In vivo effect of pinosylvin and pterostilbene in the animal model of adjuvant arthritis

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Abstract

OBJECTIVE: The aim of this study was to evaluate the effects of pinosylvin (PIN) and pterostilbene (PTE), natural substances from the stilbenoid group, on the development of adjuvant arthritis in rats.

METHODS: Adjuvant arthritis (AA) was induced by a single intradermal injection of *Mycobacterium butyricum* in incomplete Freund´s adjuvant in male Lewis rats. Our experiments included healthy intact animals as reference controls, arthritic animals without any drug administration, and arthritic animals with administration of PIN and PTE in the oral daily dose of 30 mg/kg b.w. The treatment involved administration of the substances tested from day 0, i.e. the day of immunization, to the experimental day 28. The following parameters were monitored: change of the hind paw volume (HPV) on day 14, 21 and 28, luminol-enhanced chemiluminescence (CL) of the joint and myeloperoxidase (MPO) activity in hind paw joint homogenates (day 28).

RESULTS: Arthritic animals treated with PIN showed a decrease in HPV, significantly on days 14 and 28. PIN decreased CL of the joint as well as MPO activity of the joint homogenate, in comparison with untreated animals. PTE had no effect on HPV and MPO activity in hind paw joint homogenates and exerted only a partial effect on luminol-enhanced CL.

CONCLUSIONS: On the basis of our results we conclude that the effect of PTE on CL was only partial. PIN, on the other hand, had a beneficial anti-inflammatory and antioxidant effect on oxidative stress induced biochemical changes occurring in AA, as determined by all three functional parameters.

ORIGINAL ARTICLE

INTRODUCTION

Oxidative stress and inflammation contribute to the pathogenesis of RA in an interactive mode. Neutrophils are implicated to be one of the major origins of oxidative stress in inflammatory diseases. Activation of neutrophils induces generation of ROS and release of enzymes, playing an important role in inflammatory and immune processes involved in many diseases, such as allergies, infections, active RA (Jaswal *et al.* 2003; Cross *et al.* 2006; Jančinová *et al.* 2006; Nosáľ *et al.* 2007; Drábiková *et al.* 2009), pannus-cartilage junction, and joints (Santoz *et al.* 1997; Laragione *et al.* 2007). Several authors reported increased levels of oxidants in whole blood, spleen and joint of rats with adjuvant arthritis as the result of ROS production by activated neutrophils, reflecting both local and systemic inflammatory responses of the organism. These results were in agreement with increased disease markers – HPV and relative spleen mass. Inhibition of oxidant production by natural compounds was however found to limit the development of inflammation in animal models of arthritis (Bauerová *et al.* 2006; Drábiková *et al.* 2007, 2009; Jančinová *et al.* 2007).

AA is a condition that involves systemic oxidative stress. Since the animal models of arthritis have similar pathological characteristics as human RA (such as symmetrical joint involvement, persistant joint inflammation, synovial hyperplasia and a good response to most therapies effective in rheumatoid arthritis), they are used for quantification of disease progress and assignment of suitable pharmacological treatment. (Bina & Wilder 1999).

Pinosylvin (PIN) [3´,5´-dihydroxystilbene] and pterostilbene (PTE) [3,5-dimethoxy-4´-hydroxystilbene] are natural substances from the stilbenoid group, wide-spread in a variety of plants. They are chemically related to resveratrol, which is well known by its antioxidative activity. Structural analogues of resveratrol possess some of the beneficial effects of the parent drug and may provide even further benefits. PIN has been studied for its anticancer, antifungal and antioxidative properties (Roupe *et al.* 2008). Remsberg *et al.* (2006) demonstrated the effect of PTE to scavenge DPPH radical and to decrease blood glucose. The anticancer effect of PTE is also known (Mannal *et al.* 2010). Both substances studied inhibited significantly the chemiluminescence (CL) of whole human blood and the CL of isolated human neutrophils (Perečko *et al.* 2008). Arthritic animals treated with PIN displayed reduction in blood concentration of oxidants and in neutrophil count (Jančinová *et al.* 2010). PTE efficiently protected human erythrocytes against oxidative injury (Mikstacka *et al.* 2010).

The aim of this study was to evaluate the effects of PIN and PTE on the development of adjuvant arthritis in rats.

MATERIAL AND METHODS

PIN and PTE were synthetized in the Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), HTAB (hexadecyl-3-methylamonium bromide), o-dianisidine dihydrochloride and hydrogen peroxide were purchased from Sigma- Aldrich Chemie, (Germany). All other chemicals were purchased from standard commercial sources and were of the highest quality available.

AA was induced in male Lewis rats (Breeding Farm Dobrá Voda, Slovakia), weighing 150–170 g each, by a single intradermal injection of heat-inactivated *Mycobacterium butyricum* in incomplete Freund´s adjuvant. In each experimental group, 9–10 animals were used. Our experiments included healthy intact animals as reference controls, arthritic animals without any drug administration, and arthritic animals with the administration of PIN or PTE in the oral daily dose of 30 mg/kg b.w. The treatment involved administration of the substances tested from day 0, i.e. the day of immunization, to the experimental day 28.

We monitored the clinical parameter of HPV (day 14, 21 and 28). It was calculated as the percentage increase of hind paw edema on day 28 in comparison to the beginning of the experiment (Bauerová *et al.* 2006).

On day 28, CL of the hind paw joint was monitored. ROS generation in this joint (cartilage and soft tissue without bone) was determined by a method of luminol-enhanced CL (Drábiková *et al.* 2007). Briefly, the pieces of approximately 450 mg joint wet weight were dissected. The samples were placed into preoxygenated (95% O_2 and 5% CO_2) physiological saline solution – PSS (in mM: 122 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.25 $CaCl_2$, 15.0 $NaHCO_3$ and 11 glucose, pH 7.4) and trasferred into a cuvette containing 1.0 ml PSS with luminol (final conc. 400 μM) immediately prior to assessment of ROS generation. CL responses were measured at 37 °C and recorded continuously for 10 min in a lumi-aggregometer model 500 (Chrono-log- Corp., USA). The wet weight of samples was recorded at the end of experiment. Data were evaluated as the peak of the CL curve expressed as mV/100 mg wet weight of the joint.

On day 28, MPO activity in the hind paw joint homogenates was measured by the modified method of Deodato *et al.* (1999)**.** Briefly, joint tissue was removed and snap-frozen in liquid nitrogen. To the thawed samples 500 μl of phosphate buffer saline (PBS) solution (137 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4), pH 6.0, with 0.5% hexadecyl-3-methylammonium bromide was added. Each sample was then finely chopped and sonicated (Bandelin electronic Sonopuls) for 30 s at 70% intensity at 4 °C. Subsequently the samples were frozen, thawn and twice more sonicated in the same way. Then the samples were centrifugated for 45 min at 23 000 × g at 4 °C. An aliquot of the supernatant was allowed to react

with 0.167 mg/ml o-dianisidine dihydrochloride and 0.001% H_2O_2 and the rate in absorbance was measured at 450 nm in a microplate spectrophotometer (Labsystems multiscan RC). Data were evaluated as the percentage activity of MPO released in the positive control group, i.e. rats with induced adjuvant arthritis (100%).

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). The data were analyzed by Student´s t-test and $p \leq 0.05$ was taken as significant. The arthritis group was compared with healthy control animals (*). The treated arthritis groups were compared with untreated AA animals (+).

RESULTS

We tested the efficacy of PIN and PTE (30 mg/kg b.w) in reducing the oxidative stress caused by auto-immune inflammatory processes in the rat AA model. On day 14, 21 and 28 after *Mycobacterium butyricum* induced adjuvant arthritis (AA), the clinical parameter – HPV was significantly increased in rats with AA compared with healthy controls. Arthritic animals treated with PIN showed a decrease in HPV, significantly on day 14 and 28. PTE had no effect on HPV (Figure 1).

In the second part of our study, we investigated luminol-enhanced CL of the joint in rats with AA. CL of joint in rats with AA was significantly increased in comparison with controls. PIN reduced significantly on day 28 the CL of the joint of rats with AA in comparison with untreated animals. PTE exerted only a partial effect on CL of the joint (Figure 2).

Further we found a significantly increased MPO activity in the hind paw joint homogenates of rats with AA in comparison with controls. PIN significantly decreased MPO activity in the hind paw joint homogenates of rats with AA in comparison with untreated animals. PTE had no effect on MPO activity in hind paw joint homogenates (Figure 3).

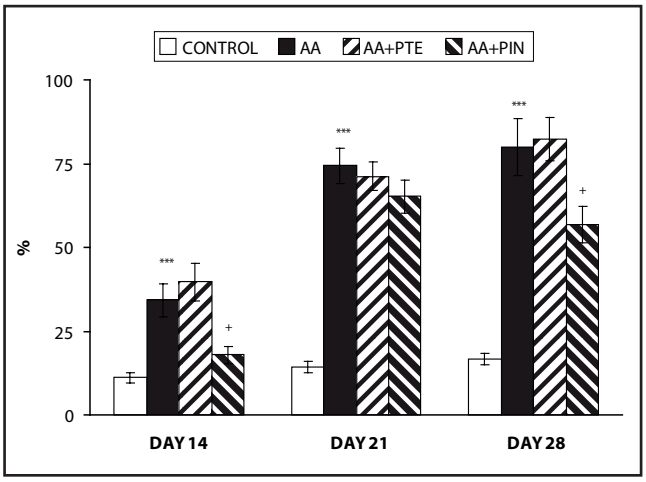

Fig. 1. Effect of PIN and PTE on hind paw volume of arthritic rats. Results are mean ± SEM, n = 9–10 animals/group. *** $p<0.001$ with respect to control healthy animals, $^{+}p<0.05$ with respect to untreated arthritic animals.

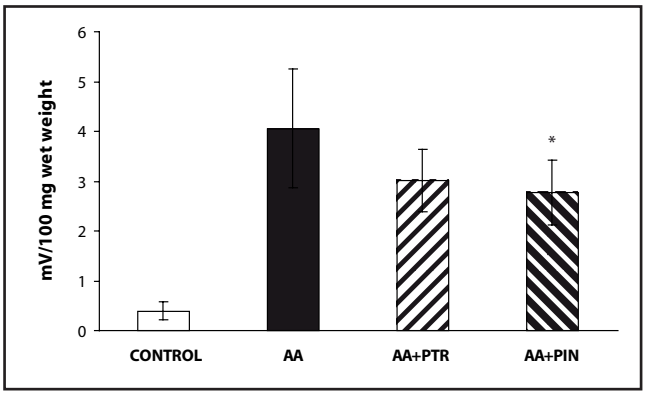

Fig. 2. Effect of PIN and PTE on joint CL of rats with AA. Values represent the mean from 9–10 peaks of CL (mV) ± SEM. $^{*}p<0.05$ with respect to control healthy animals, $^{+}p<0.05$ with respect to untreated arthritic animals.

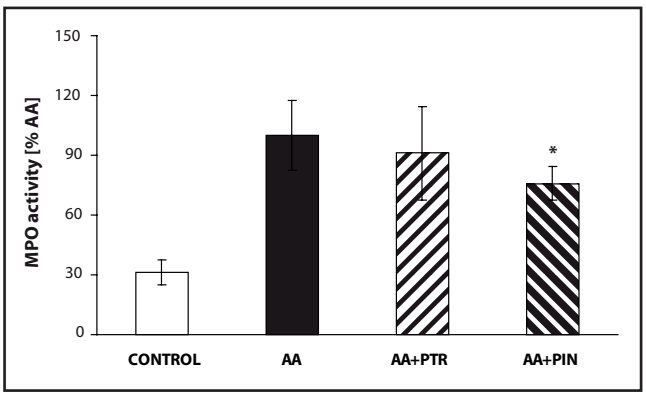

Fig. 3. Activity of MPO in hind paw joint homogenates of arthritic rats treated with PIN and PTE. Results are mean ± SEM, n = 9–10 animals/group. $^{*}p<0.05$ with respect to control healthy animals, $^{+}p<0.05$ with respect to untreated arthritic animals.

DISCUSSION

Oxidative stress has been implicated in various pathological conditions involving several diseases and aging (Valko *et al.* 2007) and as a primary factor involved in the pathogenetic changes during RA (Bauerová and Bezek 1999; Jaswal *et al.* 2003; Bohanec *et al.* 2009). The role of ROS in the etiology and pathogenesis of RA is supported by numerous studies documenting their damaging effects as well as participation in regulation of inflammatory processes (Kunsch *et al.* 2005).

AA is a type of progressive inflammatory polyarthritis displaying many features of human RA, with the prominence of synovial thickening and hypercellularity of numerous inflammatory cells including neutrophils. The animal model has been widely used to investigate pathogenic mechanisms in RA and to evaluate potential new therapeutic agents (Bostan *et al.* 2001; Laragione *et al.* 2007).

Neutrophils are the first cells arriving at the site of inflammation and under chronic inflammation like RA they interact with other cells and by production of ROS

and proinflammatory cytokines they are able to regulate the whole process. In such conditions, the mechanisms of activation and apoptosis are altered, i.e. neutrophils survive longer and release more destructive enzymes (Bauerová & Bezek 1999; Bostan *et al.* 2001; Firestein 2003*;* Fairhust *et al.* 2007; Laragione *et al.* 2007; Filippin *et al.* 2008; Bauerová *et al.* 2009). Modulation of neutrophil activity and survival seem to be very important processes in resolving RA.

With regard to the above facts, we chose PIN and PTE, which are of natural origin and could be beneficial in decreasing of ROS (Roupe *et al.* 2006; Remsberg *et al.* 2008) and thus supporting the treatment of inflammatory diseases. Significant inhibition of human blood CL was reached for PTE and PIN. In intracellular CL of isolated human neutrophils stimulated with PMA, PIN was more effective than PTE, however PTE was more effective than PIN in extracellular CL of isolated human neutrophils stimulated with PMA. None of the substances tested at concentrations of 1–100 μM did affect cell viability, measured by ATP-release test (Perečko *et al.* 2008). Both compounds dose dependently decreased superoxide generation and MPO release from PMA and fMLP activated isolated human neutrophils (Pečivová *et al.* 2010).

In our study, the natural substances PIN and PTE were evaluated by using AA – an animal model of RA, which allows monitoring of the disease processes in the acute phase on days 14–21 (clinical parameter HPV) and in the subchronic phase after day 28 (ROS generation in hind paw joint and MPO activity in hind paw joint homogenates). Bauerová *et al.* (2007) confirmed that clinical parameters, such as HPV and body weight, became significantly modified, with an onset around day 14.

In our experiment, HPV was significantly increased in the arthritic group in comparison with healthy controls, already on day 14, and this increase persisted until the end of the experiment. Arthritic animals treated with PIN in the oral daily dose of 30 mg/kg b.w. showed a decrease in HPV, significantly on days 14 and 28. PTE, in the same dosage, had no effect on HPV.

Luminol-enhanced CL of the joint was used to study the antioxidant action of PIN and PTE.

Along with improvement of the disease parameter – HPV, an inhibitory effect of PIN and PTE on CL of the joints was observed on day 28 after *M. butyricum* administration. PTE had only a partial effect on CL, however PIN significantly decreased CL of the joint.

CL was also used for detection of ROS produced by rat neutrophils. Compared to arthritic controls, animals treated with PIN (28 days, 30 mg/kg) displayed reduction in blood concentration of oxidants. Moreover, the drop in antioxidative plasma activity was less evident in the presence of PIN (Jančinová *et al.* 2010). PTE (28 days, 30 mg/kg) given to rats with AA nonsignificantly decreased the production of oxidants in blood, except day 28 (Perečko *et al.* 2010).

The development of AA in rats was accompanied also with an increase in blood neutrophil count when compared with control animals (Nosáľ *et al.* 2007; Drábiková *et al.* 2009). PIN and PTE decreased the number of neutrophils in whole blood in rats with AA (Jančinová *et al.* 2010; Perečko *et al.* 2010).

MPO, the most abundant enzyme in neutrophils, is a major NO scavenger and marker of oxidative stress (Brennan & Hazen 2003). MPO enhances the binding of leukocytes, including monocytes and neutrophil, to the endothelium (Johansson *et al.* 1997). Vascular endothelial cells are also capable of secreting various cytokines, which are potent chemoattractants for neutrophils. This both MPO and cytokines participate in the recruitment of cells into the area of inflammation. Lefkowitz *et al.* (1999) reported that MPO may be an important mediator in the inflammatory response.

We investigated MPO activity in the hind paw joint homogenate. Arthritic animals treated with PIN in the oral daily dose of 30 mg/kg b.w. showed a significantly decreased MPO activity on day 28. PTE in the same dose had no effect on MPO activity.

Our results correspond with the decreasing activity of gamma-glutamyltransferase in joint tissue on day 28.The arthrogram was modified by PIN administration on all days monitored (14,21,28), but PTE was without effect. Both compounds decreased the immunological marker MCP-1 plasmatic levels as found on day 14, yet the effect was significant only for PIN (Mihálová *et al.* 2010).

The obtained results proved PIN to be more effective than PTE in reducing the markers of oxidative stress in AA. In our experimental setting, the reduction of oxidative stress in arthritis correlated with the clinical manifestations of the disease. Inhibition of the increase in HPV was associated with inhibition of neutrophil infiltration, as assessed by MPO, and thus with lower tissue destruction. The daily oral administration of PIN effectively inhibited the increase of HPV, MPO activity measured in the joint homogenate and CL of the joint. The HPV – a local inflammatory parameter, gave very good association with MPO activity, measured in joint homogenate, and with CL of the joint. Concluding, these parameters may be a marker for AA and RA development both at the systemic and local level. The new information on the inhibitory effect of PIN and PTE on the HPV, ROS and MPO activity suggest that the protective effect of PIN may be beneficial in controlling inflammation.

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