The effect of inflammation and oxidative stress on signaling processes and stability of biological membrane systems

Ph.D. thesis

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Contents

I. Introduction

1. Inflammation: The TLR4 signal pathway. Resveratrol.

Toll-Like Receptor 4 (TLR4) plays an important role in initiating the innate immune response and its activation by the bacterial cell wall component endotoxin (lipopolysaccharide (LPS)) is responsible for chronic and acute inflammatory disorders. Uncontrolled inflammatory signaling may lead to more severe sepsis causing high mortality (septic shock).

LPS binding activates two pathways downstream of TLR4: myeloid differentiation primary response gene 88 (MyD88)-dependent and Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-β (TRIF)-dependent pathways that culminate in the activation of NF-κB, IRFs, or MAP kinases to regulate the expression of cytokines, chemokines, and type I IFNs that ultimately protect the host from microbial infection.

MyD88 recruits interleukin-1 receptor associated kinase-1 and -4 and tumor necrosis factor receptor associated factor 6 (TRAF6), leading to activation of the IκB kinase (IKK) via transforming growth factor-β activated kinase-1 (TAK1). IKK phosphorylates IκBα which leads to the nuclear translocation and DNA binding of NF-κB.

In the other branch of the TLR4 signaling pathway, Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule are recruited to TLR4, and TRIF recruits receptor interacting protein-1 (RIP1) to the proximal receptor signaling complex. It is thought that TRAF6 and RIP1 Lys63-linked polyubiquitinations both facilitate TAK1 and so NF-κB activation.

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a natural polyphenol found in some fruits and vegetables. Considerable evidence demonstrates the anti-inflammatory properties of resveratrol, including inhibition of reactive oxygen and nitrogen species (ROS and RNS) (see below I.2.) production in cells. Some molecular targets of resveratrol have been identified in TLR-mediated signaling pathways. It has been reported that resveratrol acts on NF-κB by the inhibition of IκB kinase, leading to the inhibition of LPS-induced IκBα degradation. Between TLR4 receptor and NF-κB transcription factor TRAF6 is one of the most important adaptor proteins. However, its exact role and its involvement in resveratrol-induced anti-inflammatory pathways are still controversial.

2. Oxidative stress and lipid peroxidation.

Oxidative stress caused by partially reduced forms of molecular oxygen known as reactive oxygen species (ROS), such as the superoxide anion, hydrogen peroxide, or the hydroxyl radical, may be a consequence of external stressors. ROS can cause oxidative damage both in polyunsaturated fatty acids (PUFAs) and, to a lower extent, in monounsaturated fatty acids (MUFAs) in biological membranes through a chain reaction process known as lipid peroxidation. Lipid peroxidation generates a number of by-products, including lipid hydroperoxides. Tertbutyl hydroperoxide (t-BuOOH) is an organic lipid hydroperoxide analog which is frequently used as a pro-oxidant to investigate external oxidative stress. t-BuOOH-induced toxicity is attributed to the generation of butoxyl radicals via a Fenton-type reaction. The free radicals produced in this way deplete the antioxidant systems. A number of studies have addressed the mode of biochemical action of t-BuOOH in yeast and fungi; however, the molecular mechanism of its cytotoxicity is still not completely understood, but these data suggested that the first target of t-BuOOH is the plasma membrane, the composition of which may influence the consequences of t-BuOOH treatment. Yeast cells have evolved a number of inducible adaptive responses to ROS and lipid peroxidation products, which appear to be regulated, at least in part, at the level of transcription.

II. Objectives

1. Firstly, we study the effect of resveratrol on the LPS-induced TRAF6 synthesis at both the mRNA and protein levels, as well as TRAF6 ubiquitination and downstream MAP kinases and Akt activation in RAW 264.7 mouse macrophage cells. These data can contribute to a better understanding of the involvement of TRAF6 in inflammatory mechanisms.
2. Secondly, the aim of the present study was to determine the consequences of the presence of the t-BOOH-resistant mutant hyd1-190 of *Schizosaccharomyces pombe* (*S. pombe)* on the plasma membrane composition, and also its biophysical and biological behavior. Another objective was to characterize the adverse effects of t-BuOOH as an external stressor on the plasma membrane in comparison with its parental strain hyd^+ [adaptation and colony-forming ability, membrane recruitment processes (glycerol-uptake), plasma membrane fluidity]. Information concerning oxidative stress tolerance and resistance may be useful for an understanding of the interactions between the plant, animal and human pathogenic microorganisms and the host.

III. Results

1. LPS-induced TRAF6 gene and protein expressions is inhibited by resveratrol in RAW 264.7 cells

To clarify whether TRAF6 has a role in TLR-mediated NF-κB activation, we determined mRNA and protein concentrations of TRAF6 after LPS treatment at 10, 30 and 60 minutes in the presence and absence of resveratrol LPS-induced expression of TRAF6 mRNA in RAW 264.7 cells showed a transient elevation peaking at 10 min after treatment. By 30 minutes after the stimulation, TRAF6 mRNA levels returned to a level comparable to the one before treatment. A second and less intensive expression peak of TRAF6 was detected 1 hour after the LPS treatment, and this increase returned to the control level 2 hours after the treatment. This biphasic expression pattern of TRAF6 mRNA is similar to the phosphorylation and expression pattern of IκBα found by Loniewski et al[1].

These data suggested that TRAF6 is likely involved in LPS-induced NF-κB activation, and its effect on IκBα phosphorylation and expression followed a biphasic pattern similar to what we found. In our study, resveratrol suppressed LPS-induced expression of TRAF6 mRNA both at 10 and 60 minutes after stimulation, abolishing the effect of LPS treatment.

Furthermore, we found that protein levels of TRAF6 followed the mRNA expression pattern. LPS induced two transient elevations of the protein level (10 and 60 minutes), and resveratrol suppressed both of them. These data suggest that TRAF6 may have a role in the LPS-induced inflammatory process and in the anti-inflammatory effect of resveratrol in RAW 264.7 macrophages.

2. LPS-induced TRAF6 ubiquitination (activation) is inhibited by resveratrol in RAW 264.7 cells

We examined TRAF6 ubiquitination in the presence and absence of LPS and resveratrol by immunoprecipitation. We found that LPS induced ubiquitination of TRAF6 as soon as 10 minutes after the treatment, which further increased 30 minutes after exposure to LPS and decreased close to the pretreatment level on longer LPS exposure. Ubiquitination of TRAF6 by LPS was significantly attenuated by resveratrol at all times. This showed that there could be ubiquitination-dependent and -independent IKK activation pathways downstream of TRAF6.

[1] Loniewski KJ, Patial S, Parameswaran N. Sensitivity of TLR4- and -7-induced NF-kappa B1 p105-TPL2-ERK pathway to TNF-receptor-associated-factor-6 revealed by RNAi in mouse macrophages. Mol Immunol 2007;44:3715–23.

Our results indicate that LPS not only increased the expression of TRAF6 but facilitated its functional activation, leading to activation of IKK and thereby NF-κB. Resveratrol attenuated all these effects, suggesting a functional role of TRAF6 in its anti-inflammatory effect.

3. Resveratrol inhibits LPS-induced activation of p38, JNK and Akt in RAW 264.7 cell line

Testing the functionality of LPS-induced TRAF6 expression and ubiquitination, we studied the effect of resveratrol on the downstream target MAP kinases by immunoblotting. We found that LPS induced an activation of p38, increasing in time during the studied period, and that resveratrol attenuated it in all cases. JNK activation was also elevated by LPS, although it increased until 30 min of exposure and then decreased. Similar to p38, resveratrol diminished JNK phosphorylation at all time points except for 30 minutes. In contrast to p38 and JNK, LPS-induced ERK phosphorylation diminished in time from an early elevated value and was not affected by resveratrol at any time points we studied. Moreover, we determined the phosphorylation pattern of Akt, and we found that, similarly to MAPKs, it followed the activation of TRAF6, and it was inhibited by resveratrol at all time points.

4. The t-BuOOH-resistant *S. pombe* exhibits 2-fold sensitivity to the antibiotic Amphotericin B. Characterization of t-BuOOH-resistant mutant.

The t-BuOOH-resistant mutants of the parental strain hyd^+ were obtained by the continuous cultivation of cells in the presence of increasing concentrations of t-BuOOH. One of the t-BuOOH-resistant mutants, designated hyd1-190, was selected for further investigations. Hyd1-190 carries a suspected single-gene mutation, as proved by tetrad analysis: in each case, the t-BuOOH testing of spore clones of 25 full tetrads furnished a segregation value of 2T:2N. In comparison with hyd^+, hyd1-190 exhibited the following characteristics: (i) a four-fold resistance to the oxidative stressor t-BuOOH; (ii) the same multiplication activity and generation time (4 h); (iii) the required t-BuOOH resistance was heritable for at least 30 passages on t-BuOOH-free SM plates; (iv) two-fold sensitivity to the antifungal drug Amphotericin B (AmB), which forms addition complexes with ergosterol and its precursors located in the plasma membrane.

5. Phase transition temperature of the plasma membrane of the t-BuOOH-resistant S. *pombe* is elevated and its glycerol uptake is decreased

Lipid extracts of untreated late-log phase cultures (23 hours) of hyd^+ and hyd1-190 cells were separated by TLC, and the amounts of sterol and FA components were determined by GC.

A quantitative modification of the sterol composition of hyd1-190 in comparison with that of hyd^+ was detected. Hyd1-190 cells exhibited a slight increase (7.8%) in ergosterol content, approximately two-fold increases in zymosterol, fecosterol, ergosta-5,7-dienol and episterol contents, and two-fold decreases in squalene, lanosterol and 24-methylene-24,25-dihydrolanosterol content.

The number of identified FAs in the total fatty acids (TFAs), polar lipids (PLFAs), triacylglycerols (TAGFAs) and the free FAs (FFAs) were 24, 17, 10 and 5 accordingly. The results presented here reflect the most detailed analysis of the FAs of *S. pombe* to this date. The main FAs in *S. pombe* are palmitic (~9–13%), stearic (~6–15%), and oleic acids (~42–72%) in each class except the FFAs. Significant alterations in the quantity of FAs were detected in hyd1-190 in comparison with hyd^+ as a consequence of the modification of the sterol composition in hyd1-190. The PLFAs of hyd1-190 demonstrated a 4.5% increase in the unsaturated/saturated ratio, and a 2.8% decrease in the polyunsaturated/saturated ratio.

The plasma membrane phase-transition temperatures (G) of the control hyd^+ and the hyd1-190 cells were determined with the aid of EPR spectroscopy. The phase-transition temperature breakpoints for untreated, control *S. pombe* cells were 11.68 and 19.64 °C for the hyd^+ and hyd1-190 strains, respectively. In fact, significant differences in assimilation were found between the hyd^+ and hyd1-190 strains.

No difference between the two strain was observed as concern the assimilation and growth yield of glucose, but glycerol taken up by facilitated diffusion differentiated the growth activities of the two strains. In comparison with its parental strain hyd^+, the mutant hyd1-190 required 25 hours longer to enter the logarithmic phase in the medium containing 1 M glycerol, although the biomasses of the two strains were the same at the end of the 48 h culturing.

6. Adaptation failure of the t-BuOOH-resistant was detected at the cell level

To investigate the consequences of t-BuOOH treatment on the lipid composition, mid-log phase cultures of both strains were treated with a subinhibitory (0.2 mM) concentration of t-BuOOH for 8 hours before lipid extraction. This treatment induced modifications in most of the components of the sterols and FAs in both strains. The most pronounced modifications occurred in the sterol composition of hyd^+, e.g., more than 100% increases in the concentrations of zymosterol, fecosterol, ergosta-5,7-dienol, and episterol. These alterations in sterol composition were reflected in modifications in the quantities of the various classes of FAs, and especially in the PLFAs. Eight components of hyd^+ and six components of hyd1-190 FAs exhibited significant differences (15–100%). The ratio of polyunsaturated/saturated PLFAs increased significantly in both strains.

Treatment of the cells with 0.2 mM t-BuOOH for 1 hour resulted in an increase in the breakpoint of the hyperfine splitting plot from 11.68 °C (untreated) to 15.63 °C (t-BuOOH-treated) in hyd^+, and in a decrease in the breakpoint from 19.64 °C (untreated) to 12.81 °C (t-BuOOH-treated) in hyd1-190. The inhibition of the growth of the *S. pombe* parental strain hyd^+ by various concentrations of t-BuOOH suggested the possibility of an adaptation process. The survival rates of the hyd^+ and hyd1-190 strains were determined by the treatment of cells for 60 min with 0.2, 1.0, or 2.0 mM t-BuOOH, which resulted in 25, 34, or 50% and 4, 12, or 20%, decreases in the colony-forming ability of the cell population, respectively. Nevertheless, the pretreatment of cells with subinhibitory concentration (0.2 mM) of t-BuOOH for 60 min caused a significantly increased survival rate of hyd^+, but not of hyd1-190, when the cells were subsequently treated with 1.0 or 2.0 mM t-BuOOH.

IV. Summary

1. Using RAW 264.7 macrophages, we determined the effect of resveratrol on LPS-induced TRAF6 expression, ubiquitination as well as activation of mitogen-activated protein (MAP) kinases and Akt in order to clarify its involvement in TLR4 signaling. LPS-induced transient elevation in TRAF6 mRNA and protein expressions is suppressed by resveratrol. LPS induces the ubiquitination of TRAF6, which has been reported to be essential for Akt activation and for transforming growth factor-β activated kinase-1–NAP kinase kinase 6 (MKK6)-mediated p38 and c-Jun N-terminal kinase (JNK) activation. We found that resveratrol diminishes the effect of LPS on TRAF6 ubiquitination and activation of JNK and p38 MAP kinases, while it has no effect on the activation of extracellular-signal-regulated kinase (ERK)1/2. The effect of resveratrol on MAP kinase inhibition is significant since TRAF6 activation was reported to induce activation of JNK and p38 MAP kinase while not affecting ERK1/2. Moreover, phosphorylation pattern of Akt followed the activation of TRAF6, and it was inhibited by resveratrol at all time points. Here, we provide the first evidence that resveratrol, by suppressing LPS-induced TRAF6 expression and ubiquitination, attenuates the LPS-induced TLR4–TRAF6, MAP kinase and Akt pathways that can be significant in its anti-inflammatory effects.

2. The resistant mutant hyd1-190 of the fission yeast *S. pombe* displayed four-fold resistance to tert-butyl hydroperoxide (t-BuOOH) in comparison with its parental strain hyd^+. The cells of hyd1-190 exhibited a quantitative alteration in the sterol content and hence in the fatty acid composition of the plasma membrane, reflected in a two-fold AmB sensitivity, increased rigidity of the plasma membrane, revealed by an elevated (Δ 7.9°C) phase-transition temperature, measured by means of electron paramagnetic resonance spectroscopy, and a significantly decreased uptake of glycerol. Treatment of the strains with a subinhibitory concentration (0.2 mM) of t-BuOOH induced adaptation via modification of the sterol and fatty acid compositions, resulting in increased (Δ 3.95°C) and decreased (Δ 6.83°C) phase-transition temperatures of the hyd^+ and hyd1-190 strains, respectively, in order to defend the cells against the consequences of t-BuOOH-induced external oxidative stress. However, in contrast with hyd^+, hyd1-190 lacks the ability to adapt to t-BuOOH at a cell level.

V. List of publications / Publikációk listája

1. Publications based on the thesis / A dolgozat alapjául szolgáló pulikációk

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