Investigation of the anti-inflammatory and anti-proliferative effects of resveratrol analogs

Ph.D. Thesis

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Introduction

Inflammation

Inflammation is the biological response of organisms to harmful stimuli, infections, or tissue damage. It aims the elimination of pathogens, localization of the damage and restoration of normal tissue function. Inflammation is based on the activation of innate immune mechanisms, which may, however, activate adaptive immunity. Usually it develops rapidly, remains localized and after the destruction of pathogens it is resolved without permanent consequences. However, in some cases (severe infection or trauma, defective regulation) inflammation might lead to pernicious, even fatal outcome.

Recent evidence suggest that inflammatory processes may lie at the heart of many serious pathological conditions, such as cancer, metabolic syndrome, diabetes, atherosclerosis and some neurodegenerative diseases. This fact underlines the importance of precise understanding of inflammatory mechanisms and the urge of development of potent anti-inflammatory therapies.

A typical inflammatory response consists of four components: inflammatory inducers, the sensors that detect them, the inflammatory mediators, and the effectors. The inducing stimulus might be exogenous (microorganisms, allergens, foreign particles) or endogenous (molecules released by damaged tissues, sodium urate crystals, oxidized lipoproteins, degradation products of the extracellular matrix).

Invading microorganisms are recognized by the host on the basis of their conserved molecular patterns (PAMP). Inflammatory stimuli cause the production of pro-inflammatory mediators by cells or by the conversion of plasma proteins. Cellular mediators are mainly produced by specialized leukocytes (tissue macrophages and mast cells), and to some extent by the inflamed tissue itself. Depending on the type of infection, the sensors, mediators, and effectors vary such that the appropriate type of inflammatory response is induced. In the case of bacterial infection TLR signaling generates an antibacterial response through the production of TNFα, IL-6 and PGE2.

The role of macrophages in inflammation

Macrophages are in the first line of defense against invading microbes. These cells recognize pathogen associated molecular patterns with their pattern recognition receptors. The mononuclear phagocyte system consists of monocytes and macrophages. Monocytes are small, myeloid progenitor cell-derived cells circulating in the blood. Following extravasation monocytes differentiate into macrophages, which are much larger in size and express a wide range of receptors related to immune function. Besides innate and adaptive immunity, macrophages have many other roles in maintaining normal tissue function, such as the clearance of degraded cells and production of important enzymes. One of their most significant functions is the antigen presentation to T-cells. Recent evidence suggest that apart from their pro-inflammatory function, macrophages have a critical role in the resolution of inflammation.

Although inflammation is a complex phenomenon of a whole organism, it derives from the inflammatory processes of individual cells. Macrophages have a pivotal role in both the acute and chronic forms of inflammation therefore suppression of the inflammatory activation of these cells might be a good therapeutic strategy against a wide range of diseases.

Inflammatory signaling in macrophages

Lipopolysaccharide (LPS) is the endotoxin of Gram-negative bacteria. It is released from the outer membrane during bacterial lysis due to antibiotics or immune mechanisms. Overactivation of host defense processes by large amount of LPS may lead to systemic inflammation, septic shock and, in many cases, death. As the main molecular pattern associated with Gram-negative bacteria, pure LPS can generate an immune response, therefore it is a frequently used tool in *in vivo* and *in vitro* models of inflammation.

Following LPS-binding of the TLR4 receptor-complex, MyD88-dependent signaling pathway stimulation results in the production of pro-inflammatory cytokines through the activation of MAP kinases and NFκB. MyD88 is assembled to the intracellular domain of TLR4 by the adapter protein TIRAP. MyD88 recruits IRAK-4 and IRAK-1, and the latter interacts with TRAF6. TRAF6 catalyzes the K63-linked polyubiquitination of itself, IRAK-1 and IKK.

Binding of TAB2 and TAB3 activates TAK1, which in turn phosphorylates IKKβ. Resulting phosphorylation of IκB mediates its proteasomal degradation, which gives rise to the nuclear

translocation of NFκB subunits and the increased expression of several inflammatory genes. After release from macrophages, inflammatory interleukins and TNFα expands inflammation to further sites of the organism, arachidonic acid-derived eikosanoids produced by COX-2 cause pain and fever. NO produced by iNOS aims the elimination of the pathogen, but at the same time may exert cytotoxic effect. As an important part of inflammatory signaling MAPK kinases activate p38, JNK and ERK MAPKs, which increase the expression of inflammatory genes through several transcription factors (NFκB, AP-1, etc.). Dephosphorylation of MAP kinases by MKP-1 is an important regulatory mechanism.

Another characteristic feature of macrophage activation is the so-called oxidative burst, which is the rapid production of reactive oxygen species predominantly by the NADPH oxidase complex. These phagosomal ROS have important anti-microbial effect however, after diffusion into the cytosol or the extracellular space these radicals have detrimental impact. ROS react with virtually all kinds of macromolecules: they lead to mutations through DNA damage, membrane disruption through lipid peroxidation and modification of protein structure due to the oxidation of amino acid side chains. As a consequence of these processes long-term oxidative stress results in the necrosis or apoptosis of nearby cells and in severe tissue damage.

Resveratrol

Resveratrol is a phytoalexin synthesized by several plants in response to fungal infection or environmental stress. It is particularly abundant in the skin of grapes and is held responsible for numerous beneficial effects of red wine. Recent data suggest resveratrol prevents some common pathological conditions like atherosclerosis, carcinogenesis and inflammation. Its anti-inflammatory properties include protection against reactive oxygen and nitrogen species, inhibition of the main pro-inflammatory transcription NFκB, and non-selective inhibition of cyclooxygenase enzymes. Resveratrol interferes with cell-cycle progression and tumor development through diverse molecular mechanisms. Among its targets are protein kinases, hormone receptors, ribonucleotide reductase and DNA polymerase.

Searching for potential chemopreventive agents with increased bioavailability, more powerful or more selective biological effects led to the synthesis of many derivatives of resveratrol. Chemical and biological investigations of these analogs reveal the structural determinants of the molecule's activity. Most of these structure-activity relationship studies focused on the

role of the functional groups. Less is known about the relation between the stilbenic backbone and the molecule's anti-inflammatory and antitumor properties.

Objectives

Resveratrol is a natural polyphenol with well-known anti-inflammatory and anti-proliferative effects. However, the molecular basis of its beneficial health impact is still not fully understood. Structural determinants of the molecule for biological activity are incompletely described as well. Regarding these facts the aims of our study were the following:

- To unravel further details of the anti-inflammatory effect of resveratrol in LPS-stimulated murine macrophage system.
- Identification of new resveratrol analogs with better anti-inflammatory or anti-proliferative features.
- Better understanding of the structure-activity relationship of stilbenic molecules due to the comparison of the effects of the new analogs and resveratrol.

Results

I. Anti-inflammatory effect of new paramagnetic resveratrol analogs

All of the paramagnetic analogs are weaker antioxidants than resveratrol

In our experimental model, we detected high levels of ROS in LPS-induced macrophages 24 h after the treatment, which were decreased by the paramagnetic analogs in a concentration-dependent manner (12.5-100 μM). None of the analogs exerted better antioxidant capacity than resveratrol. Compound HO4450 and HO4409 proved to possess the strongest effect among the analogs.

Several analogs inhibit nitrite-production better than resveratrol

All of the paramagnetic analogs were found to possess a concentration-dependent (12.5-100 μM) inhibitory effect against LPS-induced nitrite-production, as measured 24 h after treatment. In the case of the compounds HO4408 and HO4409 this inhibition was remarkably more powerful, as they were able to reduce the elevated nitrite-production by approximately 50 %.

Three analogs inhibit TNFα-production more powerfully than resveratrol

Not only directly damaging, but immunomodulating agents - cytokines, such as interleukins and interferons - are synthesized by activated macrophages. One of the most important cytokines is TNFα, which is basically responsible for the induction of inflammatory processes. 1.5 h after LPS challenge we measured a 10–12-fold increase in TNFα concentration in the media of the treated cells. Most of the used paramagnetic analogs (50 μM) and resveratrol (50 μM) decreased the amount of TNFα and compound HO4409, HO4450 and HO4569 exhibited the strongest effect.

II. Effect of resveratrol and its triple-bond analog (TDPA) on LPS-stimulated macrophages

Resveratrol is more potent antioxidant than TDPA

We first determined the antioxidant capacity of resveratrol and TDPA in a cell-free system and in LPS-activated RAW 264.7 macrophages. In both systems resveratrol proved to be better antioxidant than TDPA. 50 μM resveratrol scavenged 93% of peroxide radicals produced by 10 μM H_2O_2 and 60 μM EDTA-Fe^{2+} salt, whereas the same concentration of TDPA scavenged only 53%. Additionally, while both polyphenols reduced ROS production in LPS-induced macrophages, TDPA showed a significantly weaker effect.

TDPA reduces cell viability more than resveratrol

Resveratrol is known to cause cell death and cell cycle arrest in various tumor cell lines. In order to evaluate the effect of TDPA on viability of LPS-stimulated macrophages, we treated RAW Blue cells with 100 ng/ml LPS and various concentrations (12.5-100 μM) of resveratrol or TDPA for 24 hours. Following the treatment we assessed cell viability by MTT assay. LPS alone did not affect cell viability. While both compounds reduced viability in a concentration-dependent manner, TDPA produced significantly stronger effect in every concentration. 50 μM resveratrol given together with LPS decreased viability by 7%. The same amount of TDPA caused a reduction of 25%.

TDPA prevents LPS-induced activation of NFκB transcription factor more efficiently than resveratrol

RAW Blue is a reporter cell line to study the activation of NFκB transcription factor. Treatment with pattern recognition receptor agonists activates NFκB. This activation induces the production of a secreted embryonic alkaline phosphatase (SEAP) easily visualized by

addition of a detection medium. From the media of the previously described MTT assays we monitored SEAP levels. While both resveratrol and TDPA detained the activation of NFκB in a concentration-dependent manner, TDPA inhibition was more effective. Comparing the values obtained at 50 μM polyphenol concentration we found resveratrol does not suppress NFκB induction, whereas TDPA inhibits the process by 31%. Moreover, suppression of LPS-induced NFκB activation by the polyphenols appeared after 5 hours of treatment, a time when no signs of cell death were detected by MTT assay or flow cytometric propidium iodide/annexin V analysis.

TDPA prevents LPS-induced mitochondrial membrane depolarization

LPS is known to disrupt the mitochondrial membrane potential in the early phase of macrophage activation. To assess the protective effect of polyphenols on LPS-induced mitochondrial membrane depolarization we pretreated RAW 264.7 macrophages with 50 μM resveratrol or TDPA and added 100 ng/ml LPS for an additional 30 minutes. Flow cytometric JC-1 assay showed LPS-induced loss of membrane potential. Resveratrol and TDPA restrict this effect in an equal measure.

TDPA inhibits cytokine production of stimulated macrophages better than resveratrol

In order to investigate the influence of the polyphenols on the pro-inflammatory cytokine production of LPS-stimulated macrophages, RAW Blue cells were treated with 100 ng/ml LPS alone or with 100 μM resveratrol or TDPA for 6 hours. IL-1β, IL-6 and TNFα levels in the cell culture medium were measured by ELISA assay. LPS induced IL-6 and TNFα levels more than 5- and 17-fold, respectively. In the case of IL-6 resveratrol reduced cytokine levels by 67%, while TDPA reduced it by 81%. TDPA was also more effective in limiting TNFα levels. TDPA suppressed TNFα production by 82% in contrast to resveratrol, which exerted a reduction of only 46%.

TDPA and resveratrol have different impact on LPS-induced signaling events

To unravel the molecular mechanisms underlying the distinct effects of TDPA and resveratrol on inflammatory processes in macrophages, we studied intracellular signaling pathways in RAW Blue macrophages following LPS and resveratrol or TDPA treatment. Resveratrol and its triple-bond analog influence the phosphorylation or the amount of several important signaling proteins differently. TDPA was more powerful than resveratrol in suppressing LPS-

induced phosphorylation of IκBα, p65 subunit of NFκB and IKK after 30 minutes, and the amount of COX-2 after 3 hours of treatment. However, it increased the phosphorylation of p38 and JNK MAP kinases, even when compared to the LPS-treated group. TDPA and resveratrol inhibited ERK phosphorylation in a similar extent. While both compounds decreased the amounts of MKP-1 protein after 3 hours, TDPA had a stronger inhibitory effect.

III. Cytotoxic and anti-proliferative effect of resveratrol and its triple-bond analog (TDPA) on tumor cell lines

TDPA shows stronger cytotoxic effect on several cell lines in MTT assay

We investigated the effect of the polyphenols on the viability of seven different tumor cell lines after 24-hour treatment, by MTT-assay. On all cell lines (RAW 264.7 murine macrophage, HepG2 human hepatocellular carcinoma, U251 human glioblastoma, A549 human lung carcinoma, HeLa human cervical carcinoma, PC12 rat phaeochromocytoma, B16-F10 murine melanoma) we found that TDPA reduces cell viability stronger than resveratrol.

TDPA and resveratrol inhibit the colony formation of U251 and HepG2 equally

In the colony formation test cells seeded at very low density (500 cells/well) were treated with the compounds for seven days, then were stained. The number and size of the colonies reflect the proliferation rate of the cells. Both of the studied cell lines (U251 and HepG2) showed that resveratrol and TDPA inhibit the cell proliferation in a comparable manner.

TDPA is not more effective than resveratrol in cell cycle arrest

We assessed the cell cycle distribution of two human cell lines (HepG2 and U118) after 24-hour polyphenol treatment. Using flow cytometric approach cells in different stages of cell cycle (G0/G1; S; G2/M) can be distinguished based on their DNA content. Both compounds arrested cells at the G1→S transition in the case of U118 cell line. Neither of the polyphenols had the same effect on HepG2 cells, however, they inhibited progression through the S→G2 transition, with resveratrol having much stronger effect.

Resveratrol and TDPA induces apoptosis of HepG2 cells in a similar manner

Investigation of the features of cell death (apoptosis vs necrosis) was carried out by flow cytometric annexin V/7-AAD staining. After 24 hours both polyphenols (75 μM) reduced the

number of healthy cells by 14 % compared to the control. The compounds increased the proportion of early apoptotic cells, while no signs of necrotic cell death were detectable.

TDPA and resveratrol reduces cell number equally according to sulphorhodamine B assay

In order to strengthen our MTT results we investigated the cytotoxic effect of the compounds by another cell viability assay, sulphorhodamine B. SRB assay was carried out in the same experimental setup as the MTT assay. Evaluating the experimental results it is apparent that resveratrol and TDPA reduce cell viability in the same manner, and the MTT assay underestimates the cytotoxic effect of resveratrol. Our polyphenols did not react with SRB or MTT stains in cell-free systems.

Resveratrol is stronger antioxidant than TDPA in tumor cell lines

The antioxidant activities of resveratrol and its triple bond analog were compared in HepG2 and U251 cells. On both cell lines resveratrol showed stronger antioxidant capacity than TDPA after 24 hours of treatment.

Summary

In the thesis I present the results of investigation of resveratrol's and its derivatives' anti-inflammatory and anti-proliferative effects. Paramagnetic, nitrogen-containing analogs were studied in the first phase of the project and a triple-bond resveratrol analog in the second. Our experiments showed that several analogs inhibited LPS-induced activation of macrophages stronger than resveratrol. Our results provide important information on the structure-activity relationship of stilbenic compounds. Based on these information it is possible to develop new therapeutic agents, exploiting the multi-target characteristic of polyphenols.

Additionally our results suggest that the ability of MTT assay for the estimation of cytotoxic effect of resveratrol is limited, possibly due to the strong antioxidant effect of the polyphenol. Instead of MTT, we suggest the use of SRB assay to investigate cell viability in experiments using resveratrol. Regarding the fact that MTT assay is still a widely applied method in the field of resveratrol research, our results have significant impact on the interpretation of the results of scientific papers in the field and also on the design of our future experiments.

List of publications/Publikációk listája

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