Formation of reactive oxygen and nitrogen species in the presence of pinosylvin – an analogue of resveratrol

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Abstract

OBJECTIVE: Formation of reactive oxygen species in neutrophils of rats with adjuvant arthritis and generation of nitric oxide in RAW 264.7 macrophages were analysed in the presence of pinosylvin.

METHODS AND RESULTS: The method of chemiluminescence was used for the detection of reactive oxygen species in blood of rats with adjuvant arthritis. Pinosylvin (50 mg/kg, daily, p.o.) and methotrexate (0.4 mg/kg, twice a week, p.o.) were applied separately or in a combination over a period of 28 days from the day of immunisation. Adjuvant arthritis was accompanied by a significantly increased number of neutrophils, by elevated concentration of oxidants in blood and by excessive responsiveness of neutrophils to stimulation with PMA. In rats treated with methotrexate, all these changes were significantly reduced and the inhibition became more pronounced when methotrexate was applied in the combination with pinosylvin; the monotherapy with pinosylvin did not induce any detectable changes in the parameters tested. Under *in vitro* conditions, pinosylvin inhibited formation of nitric oxide (NO) in macrophages, as demostrated by the decreased concentration of nitrite – the end-product of NO metabolism (assessed by Griess' method), by the reduced expression of inducible NO synthase (detected by Western blot), and by the failure of pinosylvin to scavenge nitric oxide (measured amperometrically in cell-free system).

CONCLUSION: The observed ability of pinosylvin to decrease concentration of reactive oxygen and nitrogen species, along with its capacity to enhance the efficacy of methotrexate in arthritis treatment may shed more light into the pharmacological potential of this prospective natural substance.

ORIGINAL ARTICLE

Abbreviations:

NO	- nitric oxide
iNOS	- inducible NO synthase
NFκB	- nuclear factor κB
PMA	- 4β-phorbol-12β-myristate-α13-acetate
RLU	- relative luminescence units

INTRODUCTION

Reactive oxygen and nitrogen species, produced by neutrophils and other inflammatory cells, participate actively in the initiation and development of many pathological states (Witko-Sarsat *et al.* 2000; Cascao *et al.* 2010). In rheumatoid arthritis, the oxidants can induce cartilage degradation, depolymerise hyaluronan and decrease its lubricative properties, they can reduce the protective antioxidant and antiproteinase capacity of synovial fluid and participate in this way in joint erosion (Edwards & Hallett 1997; Cross *et al.* 2006; Cascao *et al.* 2009). These facts focussed attention on antioxidative and neutrophil targeting substances as they may increase the effectiveness and minimise unwanted side effects of disease-modifying antirheumatic therapy. Several compounds of plant or microbial origin were found to be promising from this perspective (Drábiková *et al.* 2009; Jančinová *et al.* 2009a; Rovenský *et al.* 2009).

Pinosylvin (3,5-dihydroxystilbene), one of the naturally occurring resveratrol (3,4',5-trihydroxystilbene) analogues, is formed constitutively and after UV irradiation or microbial attack in the wood and needles of *Pinus* species. The majority of the available data characterises antifungal, antibacterial and anticancer activities of pinosylvin (Lee *et al.* 2005; Roupe *et al.* 2006a; Simard *et al.* 2008), yet little is known about its antioxidant and antiinflammatory effects (Park *et al.* 2004; Adams *et al.* 2005; Lee *et al.* 2006). Previously, we found that incubation of human neutrophils with this stilbene reduced production of reactive oxygen species and this effect involved the inhibition of protein kinase C isoforms α and βII (Perečko *et al.* 2008; Jančinová *et al.* 2009b). In the present study, its interference with neutrophils was further tested under *in vivo* conditions – in rats with adjuvant arthritis, and formation of the inflammatory mediator nitric oxide in macrophages was also analysed.

MATERIALS AND METHODS

Materials

Pinosylvin was synthesised at the Institute of Organic Chemistry and Biochemistry AS CR (Praha, Czech Republic), methotrexate was from Pharmachemie (Haarlem, Netherlands), Griess' reagent, lipopolysaccharide from *Escherichia coli*, luminol, and PMA from Sigma-Aldrich Chemie (Deisenhofen, Germany), murine RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA).

Formation of reactive oxygen species – effects of pinosylvin in arthritis

Adjuvant arthritis was induced in male Lewis rats by a single intradermal injection of heat-killed *Mycobacterium butyricum* (Poništ *et al.* 2010). The study was performed in compliance with Principles of Laboratory Animal Care and was approved by the local Ethics Committee and by the State Veterinary and Food Administration of the Slovak Republic. Pinosylvin (50 mg/kg, daily, p.o.) and methotrexate (0.4 mg/kg, twice a week, p.o.) were applied separately or in a combination over a period of 28 days after arthritis induction. Then neutrophil count and concentration of reactive oxygen species in blood were assessed. The production of oxidants (spontaneous or stimulated with 0.05 μmol/l PMA) was estimated on the basis of luminol-enhanced chemiluminescence and presented as the mean integral values over 3600 s (Nosáľ *et al.* 2007; Jančinová *et al.* 2009a).

Formation of nitric oxide – effects of pinosylvin in vitro

Generation of reactive nitrogen species was determined indirectly as the accumulation of nitrites – end products of nitric oxide (NO) metabolism. The detection was performed spectrophotometrically by Griess' method in the supernatant of murine macrophages RAW 264.7, which were pre-incubated for 1 h with pinosylvin and after that stimulated for 24 h with lipopolysaccharide (LPS). The concentrations of nitrites were derived by regression analysis using serial dilutions of sodium nitrite as a standard (Králová *et al.* 2008). The cell fractions of these samples were used for the detection of inducible NO synthase expression by Western blot. Relative protein levels were quantified by scanning densitometry using the Image J programme (Ambrožová *et al.* 2010). The NO scavenging activity of pinosylvin was measured amperometrically using an ISO-NO Mark II NO meter and NO standard solutions. Changes in electrical current were recorded and the index of scavenging was calculated by dividing the maximum height and width of the obtained curves (Číž *et al.* 2008).

Statistical analysis

Statistical significance of differences between means was established by one-way analysis of variance (ANOVA); *p*-values below 0.05 were considered statistically significant.

RESULTS

Adjuvant arthritis was accompanied by a significantly increased number of neutrophils and by massive formation of oxidants, which was manifested by elevated chemiluminescence of blood (Table 1). Stimulated chemiluminescence was increased seven times and it rose more rapidly than the other parameters tested. This indicates that neutrophils of arthritic rats responded to PMA by an excessive production of radicals, similarly as found by hyper-reactive „primed" neutrophils

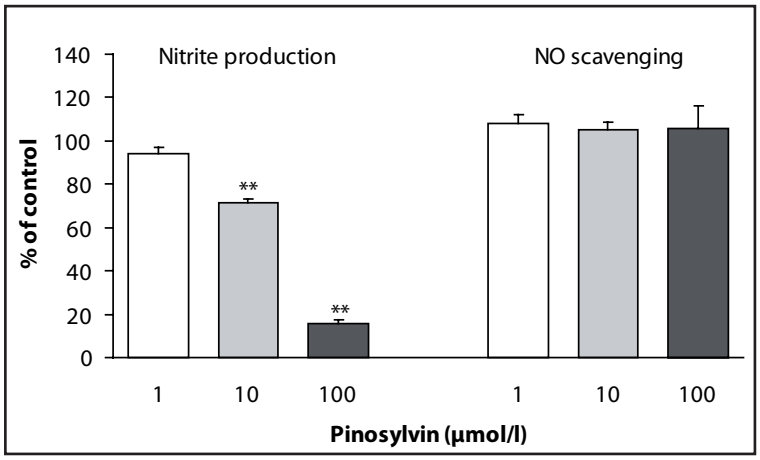

Fig. 1. Nitrite production and nitric oxide (NO) scavenging assessed in the presence of pinosylvin.
Concentration of nitrites was measured spectrophotometrically in supernatants of RAW 264.7 cells stimulated with LPS; the NO scavenging effect of pinosylvin was determined amperometrically in a cell-free chemical system. Pinosylvin-induced changes in nitrite concentration or in the scavenging index were expressed as percentage of controls (i.e. samples incubated in the absence of pinosylvin). Mean ± SEM, n = 3, ** $p<0.01$ (vs Control).

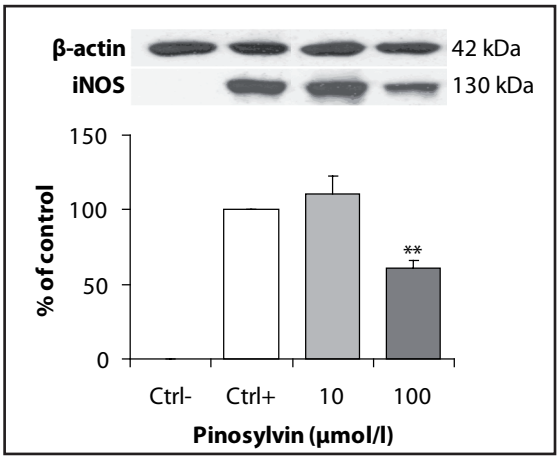

Fig. 2. Effect of pinosylvin on the expression of inducible nitric oxide synthase (iNOS).
Densitometric analysis and representative Western blot of iNOS protein expression in RAW 264.7 cells treated with 10 and 100 μmol/l pinosylvin and stimulated with LPS. Unstimulated (Ctrl–) and LPS-stimulated (Ctrl+) control cells were used to express effects of the stimulus and of pinosylvin, respectively. The immunoblotting of β-actin verified the equal loading of proteins. Mean ± SEM, n = 3, ** $p<0.01$ (vs Ctrl+).

of patients with rheumatoid arthritis (Fairhurst *et al.* 2007). In rats treated with methotrexate, all the arthritis-induced changes were significantly reduced and this inhibition became more pronounced when methotrexate was applied along with pinosylvin. Methotrexate alone decreased neutrophil count, spontaneous and stimulated chemiluminescence by 28%, 41% and 43%, respectively, whereas in combination with pinosylvin, it inhibited these parameters by 59%, 69% and 63%. The monotherapy with pinosylvin failed to induce any detectable changes either in the number of neutrophils or in oxidant concentration.

Considering the fact that overproduction of nitric oxide is involved in rheumatoid arthritis, and with the aim to find further mechanism(s) potentially involved in the antiinflammatory effect of pinosylvin, the interference of this stilbene with macrophage activity and with NO formation was analysed (Figures 1 and 2). Pinosylvin significantly reduced the quantity of NO produced by stimulated RAW 264.7 macrophages, as evidenced by the decreased accumulation of nitrites in supernatants of these cells. The reduced expression of inducible NO synthase and the absence of NO scavenging indicated that this inhibition resulted from diminished generation of nitric oxide.

Tab. 1. Effect of pinosylvin (PIN) and methotrexate (MTX), applied separately or in combination, on spontaneous and stimulated chemiluminescence and neutrophil count in blood of arthritic rats.

	Chemiluminescence (RLU*s)		Neutrophil count
	Spontaneous	PMA stimulated	in 1 μl of blood
Healthy control	41 802±2 452	150 789±9 159	12 174±747
Arthritis	168 203±12 815	1 165 603±94 470	40 260±3 325
Arthritis + PIN	190 157±32 580	1 132 747±104 750	44 002±4 398
Arthritis + MTX	98 806±8 136**	664 618±68 695**	29 180±1 903*
Arthritis + PIN + MTX	51 739±3 437++	429 813±45 494++	16 635±2 157++

Mean ± SEM, n = 8-10, *$p<0.05$, **$p<0.01$ (vs Arthritis) ++$p<0.01$ (vs Arthritis + MTX), PMA – 4β-phorbol-12β-myristate-α13-acetate; RLU – relative luminescence units

DISCUSSION

The recently confirmed active involvement of neutrophils in the initiation and development of rheumatoid arthritis (Cascao *et al.* 2009 and 2010; Wright *et al.* 2010) focussed attention on substances which are able to repress the activity of these cells both *in vitro* (Adams *et al.* 2005; Perečko *et al.* 2008; Jančinová *et al.* 2009b) and *in vivo* (Nosáľ *et al.* 2007; Drábiková *et al.* 2009; Jančinová *et al.* 2009a; Rovenský *et al.* 2009; Poništ *et al.* 2010). Elimination of the inflammatory reaction caused by neutrophils and resulting in tissue damage may significantly potentiate the effectiveness of antirheumatic therapy. Moreover, co-application of natural medicines which are able to reduce harmful effects of neutrophils could lower the dosage of antirheumatic drugs and thus decrease their toxicity.

The presented results indicate that pinosylvin may be considered a candidate for the combined therapy of rheumatoid arthritis. This stilbene potentiated the suppressive effect of methotrexate on neutrophil activity and concentration of oxidants in blood. Moreover, the rats treated with the combined therapy showed reduced hind paw swelling in comparison with those treated with methotrexate alone (Bauerová *et al.* 2010). The antiinflammatory activity of methotrexate arises from its ability to inhibit T cell proliferation and cytotoxicity, to decrease recruitment of monocytes and other cells to the inflamed joint and from the increased release of the endogenous anti-inflammatory mediator adenosine (Cronstein 2005). All these alterations could lead to a decreased number and activity of neutrophils and to reduced whole blood chemiluminescence. The interference of pinosylvin with inflammation seems to be related to other mechanisms. As found for resveratrol – a compound structurally similar to pinosylvin, the stilbene-induced downregulation of the inflammatory response involves reduced synthesis and release of pro-inflammatory mediators, modified eicosanoid synthesis, decreased activity of immune cells and suppressed activation of nuclear factor κB – NFκB (Alarcón de la Lastra & Villegas 2005; Khanna *et al.* 2007). Moreover, resveratrol or its oligomeric derivative α-viniferin induced apoptosis of human rheumatoid arthritis synovial cells (Nakayama *et al.* 2010) and suppressed tissue destruction in model arthritis (Lee *et al.* 2004; Elmali *et al.* 2005). Some of these effects have been already observed in the presence of pinosylvin – e.g. reduced production of pro-inflammatory mediators (tumour necrosis factor α, interleukin-8, prostaglandin E_2 and leukotriene B_4) in neutrophils and macrophages as well as the suppressed cyclooxygenase-2 protein and gene expression resulting from inhibition of NFκB activation (Park *et al.* 2004 and 2005; Adams *et al.* 2005; Lee *et al.* 2006). Contrary to *in vitro* experiments (Perečko *et al.* 2008; Jančinová *et al.* 2009b), orally administered pinosylvin did not alter the activity of neutrophils when applied in monotherapy. All the beneficial activities of pinosylvin might intensify the anti-inflammatory activity of methotrexate, although by itself it was not sufficient to induce any detectable changes. This might be due to the lower oral bioavailability of pinosylvin (Roupe *et al.* 2006b) and/or to the period of administration – not sufficient to achieve an effective concentration in neutrophils.

Besides neutrophils and reactive oxygen species, the benefit of pinosylvin applied in combined therapy might involve interference of this stilbene with other inflammatory cells and mediators. Under *in vitro* conditions, pinosylvin decreased the activity of stimulated RAW 264.7 macrophages and reduced the concentration of nitric oxide in the supernatant of these cells. Šmidrkal *et al.* (2010) observed similar effects in murine peritoneal macrophages. In the present study, this inhibition was in parallel with the decreased expression of inducible NO synthase protein, indicating an interference of pinosylvin with nitric oxide formation. The mechanism of this effect may be related to the ability of this stilbene to suppress NFκB activation and iNOS gene expression, documented in cancer cells (Park *et al.* 2005). In contrast to flavonoids (Číž *et al.* 2008), pinosylvin did not possess any NO scavenging activity, as confirmed by amperometrical measurement in cell-free system. Considering the fact that in the presence of neutrophils and superoxide, nitric oxide is transformed into highly reactive peroxynitrite which activates pro-inflammatory signalling (Gao 2010) and contributes to the pathogenesis of arthritis (Abramson 2008), the ability of pinosylvin to decrease the concentration of reactive oxygen and nitrogen species can be considered beneficial and may be involved in the antiinflammatory activity of this prospective natural substance.

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