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Attenuation of interleukin-1β secretion in mice by post-inflammatory treatment with azithromycin and clarithromycin

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ABSTRACT

The literature indicates that, in addition to anti-bacterial effects, certain macrolide antibiotics, such as azithromycin and clarithromycin, also exert anti-inflammatory and immunomodulatory activity, by accumulating in inflammatory cells and inhibiting the secretion of pro-inflammatory cytokines. Previous reports have shown that pre-treatment with these macrolides attenuates induced immediate skin inflammatory reactions in mice. However, the anti-inflammatory activity of the post-inflammatory induction of macrolide antibiotics in this experimental model has not been investigated. The aim of this study was to explore whether the anti-inflammatory activity of azithromycin and clarithromycin, applied transdermally, 30 min after a phorbol 12-myristate 13-acetate (PMA)-induced immediate skin inflammation in mice, could attenuate a Th1 inflammatory reaction. The capacity of azithromycin and clarithromycin (500 μg/ear) to exert anti-inflammatory effects, similar to those of dexamethasone (50 μg/ear), was confirmed by the inhibition by all three agents of ear swelling and interleukin-1β concentration in the ear tissue of PMA-treated mice, supporting this clinically relevant treatment mode.

Key words: anti-inflammatory activity, IL-1β, macrolide antibiotic, mouse, skin inflammation

Introduction

Macrolide antibiotics are a commonly used class of antimicrobial agents, isolated from Streptomycetes, and have been used for decades in the treatment of moderate to severe bacterial infections (ZHANEL et al., 2001). Published results from *in vitro* and *in vivo* experiments demonstrate that, in addition to their antibacterial effects, certain macrolide antibiotics also exert anti-inflammatory and immunomodulatory activity (CAREVIĆ and DJOKIĆ, 1988; ČULIĆ et al., 2001; IVETIĆ TKALČEVIĆ et al., 2006; 2008; BOSNAR et al., 2009). These macrolides accumulate in inflammatory cells, especially neutrophils and

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macrophages (GLADUE et al., 1989; WILDFEUER et al., 1996), inhibiting the synthesis of reactive oxygen species and the secretion of cytokines and chemokines (KHAN et al., 1999; IANARO et al., 2000; TERAO et al., 2003).

At the moment, corticosteroids are very frequently used as anti-inflammatory substances for chronic inflammatory conditions, since they display excellent anti-inflammatory activity. However, the side effects observed, such as metabolic disorders, increased susceptibility to infection, myopathy, ecchymosis, behavioral disturbances and skin atrophy (SCHACKE et al., 2002), following long-term treatment, restricts the application of corticosteroids in chronic inflammatory disorders. Therefore, different chemical entities with anti-inflammatory effects, such as macrolides, are being extensively investigated.

A recently published report on the molecular and cellular mechanism of inflammatory reaction in phorbol 12-myristate 13-acetate (PMA) induced skin inflammation, showed that topically applied PMA triggers Th1-mediated inflammatory reaction, with the increased secretion of interleukin (IL)-1β and tumor necrosis factor (TNF)-α in ear tissue (IVETIĆ TKALČEVIĆ et al., in press). In the same report, azithromycin and clarithromycin exerted anti-inflammatory effects by decreasing the concentration of Th1 cytokines. Similarly, several reports have shown that oral and transdermal macrolides inhibit immediate skin inflammation, observed as ear and paw oedema, induced by different inflammatory agents, such as croton oil, cantharidin, oxazolone, picryl chloride and carragenin (TARAYRE et al., 1987; AGEN et al., 1993; SCAGLIONE and ROSSONI, 1998). However, in these experimental models, macrolides were administered as a preventive treatment before the inflammation was induced.

Since treatment with macrolides after inflammation induction seems to be effective in oxazolone (OXA)-induced allergic skin inflammatory reactions (IVETIĆ TKALČEVIĆ et al., in press), we aimed in this study to investigate whether the anti-inflammatory effects of transdermally applied azithromycin and clarithromycin administered after inflammation induction would also attenuate Th1-mediated PMA-induced immediate skin inflammation, and to compare it to the effects of standard anti-inflammatory corticosteroid dexamethasone.

Materials and methods

Study design. PMA induction of immediate skin inflammation was conducted according to IVETIĆ TKALČEVIĆ et al. (in press) with some modifications. Animals were anaesthetized by the inhalation of Isoflurane, delivered in an anaesthesia induction chamber (Stoelting Co., USA). Gas scavenging was provided using the Fluovac 240V system (International Market Supply, England). The fully anaesthetized animal was topically treated on the inner surface of both ears with 12 μL of 0.01% PMA solution in

acetone. Negative control animals were treated with the same volume of acetone. Thirty minutes after inflammation induction, azithromycin (500 μg/15 μL/ear), clarithromycin (500 μg/15 μL/ear) and dexamethasone (50 μg/15 μL/ear) dissolved in a trans-phase delivery system (TPDS) (acetone : isopropanol : benzyl alcohol = 40% : 50% : 10%) were transdermally applied to the inner surface of both ears. The negative and positive controls were treated with the same volume of TPDS vehicle. Six hours after PMA administration, animals were anaesthetized as already described and, using a digital caliper (Mitutoyo IP63; type 209-530, Japan), connected to the measuring system Plexx (Netherlands) compound of the computer programme "Windows Hyper Terminal" and a pedal, the ear thickness of each animal was measured. In order to determine IL-1β concentration in ear tissue, the animals were then euthanized using intraperitoneal Thiopental injections, 8 hours after inflammation induction. Ear tissue was collected by cutting off both ears at the base. For the ELISA analysis, both ears of each animal were transferred into Eppendorf tubes and frozen at -20 °C.

Animals. PMA-induced skin inflammation was performed on male CD1 mice, 15 weeks old, obtained from Charles River, France. Eight mice per group were kept on wire mesh floors with irradiated maize granulate bedding (Scobis Due, Mucedola, Italy) and maintained under standard laboratory conditions (temperature 23 - 24 °C, relative humidity 60 ± 5%, approx. 15 air changes per hour, artificial lighting with a circadian cycle of 12 h). Food (Standard food for mice and rats, 4R21, Mucedola, Italy) and tap water were provided *ad libitum*. Mice were allowed to acclimatize for 10 days before the beginning of each experiment.

All procedures on the animals were performed in accordance with (a) the EEC Council Directive 86/609 of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes; and (b) the Constitution of the Republic Croatia, Animal Welfare Act, OG 135/06 of 13 December 2006.

Materials: drugs, chemicals and antibodies. Azithromycin was purchased from PLIVA Inc., Croatia, and clarithromycin and dexamethasone from Sigma Chemical Co., USA. PMA was obtained from Alexis biochemicals, USA. The inhalation anaesthetic Isoflourane was purchased from Forane, Abbott Laboratories Ltd., UK while Thiopental used for the euthanasia of experimental animals was from Nycomed, Germany. Organic diluents (acetone, isopropanol and benzyl alcohol) were from Kemika, Croatia. IL-1β antibodies for enzyme-linked immunosorbent assay (ELISA) were from R&D Systems, USA. All other reagents were from Sigma Chemical Co., USA.

ELISA analysis. The concentration of IL-1β in ear tissue was determined by sandwich ELISA, using capture and detection antibodies, according to the manufacturer's recommendations. Prior to analysis, both ears from the same animal were homogenized

in 1 mL of phosphate buffered saline (PBS), to which protease inhibitors were added (pepstatin A, phenylmethanesulfonyl fluoride, leupeptin and aprotinin). The homogenate was centrifuged (10000 ×g) for 10 min at 4 °C. The resulting supernatant was then diluted 10-fold. The final concentrations were expressed as pg/mg of total protein in the ear tissue previously determined by biochemical analyzer (Olympus AU 400, UK).

Data analysis and statistical evaluation. Statistical analysis was conducted using GraphPad InStat v. 4.0 (GraphPad Software Inc., USA). A P-value <0.05 was regarded as significant. For multiple comparisons, Kruskal-Wallis ANOVA with Dunn post-tests were used.

Results

Topically applied PMA induced ear swelling 6 hours after administration (Fig. 1). Treatment with azithromycin and clarithromycin at 500 µg/ear, and dexamethasone at 50 µg/ear after inflammation induction significantly reduced ear thickness (Fig. 1). There was no marked difference in the anti-inflammatory effects of the tested macrolides and dexamethasone.

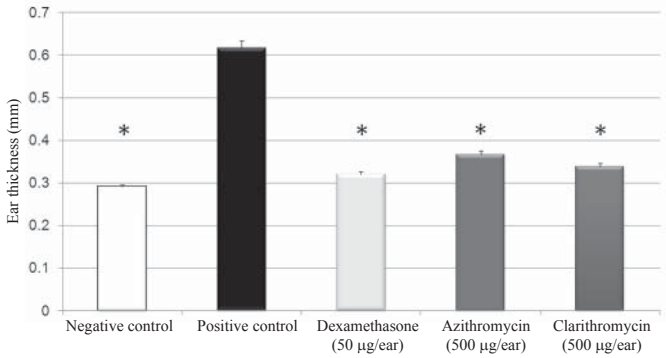

Fig. 1. Ear thickness 6 hours after topical PMA administration. Dexamethasone, azithromycin and clarithromycin were applied transdermally 30 min after inflammation induction. Data are presented as group averages ± SEM. The symbol * indicates significant divergence from the positive control (P<0.05, Kruskal-Wallis ANOVA with Dunn post-tests, n = 8).

As previously reported (IVETIĆ TKALČEVIĆ et al., in press), the concentration of IL-1β in ear tissue reaches its peak 8 hours after PMA application. Therefore the investigation of the effects of azithromycin, clarithromycin and dexamethasone on PMA-induced IL-1β production was limited to that time frame. Like dexamethasone (50 µg/ear), both tested macrolides (500 µg/ear) applied 30 min after inflammation induction significantly reduced IL-1β concentration in ear tissue (Fig. 2).

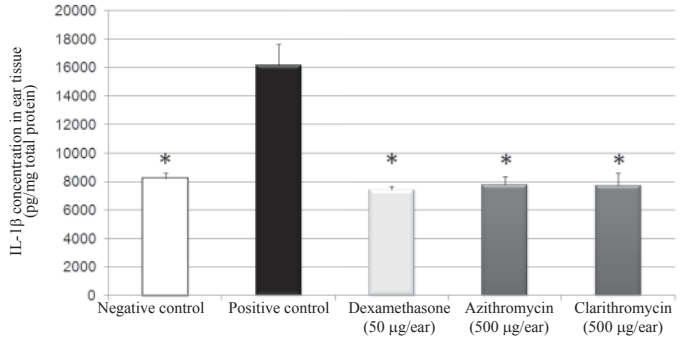

Fig. 2. Effects of dexamethasone, azithromycin and clarithromycin on interleukin (IL)-1β concentration in mouse ear tissue 8 hours after topical PMA administration. Substances were administered transdermally 30 min after inflammation induction. Data are presented as group averages ± SEM. The symbol * indicates significant divergence from positive control ($P<0.05$, Kruskal-Wallis ANOVA with Dunn post-tests, n = 8).

Discussion

In this study of mouse PMA-induced immediate skin inflammation, we initially confirmed that PMA induces Th1 inflammatory reaction. Like dexamethasone, transdermally applied macrolides, azithromycin and clarithromycin, inhibit acute inflammatory responses, including ear swelling and IL-1β production, when administered after inflammation induction.

Earlier investigations have indicated the anti-inflammatory ability of commercially available macrolide antibiotics to attenuate ear and paw oedema induced by different inflammatory agents when administered before inflammation induction. TARAYRE et al. (1987) have shown that a single dose of transdermally applied erythromycin (0.250 - 1 mg/ear) markedly decreases ear oedema induced by croton oil, cantharidin and picryl-chloride. In the experiment performed by AGEN et al. (1993) a single oral dose of roxithromycin at 5 and 20 mg/kg significantly reduced carrageenin and poly-L-arginin induced paw oedema and croton oil induced ear oedema. Also, SCAGLIONE and ROSSONI (1998) have indicated that a single oral pretreatment with roxithromycin, azithromycin and clarithromycin at 20 mg/kg markedly inhibits carragenin induced paw oedema.

Our group recently demonstrated the ability of standard macrolide antibiotics to attenuate allergic inflammatory skin reactions when administered after induction. In contrast to the inflammatory scheme, post-inflammatory treatment with azithromycin and clarithromycin (both at 2 mg/ear) reduced inflammatory cell accumulation and IL-4 concentration in ear tissue in OXA-induced delayed type hypersensitivity (DTH) in mice (IVETIĆ TKALČEVIĆ et al., in press).

Although the mechanism of macrolide anti-inflammatory activity remains unknown, the results from different *in vivo* models demonstrate that they inhibit Th1 mediated inflammation by reducing the secretion of pro-inflammatory cytokines such as IL-1β, interferon (IFN)-γ, TNF-α and granulocyte monocyte colony-stimulating factor (GM-CSF) (IVETIĆ TKALČEVIĆ et al., 2006; 2008; BOSNAR et al., 2009).

The epicutaneous application of PMA results in vascular leakage, leukocyte infiltration, epidermal hyperplasia, activation of protein kinase C and the increased release of arachidonic acid and its metabolites (YOUNG and DE YOUNG, 1989). In a mouse model of PMA-induced ear oedema, topically applied PMA induced the accumulation of predominantly polymorphonuclear (PMNL) and few mononuclear cells in ear tissue, which are the major sources of pro-inflammatory Th1 cytokines IL-1β and TNF-α (IVETIĆ TKALČEVIĆ et al., in press). Macroscopically, inflammation is observed as ear swelling. Since PMA induces a local skin inflammatory reaction which, in terms of the accumulation of neutrophils, resembles psoriasis (DeYOUNG et al., 1989; FRETLAND et al., 1990), in our experiment azithromycin and clarithromycin were administered transdermally after inflammation induction, in order to investigate whether macrolides applied during the treatment regimen described had anti-inflammatory effects on such skin inflammatory reactions.

Administered before inflammation induction, at 500 μg/ear, azithromycin and clarithromycin attenuated PMA-induced inflammatory cell accumulation and thus decreased ear IL-1β and TNF-α concentration and ear swelling (IVETIĆ TKALČEVIĆ et al., in press). Since treatment with azithromycin and clarithromycin after inflammation induction in our experiment reduced ear swelling and IL-1β concentration in ear tissue, and since accumulated PMNL cells are the cellular source of IL-1β (IVETIĆ TKALČEVIĆ et al., in press), it can be presumed that azithromycin and clarithromycin administered after inflammation induction inhibited the accumulation of PMNL cells in ear tissue and thereby reduced IL-1β concentration, along with ear swelling. Interestingly, in comparison to the activity observed after the preventive treatment (IVETIĆ TKALČEVIĆ et al., in press), macrolide anti-inflammatory treatment after inducing inflammation was more similar to the effects of dexamethasone in terms of its activity on IL-1β secretion and ear swelling. This result is in accordance with previous observations made in an OXA-induced DTH model, in which azithromycin and clarithromycin demonstrated more potent anti-inflammatory activity on cellular accumulation and cytokine secretion when administered after an OXA challenge (IVETIĆ TKALČEVIĆ et al., in press).

Conclusion

It can be concluded that, in addition to their effects on allergic skin reaction, the anti-inflammatory activity of commercially available macrolide antibiotics, administered after

the induction of inflammatory reaction, also attenuates immediate skin inflammation by inhibiting Th1-mediated inflammatory processes. There is no significant difference in the anti-inflammatory activity of the tested doses of macrolides and dexamethasone after post-inflammatory treatment in PMA-induced immediate skin inflammation. This finding encourages the further testing of macrolide anti-inflammatory activity in a clinically relevant treatment mode.

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IVETIĆ TKALČEVIĆ, V., V. ERAKOVIĆ HABER: Smanjeno oslobađanje interleukina-1β u miševa nakon poslijeupalnog davanja azitromicina i klaritromicina. Vet. arhiv 82, 201-209, 2012.

SAŽETAK

Literaturni podaci pokazuju da odgovarajući makrolidni antibiotici, poput azitromicina i klaritromicina, osim protubakterijskih učinaka posjeduju i protuupalna i imunomodulacijska svojstva koja se očituju njihovim nakupljanjem u upalnim stanicama i sprječavanjem oslobađanja proupalnih citokina. Dosadašnja istraživanja pokazuju da davanje navedenih makrolida u miševa, prije izazivanja upale, umanjuje neposrednu upalnu reakciju kože. Međutim, njihova protuupalna svojstva nakon poslijeupalnog davanja u ovom pokusnom modelu nisu istražena. Cilj ovog istraživanja bio je istražiti da li azitromicin i klaritromicin, naneseni na površinu uške miševa 30 minuta nakon izazivanja neposredne upale kože s forbol 12-miristat 13-acetatom (PMA), svojim protuupalnim djelovanjem umanjuju Th1 upalni odgovor. Protuupalni učinci azitromicina i klaritromicina (500 μg / uški) bili su podjednaki protuupalnom djelovanju deksametazona (50 μg / uški), u smislu smanjenja otekline uške i koncentracije interleukina-1β u tkivu uške miševa obrađivanih s PMA-om, što ide u prilog klinički relevantnom načinu liječenja.

Ključne riječi: protuupalno djelovanje, IL-1β, makrolidni antibiotici, miš, upala kože