability to inhibit all three MAPKs. These findings are in agreement with another study that reported anti-inflammatory properties of myrcene in a mouse model of pleurisy induced by zymosan and bacterial lipopolysaccharide where it inhibited the production of NO and inflammatory cytokines (Souza et al., 2003). Furthermore, myrcene, but not limonene, caused a net increase in the expression of the anti-catabolic genes, TIMP-1 and -3, which in combination with the decrease in MMP-1 and -13 expression can cause a significant reduction of the catabolic milieu characteristic of OA.

On the other hand, myrcene also completely prevented the increase in collagen I induced by IL-1β. Collagen I is not normally found in articular cartilage and its expression increases in OA and in association with chondrocyte dedifferentiation, a process that involves several alterations of chondrocyte gene expression and morphology and leads to the formation of fibrocartilage (Martin et al., 2001). Therefore, even though it did not increase the specific anabolic responses of human chondrocytes, myrcene may be effective in preventing chondrocyte dedifferentiation associated with increased collagen I expression, while decreasing inflammatory and catabolic processes directly involved in cartilage destruction.

Reports on pharmacological properties of limonene are scarce, but it has been shown to have antimicrobial properties (Bevilacqua et al.,...
and anti-inflammatory effects in a mouse model of LPS-induced acute lung injury by suppressing MAPK and NF-κB pathways (Chi et al., 2012). The results presented here only partially agree with this study, since limonene inhibited NF-κB and p38 activation, but did not affect IL-1β-induced JNK and actually potentiated ERK1/2 activation, suggesting that this compound has cell- and/or stimulus-specific effects. On the other hand, ERK1/2 is required for a number of cellular processes, including activation of c-fos expression which, among other functions, is involved in cell survival (Karin et al., 1997; Shaulian and Karin, 2002). Whether increased activation of ERK1/2 by limonene contributes to enhance chondrocyte survival was not addressed in this study, but is an interesting possibility to study further, as increased chondrocyte death is a relevant feature of OA (Johnson et al., 2008). Nonetheless, since ERK1/2 has also been shown to inhibit proteoglycan synthesis and to promote inflammatory and catabolic responses in chondrocytes (Scherle et al., 1997), the net effect resulting from its induction by limonene is likely undesirable, compromising its potential utility as a therapeutic agent in OA.

Form another point of view, it is intriguing that limonene induced ERK1/2 activation while decreasing p38 and not affecting JNK activation. This is even more puzzling as myrcene was able to inhibit the activation of all three MAPKs. Even though we cannot provide an explanation for the differential effects of both compounds, it seems reasonable to admit that they may act on distinct molecular targets. Although the exact signaling pathways that link IL-1β binding to its receptor to downstream events are still incompletely understood, the immediate upstream activators of each MAPK have been identified. While some of those, namely the mitogen-activated protein kinase kinase 4 (MKK4 or MEK4), can activate both JNK and p38, others specifically activate each of these MAPK. MKK3 and MKK6 are p38 activators while MKK7 activates JNK and MKK1 activates ERK1/2 (Weber et al., 2010). Thus, limonene may inhibit MKK3 or MKK6 without affecting MKK4 or MKK7 or any other upstream intermediate, while inducing MKK1 activation, either direct or indirectly. On the other hand, myrcene may act on another intermediate common to the three MAPK and also to NF-κB or instead it may independently inhibit the MAPK and NF-κB signaling pathways. Clearly, further studies are required to identify the specific molecular targets of myrcene and limonene.

Nonetheless, this may be a difficult task given the complexity of each of these pathways and the extensive cross-talk between them (Virtue et al., 2012; Weber et al., 2010).

In conclusion with (+)-α-pinene that we previously reported to have anti-inflammatory and anti-catabolic properties in human chondrocytes (Rufino et al., 2014b), myrcene shows potential advantages since, besides inhibiting iNOS expression and activity and NF-κB and JNK activation to a similar extent but at lower concentrations, it further decreased ERK1/2 and p38 activation and increased anti-catabolic responses, namely TIMP-1 and -3 expression. Moreover, myrcene can also promote the maintenance of the differentiated chondrocyte phenotype, as it also decreased collagen I expression. Nonetheless, none of the compounds tested, nor (+)-α-pinene, seem effective in promoting the expression of articular cartilage matrix-specific genes and, thus, are unlikely to promote the repair of areas already damaged. Moreover, the potency of myrcene is relatively low which may also hinder its therapeutic efficacy.

5. Conclusions

Myrcene has significant anti-inflammatory and anti-catabolic properties in vitro suggesting that it may be useful to halt or, at least, slow down cartilage destruction and, thus, OA progression. Future studies in in vivo models of OA are thus warranted to evaluate its potential as a disease-modifying osteoarthritis drug.

Acknowledgment


Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2015.01.018.

References


