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Anti-inflammatory effects of nortrachelogenin in murine J774 macrophages and in carrageenan-induced paw edema model in the mouse

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Abstract

Nortrachelogenin is a pharmacologically active lignan found in knot extracts of *Pinus sylvestris*. In previous studies, some lignans have been shown to have anti-inflammatory properties which made nortrachelogenin an interesting candidate for our study. In inflammation, bacterial products and cytokines induce the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1). These enzymes synthesize factors which, together with proinflammatory cytokines, are important mediators and drug targets in inflammatory diseases.

The effects of nortrachelogenin on the expression of iNOS, COX-2 and mPGES-1 as well as on the production of nitric oxide (NO), prostaglandin E_2 (PGE_2) and cytokines IL-6 and MCP-1 were investigated in murine J774 macrophage cell line. In addition, we examined the effect of nortrachelogenin on carrageenan-induced paw inflammation in mice.

Interestingly, nortrachelogenin reduced carrageenan-induced paw inflammation in mice and inhibited the production of inflammatory factors NO, PGE_2, IL-6 and MCP-1 in J774 macrophages *in vitro*. Nortrachelogenin inhibited mPGES-1 protein expression but had no effect on COX-2 protein levels. Nortrachelogenin had also a clear inhibitory effect on iNOS protein expression but none on iNOS mRNA levels, and the proteasome inhibitor lactacystin reversed the effect of nortrachelogenin on iNOS expression suggesting a post-transcriptional mechanism of action. The results revealed hitherto unknown anti-inflammatory properties of nortrachelogenin which could be utilized in the development of anti-inflammatory treatments.

Keywords: nortrachelogenin, lignan, iNOS, inflammation, *Pinus sylvestris,* Pinaceae

Abbreviations: COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; IL-6, interleukin-6; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; mPGES-1, microsomal prostaglandin E synthase-1; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; XTT, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate

Introduction

Inflammation is a complex and tightly regulated reaction to injurious, irritating or pathogenic factors. Its purpose is to eliminate these harmful factors and to induce the regenerative processes to repair the tissue damage. In chronic inflammatory diseases or autoimmune disorders, the inappropriate, prolonged and/or poorly coordinated inflammatory response results in the symptoms and signs observed in patients. Excessively increased activation of inflammatory and immune cells leads to over-production of inflammatory cytokines and other factors involved in inflammation which cause harm to the host and, therefore, serve as powerful targets for anti-inflammatory treatments.[1]

Nitric oxide (NO) and prostaglandins (PGs) play an important role in the generation of the inflammatory response as well as in regulating several physiological responses including vascular tone and blood clotting [2,3]. Their biosynthesis is significantly increased in inflamed tissue, and they contribute to the development of the cardinal signs of acute inflammation.

The first PGE_2 synthase, namely mPGES-1, was identified 1999 by Jakobsson et al [4]. It is induced by many proinflammatory cytokines and it seems to have a role in the pathophysiology of several diseases. mPGES-1 is a potential new target for the drug development especially for inflammatory diseases. [5]

Nitric oxide (NO) is a small gaseous signalling molecule which acts as a regulatory and effector molecule in inflammation. NO is produced by three different forms of NOS, namely neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). iNOS expression is induced in inflammatory and tissue cells in response to bacterial products and inflammatory cytokines, and it is responsible for prolonged production of NO in high concentrations [2]. In animal studies iNOS inhibitors have had

beneficial effects in several models of acute and chronic inflammation [6]. However, the results in clinical studies have not been so promising [7]. Nevertheless, it is evident that NO plays an immunoregulatory role in the induction and resolution of inflammation in a concentration dependent manner, and it is likely that NO is functionally relevant to host defence. [7-9]

Interleukin-6 (IL-6) is a 184 amino acid glycosylated protein that mediates inflammation, immune response and hematopoiesis via specific receptor, IL-6R. Because of wide range of biological activities and pathological role of IL-6 in several diseases, targeting IL-6 in drug development has become important. Tocilizumab, a humanized anti-IL-6R monoclonal antibody is in the market for the treatment of rheumatoid arthritis in more than 100 countries. [10] Besides cytokines also chemokines are released in the early phase of inflammation. Monocyte chemoattractant protein-1 (MCP-1) is a central chemokine in the inflammatory response that serves as a chemoattractant for monocytes and macrophages, and plays key roles in many immune processes. [11]

The lignans are a group of polyphenolic compounds found in plants. They are basically formed by oxidative coupling of two phenylpropane units. Coniferous trees present a rich source of lignans. [12] Considerable health benefits have been implicated with lignan-rich diet, including prevention of cancers and cardiovascular diseases [13,14]. In previous studies, some lignans have shown to have anti-inflammatory properties [15] which made nortrachelogenin (Fig. 1) an interesting candidate for our study.

In our preliminary search of bioactive compounds from knot extracts of *Pinus sylvestris*, the anti-inflammatory compound nortrachelogenin was identified. Nortrachelogenin is a pharmacologically active lignan which was first isolated from *Wikstroemia indica* in 1979 by Kato et al [16]. In addition, it is found in several other resources from nature like *Carissa spinarum*, *Daphne oleoides*, *Juniperus*

rigida and *Trachelospermum jasminoides* [17-20]. According to our knowledge, only a limited amount of data is available on the biological or pharmacological effects of nortrachelogenin *in vitro* and *in vivo*. In previous studies, nortrachelogenin has been shown to have anti-plasmoidial activity *in vitro* and antileukemic properties *in vivo* but it did not inhibit the growth of the DMBA-induced mammary tumors in rats [21-23]. It was also moderately active against HIV-1 *in vitro* [24]. In a recent study, nortrachelogenin was shown to enhance tumor necrosis factor related apoptosis-inducing ligand and to inhibit Akt signalling [25] but its anti-inflammatory potential remains practically unexplored.

In the presence study, we investigated the anti-inflammatory properties of nortrachelogenin in activated macrophages *in vitro* by measuring its effects on the expression of inflammatory enzymes iNOS, COX-2 and mPGES-1 and on the production of inflammatory mediators NO, PGE_2, IL-6 and MCP-1. Further, we were interested if the anti-inflammatory properties found in those *in vitro* studies are also translated to the *in vivo* situation and measured the effects of nortrachelogenin on carrageenan-induced paw inflammation in the mouse.

Results

NO production and iNOS expression were not detectable in resting J774 macrophages. When the cells were activated through Toll-like receptor 4 (TLR4) pathway by exposing them to bacterial lipopolysaccharide (LPS), iNOS expression and NO production were significantly enhanced. Nortrachelogenin decreased iNOS protein expression and NO production in a dose-dependent manner (Fig. 2A,B). Reduction of iNOS protein levels was about 50 % at 1 µM concentration and over 90 % at 30 µM concentration (EC50 value 1 µM). Nortrachelogenin inhibited also NO production but the effect was smaller: 49 % inhibition was found when nortrachelogenin was given to the cells at 30 µM concentration. The effect was not stimulus-specific as nortrachelogenin (30 µM) also inhibited NO production induced by interferon gamma (IFN-γ) alone or in combination with interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) ($p<0.01$) (Fig. 2C,D).

Next, we examined the effects of nortrachelogenin on iNOS mRNA levels. J774 cells were cultured with LPS in the presence and absence of nortrachelogenin and mRNA levels at time points 3 h, 6 h, 12 h and 24 h were measured. The maximal iNOS mRNA levels were found following 6 h incubation, and thereafter the mRNA levels decreased rapidly. Nortrachelogenin had no effect on iNOS mRNA levels at any time point measured (Fig. 3) suggesting that the effect of nortrachelogenin on iNOS protein expression (and subsequent NO production) is mediated trough post-transcriptional mechanisms.

There is previous evidence showing that iNOS protein is degraded by the proteasome pathway and some pharmacological compounds enhance that effect [26]. Therefore we investigated the effect of nortrachelogenin on LPS-induced iNOS expression in the presence of the proteasome inhibitor

lactacystin [27,28]. Lactacystin was added to the cells after 8 h incubation with LPS or with the combination of LPS and nortrachelogenin. As expected, iNOS protein levels measured after 24 h incubation were higher in the cells treated with LPS and lactacystin than in cells treated with LPS only (Fig. 4). Interestingly, nortrachelogenin had no effect on iNOS protein levels in LPS treated macrophages in the presence of lactacystin while it significantly inhibited iNOS expression in the absence of the proteasome inhibitor. The findings sustained our hypothesis that nortrachelogenin is likely to inhibit iNOS expression and inducible NO production by enhancing iNOS protein degradation through proteasome.

To find out whether nortrachelogenin has effects on other important inflammatory mediators we investigated its effects on the production of chemokine MCP-1 and proinflammatory cytokine IL-6 (Fig. 5) in J774 macrophages activated with LPS. Nortrachelogenin reduced both MCP-1 and IL-6 production in a dose-dependent manner with EC50 values 7 µM for MCP-1 and 25 µM for IL-6, respectively. The highest concentration used (30 µM) caused about 60 % inhibition on MCP-1 and 55 % inhibition on IL-6 production.

We also studied the effects of nortrachelogenin on the expression of COX-2 and mPGES-1 in LPS-stimulated J774 macrophages. Interestingly, nortrachelogenin reduced mPGES-1 protein levels and, accordingly, inhibited the synthesis of its product PGE_2 (EC50 values 14 µM for mPGES-1 and 17 µM for PGE_2). However, nortrachelogenin had no effect on COX-2 expression (Fig. 6).

As nortrachelogenin proved to have anti-inflammatory effects *in vitro*, we wanted to investigate if those effects are also translated to *in vivo* and studied the effects of nortrachelogenin in carrageenan-induced paw inflammation in the mouse. Intraperitoneal administration of nortrachelogenin (100 mg/kg) reduced carrageenan-induced paw edema at 3 h by 53 % and at 6 h by 50 % as seen in Figure

7, whereas the known anti-inflammatory glucocorticoid dexamethasone (2 mg/kg) decreased carrageenan-induced paw inflammation by about 80 %.

Discussion

In the present study, we evaluated anti-inflammatory effects of nortrachelogenin, a lignan ingredient found in knot extracts of *Pinus sylvestris*. The results showed that nortrachelogenin suppressed iNOS expression and NO production by enhancing iNOS protein degradation through proteasome pathway. Nortrachelogenin decreased also the production of pro-inflammatory factors PGE_2, IL-6 and MCP-1 in J774 macrophage cell line. More importantly, nortrachelogenin inhibited significantly carrageenan-induced paw edema in the mouse. To our knowledge this is the first report showing that nortrachelogenin has anti-inflammatory effects; and they were evident both *in vitro* and *in vivo*.

Carrageenan-induced paw edema is a commonly used model in inflammation research representing features of acute inflammation and innate immunity. Carrageenan-induced inflammation is mediated by inflammatory cells especially macrophages and neutrophils. The development of edema is described as a biphasic event [29]. The later phase (3-6 h after carrageenan injection) is strongly associated with increased expression of iNOS and COX-2 and local production of nitric oxide and prostaglandins. During the later phase IL-6, IL-1β, TNF-α and MCP-1 levels are also enhanced [30,31]. Recently it has been shown that also transient receptor potential ankyrin 1 (TRPA1) mediates carrageenan-induced inflammation as carrageenan-induced response was found to be attenuated in TRPA1 deficient mice. [32]

Nortrachelogenin decreased carrageenan-induced paw edema possibly by reducing cytokine formation and by down-regulating iNOS / NO and mPGES-1 / PGE_2 pathways as was shown in the macrophage cell model. The response of nortrachelogenin was parallel to that of the control compound dexamethasone which is a very efficacious anti-inflammatory drug that also inhibits iNOS expression [33]; however, dexamethasone was more potent than nortrachelogenin and it was used at a clearly lower dose. Interestingly, also selective iNOS inhibitors have been shown to be very effective in carrageenan-induced paw edema model [34]. Nortrachelogenin also inhibited MCP-1 production. This could lead to decreased amount of inflammatory cells to be recruited to the inflammation site.

Most known iNOS suppressing compounds regulate iNOS expression at the transcriptional level but some compounds, e.g. PPARα agonists, natural compound curcumin and lignan compound arctigenin have been reported to promote degradation of iNOS protein through proteasome pathway [26,35,36]. In the present study, nortrachelogenin had no effect on iNOS mRNA levels but, interestingly, the proteasome inhibitor lactacystin reversed the effect of nortrachelogenin on iNOS protein expression. Accordingly, when measured at the same time point (after 24h incubation), nortrachelogenin had a greater inhibitory effect on iNOS protein levels than on nitrite levels (the latter reflecting the cumulative NO production during the entire incubation). These data together support the idea that nortrachelogenin enhances iNOS protein degradation through proteasome pathway leading to reduced iNOS levels and suppressed NO production. Additional studies are needed to clarify in further detail which proteasome subcomponents are targets of nortrachelogenin. At least 26S and 20S have been reported to be important for the degradation of iNOS [27,28].

Lignans are plant polyphenols traditionally classified into two types, classical lignans and neolignans. Classical lignans are formed from two phenylpropanes linked in a β-β' (8–8') fashion, while neolignans are those dimers whose coupling patterns differ from β-β'linkage. Nortrachelogenin is a classical lignan related to enterolactone also grouped to dibenzylbutyrolactone lignans. [37] Formerly it has been shown that nortrachelogenin has a moderate inhibitory effect on TNF-α production in peripheral blood stimulated with LPS but in the same study the effect on IL-1 biosynthesis was inconsistent [19]. Bis-5,5-nortrachelogenin has also been shown to inhibit NO production in RAW 264.7 murine macrophage-like cell line [38]. The present study extends the previous knowledge by showing that nortrachelogenin has anti-inflammatory properties *in vivo* and inhibits iNOS and mPGES-1 expression and NO, PGE_2, IL-6 and MCP-1 production in activated macrophages *in vitro*. In a previous study of During et al. [39] pinoresinol had strongest anti-inflammatory properties of six studied lignans i.e. secoisolariciresinoldiglucoside, secoisolariciresinol, pinoresinol, lariciresinol, matairesinol and hydroxymatairesinol; pinoresinol was found to reduce IL-6 and MCP-1 production in Caco-2 cells stimulated with IL-1β with IC50 values of 12.5 μM and 100 μM, respectively [39]. Those findings support the results of our study, even though pinoresinol has a different furofuran structure than nortrachelogenin and it is known to convert to enterolactone in the colon [40].

In conclusion, we showed here, for the first time, that nortrachelogenin has anti-inflammatory properties *in vitro* and *in vivo* by down-regulating inflammatory gene expression in macrophages and by attenuating the carrageenan-induced paw edema in the mouse, likely through multiple independent or cross-talking mechanisms. Nortrachelogenin is a promising new anti-inflammatory compound for interfering iNOS and mPGES-1 expression and cytokine production in various inflammatory conditions.

Materials and Methods

Materials

Nortrachelogenin (purity > 95%) was purchased from Arbonova and dexamethasone (purity > 97%) from Sigma Chemical Co. Rabbit polyclonal iNOS (sc-650), COX-2 (sc-1745) and β-actin (sc-1615-R) antibodies and HPR-conjugated goat polyclonal anti-rabbit antibody and donkey polyclonal anti-goat antibody were purchased from Santa Cruz Biotechnology Inc and rabbit polyclonal mPGES-1 (AS03031) from Agrisera. All other reagents were from Sigma Chemical Co unless otherwise stated.

Cell culture

Murine J774 macrophages (American Type Culture Collection) were cultured at 37° C in 5% CO_2 atmosphere and grown in Dulbecco's Modified Eagle's Medium (DMEM) with glutamax-I containing 10 % heat-inactivated foetal bovine serum, penicillin (100 units/mL), streptomycin (100 µg/mL) and amphotericin B (250 ng/mL) (Invitrogen). Cells were seeded on 96 well plates for XTT-test, and on 24 well plates to measure NO, PGE_2, MCP-1 and IL-6 production or iNOS, mPGES-1 and COX-2 expression. Cell monolayers were grown for 72 h to confluence before the experiments were started and the compounds of interest were added in fresh culture medium. Cytotoxicity of nortrachelogenin was ruled out by measuring cell viability using Cell Proliferation Kit II (Roche Diagnostics).

Nitrite Assays

NO production was determined by measuring the accumulation of nitrite, a stable metabolite of NO in aqueous milieu, by Griess reaction [41].

Western Blot Analysis

At the indicated time points, cells were rapidly washed with ice-cold phosphate-buffered saline (PBS) and solubilized in cold lysis buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodiumorthovanadate, 20 µg/mL leupeptin, 50 µg/mL aprotinin, 5 mM sodium fluoride, 2 mM sodium pyrophosphate and 10 µM *n*-octyl-β-D-glucopyranoside. After incubation for 15 min on ice, lysates were centrifuged (12,000 g, 4° C for 10 min), and supernatants were collected and stored in SDS sample buffer at -20° C. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method [42].

Protein samples (20 µg of lysates) were analyzed according to standard Western blotting protocol as described previously [43]. The membrane was incubated with the primary antibody in the blocking solution overnight at 4º C, and with the secondary antibody in the blocking solution for 1 h at room temperature. Bound antibody was detected using SuperSignal West Pico or Dura chemiluminescent substrate (Pierce) and Image Quant LAS 4000 mini imaging system (GE Healthcare Bio-Sciences AB). The quantitation of the chemiluminescent signal was carried out with the use of Imaging Quant TL software (GE Healthcare Bio-Sciences AB).

RNA extraction and quantitative RT-PCR

Primers and probes for quantitative reverse transcription polymerase chain reaction (RT-PCR) were obtained from Metabion International AG. At the indicated time points, culture medium was removed

and total RNA was extracted with GenElut Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions and as previously described [44]. Total RNA was reverse-transcribed to cDNA using TaqMan Reverse Transcription reagents and random hexamers (Applied Biosystems). cDNA obtained from the RT-reaction was diluted 1:20 with RNAse-free water and subjected to PCR using TaqMan Universal PCR Master Mix and ABI PRISM 7000 Sequence detection system (Applied Biosystems). The primers and probes were the following: mouse iNOS forward 5'-CCTGGTACGGGCATTGCT-3' (300 nM), mouse iNOS reverse 5'-GCTCATGCGGCCTCCTT-3' (300 nM), mouse iNOS probe 5'-CAGCAGCGGCTCCATGACTCCC-3' (150 nM), mouse GAPDH forward 5'-GCATGGCCTTCCGTGTTC-3' (300 nM), mouse GAPDH reverse 5'-GATGTCATCATACTTGGCAGGTTT-3' (300 nM), mouse GAPDH probe 5'-TCGTGGATCTGACGTGCCGCC-3' (150 nM). The primer and probe sequences and concentrations were optimized according to the manufacturer's guidelines in TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. PCR reaction parameters were as follows: incubation at 50º C for 2 min, incubation at 95º C for 10 min, and thereafter 40 cycles of denaturation at 95º C for 15 s and annealing and extension at 60º C for 1 min. Each sample was determined in duplicate. A standard curve method was used to determine the relative mRNA levels.

Enzyme-linked immunosorbent assay

IL-6, MCP-1 and PGE_2 were measured in the culture medium by enzyme linked immunosorbent assay (ELISA) using reagents from R&D Systems Europe Ltd (IL-6 and MCP-1) and Cayman Chemicals (PGE_2).

Carrageenan-induced paw edema in mice

Anti-inflammatory effects were studied by measuring carrageenan-induced paw edema in male C57BL/6 mice (Harlan Laboratories BV). The study was carried out in accordance with the legislation for the protection of animals used for scientific purposes (directive 2010/63/EU) and The Finnish Act on Animal Experimentation (62/2006). The study was authorized in Finland by the national Animal Experiment Board, license number ESLH-2009-07700/Ym-23 (granted September 23, 2009). Paw edema was induced under anesthesia and all efforts were made to minimize suffering. Mice were housed under conditions of optimum light, temperature and humidity (12:12 h light:dark cycle, 22±1° C, 50-60 %) with food and water provided *ad libitum*. Male mice aged 10 weeks were divided into three groups: control group, nortrachelogenin (100 mg/kg) group and dexamethasone (2 mg/kg) group. The doses of nortrachelogenin and dexamethasone based on our preliminary experiments. Mice were dosed with 150 µL of PBS-10 % DMSO vehicle or the tested compound by intraperitoneal injection 2 h before carrageenan was applied. The mice were anesthesized by intraperitoneal injection of 0.5 mg/kg of medetomide (Domitor 1 mg/mL, Orion Oyj) and 75 mg/kg of ketamine (Ketalar 10 mg/mL, Pfizer Oy Animal Health), and thereafter the mice received 30 µL injection of sterile saline containing 1.5 % of λ-carrageenan (w/v) in one hind paw. The contralateral paw received 30 µL of saline and it was used as a control. Edema was measured before and 3 and 6 h after carrageenan injection with plethysmometer (Ugo Basile). Edema is expressed as the difference, in µL, between the volume changes of the carrageenan treated paw and the control paw.

Statistics

Results are expressed as the mean + standard diviation (SD). Statistical significance of the results was calculated by one-way ANOVA with Dunnett's post test (dose curves) or Bonferroni´s post test

(multiple comparisons) by using GraphPad InStat 3 for Windows XP (GraphPad Software). Differences were considered significant at *$p < 0.05$, **$p<0.01$ and ***$p<0.001$. EC50 values were calculated with GraphPad Prism version 7.01 for Windows (GraphPad Software).

Conflict of interest statement

The authors declare no conflicts of interests.

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Figure legends

Fig. 1. Chemical structure of nortrachelogenin.

Fig. 2. Effects of nortrachelogenin on LPS induced iNOS protein expression and NO production in J774 macrophages. iNOS expression (A) was measured by western blot and NO production (B,C,D) as its stable metabolite nitrite by Griess reaction after 24 h incubation. Values are expressed as mean + SD, n=4, *p<0.05 and **p<0.01 as compared to cells cultured with LPS (A,B), IFN-γ (C) or cytomix (D). Cytomix is a combination of IFN-γ (10 ng/mL), TNF-α (20 ng/mL) and IL-1β (10 ng/mL).

Fig.3. Effects of nortrachelogenin on iNOS mRNA expression. J774 macrophages were cultured with LPS alone or with LPS and nortrachelogenin and RNA was extracted at time points 3 h, 6 h, 12 h and 24 h. iNOS mRNA expression was measured by quantitative RT-PCR. The results were normalised against GAPDH mRNA and are expressed as mean + SD, n=4.

Fig. 4. Effects of the proteasome inhibitor lactacystin and nortrachelogenin on iNOS expression in J774 macrophages. Cells were stimulated with LPS in the presence and in the absence of nortrachelogenin. After 8 h incubation the proteasome inhibitor lactacystin was added into the culture. Proteins were extracted after 24 h incubation and iNOS protein levels were measured by western blot. Values are expressed as mean + SD, n=4, **p<0.01 and ns=not significant as compared to cells cultured with LPS only.

Fig. 5. Effects of nortrachelogenin on MCP-1 (A) and IL-6 (B) production. J774 macrophages were stimulated with LPS in the presence of increasing concentrations of nortrachelogenin for 24 h

before the incubations were terminated and MCP-1 and IL-6 concentrations in the culture media were determined by ELISA. Results are expressed as mean + SD, n=4, *p<0.05 and **p<0.01 as compared to cells cultured with LPS only.

Fig. 6. Effects of nortrachelogenin on LPS-induced PGE_2 production and COX-2 and mPGES-1 expression. PGE_2 production (A), COX-2 protein expression (B) and mPGES-1 protein expression (C) were measured in J774 macrophages after 24 h incubation. COX-2 and mPGES-1 protein levels were measured by western blot and PGE_2 production by ELISA. Values are expressed as mean + SD, n=4, **p<0.01 as compared to cells cultured with LPS only.

Figure 7. Effects of nortrachelogenin and the anti-inflammatory steroid dexamethasone on carrageenan-induced paw inflammation model in the mouse. Nortrachelogenin (100 mg/kg) and dexamethasone (2 mg/kg) were administered i.p. 2 h prior to carrageenan (1.5 %) was injected into the paw. Paw edema was measured before, and 3 and 6 h after carrageenan injection with a plethysmometer. Edema is expressed as the difference in volume changes between the carrageenan treated paw and the contralateral vehicle-injected paw. Results are expressed as mean + SD, n=6, ***p<0.001 as compared to mice without drug treatment.

Fig.1

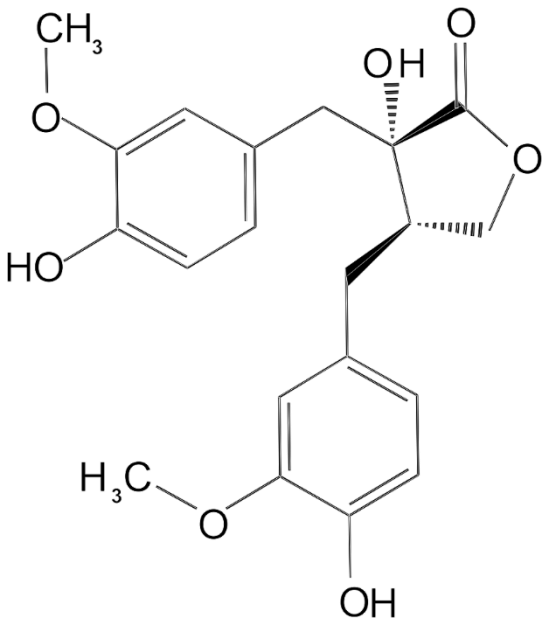


Fig. 2

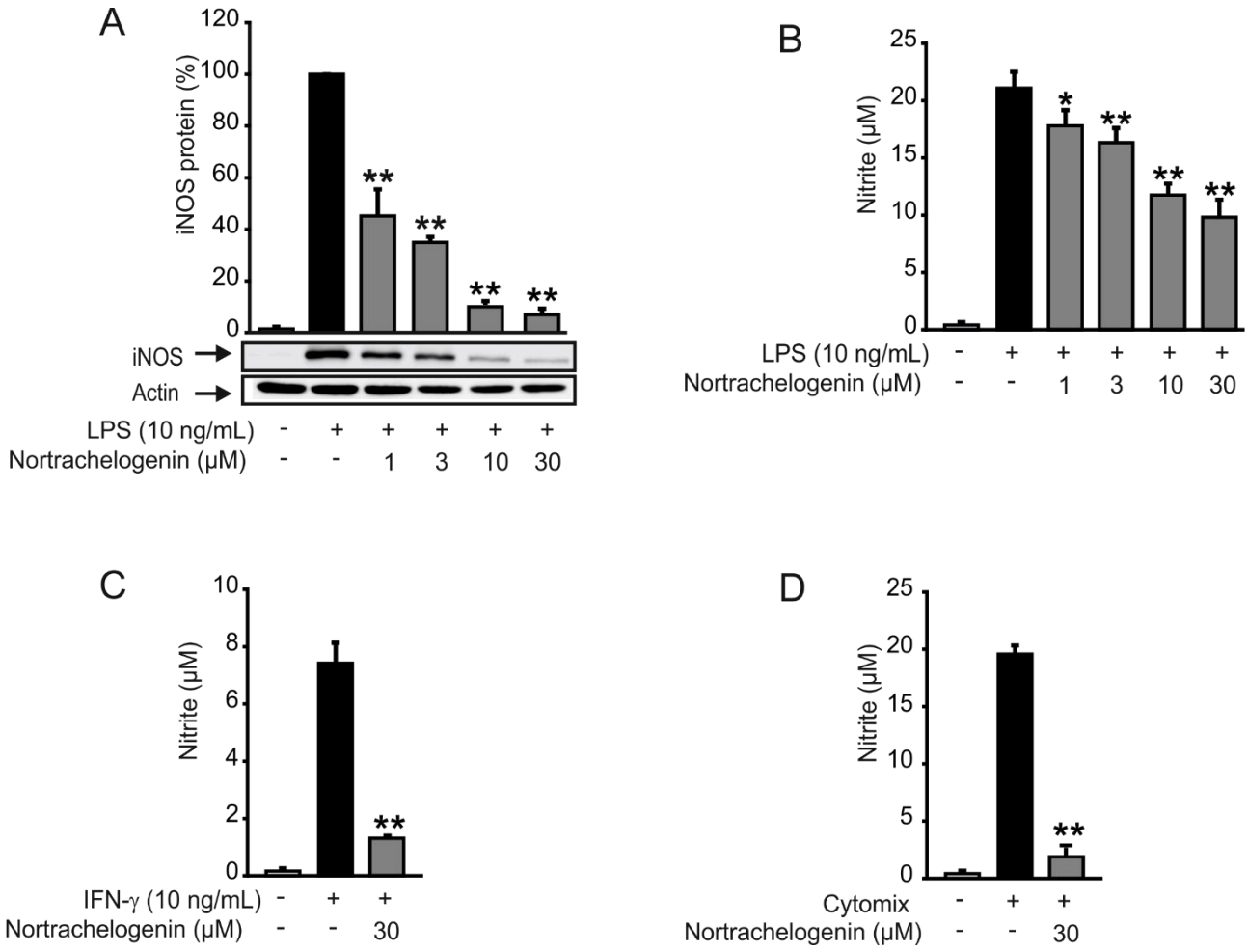

A
iNOS protein (%)
120
100
80
60
40
20
0
**
**
**
**
iNOS
Actin
LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30
B
Nitrite (μM)
25
20
15
10
5
0
*
**
**
**
LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30
C
Nitrite (μM)
10
8
6
4
2
0
**
IFN-γ (10 ng/mL) - + +
Nortrachelogenin (μM) - - 30
D
Nitrite (μM)
25
20
15
10
5
0
**
Cytomix - + +
Nortrachelogenin (μM) - - 30

Fig. 3

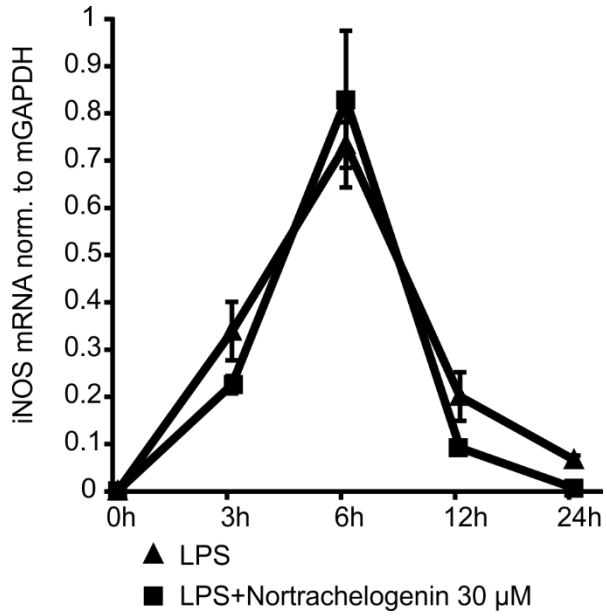

iNOS mRNA norm. to mGAPDH
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0h
3h
6h
12h
24h
LPS
LPS+Nortrachelogenin 30 μM

Fig. 4

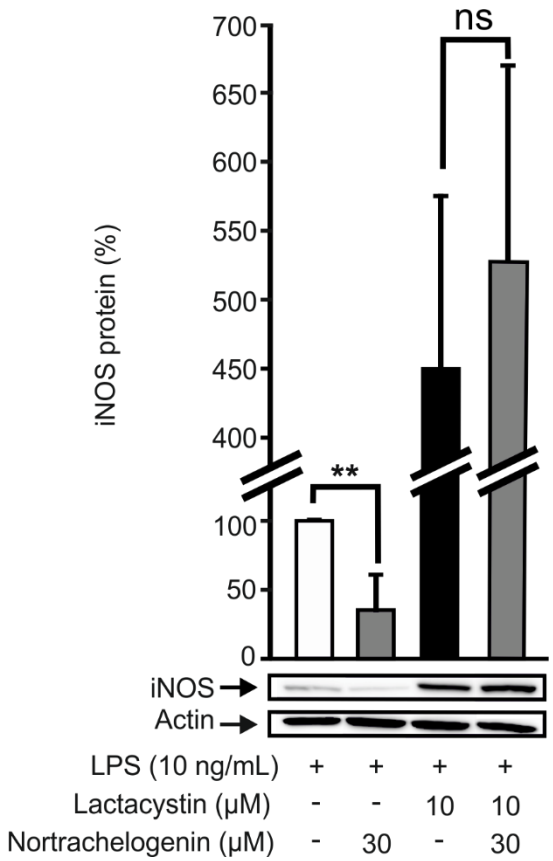

ns
700
650
600
550
500
450
400
**
100
50
0
iNOS protein (%)
iNOS
Actin
LPS (10 ng/mL) + + + +
Lactacystin (μM) - - 10 10
Nortrachelogenin (μM) - 30 - 30

Fig. 5

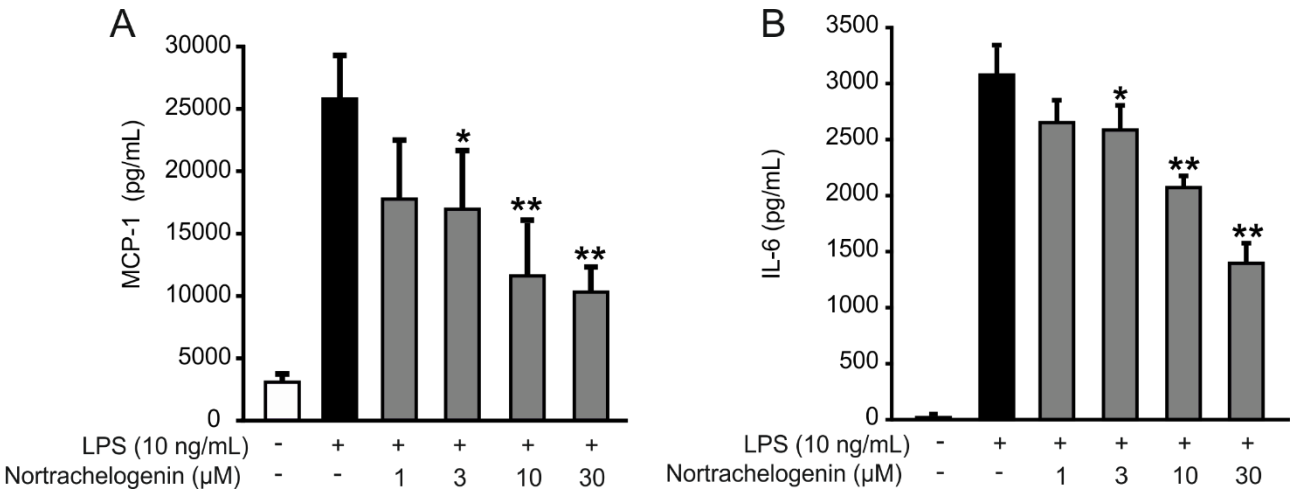

A
MCP-1 (pg/mL)
30000
25000
20000
15000
10000
5000
0
*
**
**
LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30
B
IL-6 (pg/mL)
3500
3000
2500
2000
1500
1000
500
0
*
**
**
LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30

Fig. 6

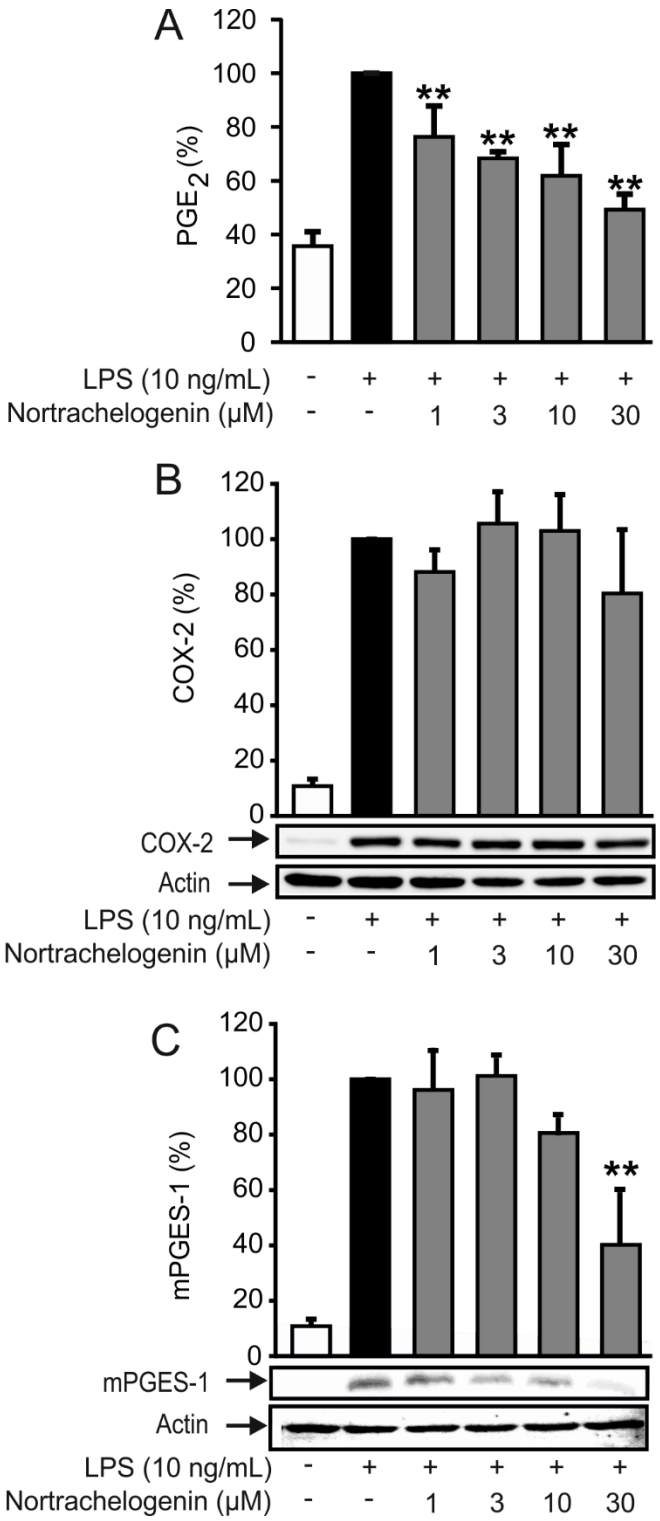
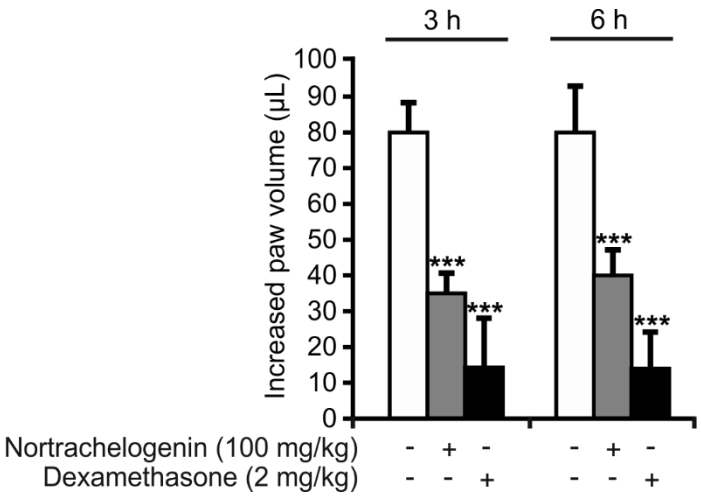

A
120
100
80
60
40
20
0
PGE2 (%)
**
**
**
**
LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30
B
120
100
80
60
40
20
0
COX-2 (%)
COX-2
Actin
LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30
C
120
100
80
60
40
20
0
mPGES-1 (%)
**
mPGES-1
Actin
LPS (10 ng/mL) - + + + + +
Nortrachelogenin (μM) - - 1 3 10 30

Fig. 7


3 h
6 h
Increased paw volume (µL)
100
90
80
70
60
50
40
30
20
10
0
***
***
***
***
Nortrachelogenin (100 mg/kg) - + - - + -
Dexamethasone (2 mg/kg) - - + - - +