Inhibitory potential of rutin on lipopolysaccharide-induced toxicity and inflammatory response of raw U937 cells and macrophages

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Abstract

Rutin is an important flavonoid found in plants with enormous pharmacological activities in various experimental models while lipopolysaccharide is an amphipathic glycolipid with potent inflammatory activity. The protective effect of rutin on lipopolysaccharide-mediated cytotoxicity and inflammatory effect on U937 cells and macrophages was investigated. U937 cells were incubated with or without rutin (50 - 200 µM) and later exposed to lipopolysaccharide (5 µg/mL). Cell viability and the production of reactive oxygen species were later analyzed. In the other experiment, the cells were differentially-induced to macrophages and incubated with or without rutin before lipopolysaccharide exposure. The secretion of cytokines and expression of some transcription factors and enzymes were analyzed. It revealed that incubating cells with lipopolysaccharide alone caused significant cell death and production of reactive oxygen species which were reduced when cells were pre-incubated with rutin. Exposure of macrophages to lipopolysaccharide also resulted in significant secretion of both TNF-α and IL-6 which was reduced by rutin. Endotoxin also enhanced the expression of the transcription factors (NF-κB and iNOS) while reduced the expression of the antioxidant enzymes superoxide dismutase and catalase. The lipopolysaccharide-induced alterations in transcription were significantly reduced when macrophages were pre-incubated with rutin. Implications of the findings are discussed.

Keywords: cytotoxicity; cytokines; lipopolysaccharide; macrophages; rutin; transcription factors

Introduction

The consumption of plants/plant products for medicinal purposes has been a routine practice for decades if not for centuries. It is believed that the medicinal potentials of these plants stem from important phytochemicals which are often products of secondary metabolism. Polyphenols, especially flavonoids have attracted serious attention because of their immense bioactivities in both in vivo and in vitro studies (Terao, 2009; Pietra, 2010). Rutin (also known as vitamin P or rutoside) is an important flavonoid found in plants such as tea leaves, apples etc with enormous pharmacological properties (Al-Dhabi et al., 2015; Ganeshpurkar and Saluja, 2017). Known pharmacological activities of rutin in experimental models include anti-neurodegradative, anti-hepatotoxic, anti-inflammatory, hypouricemic, antioxidant etc. (Al-Dhabi et al., 2015; Iriti et al., 2017; Enogieru et al., 2018).

Lipopolysaccharide (also known as endotoxin) is located at the outer leaflet of the outer membrane of Gram-negative bacteria (Wang and Quin, 2010). Lipopolysaccharide is an amphipathic glycolipid which gives
Gram-negative bacteria a strong permeability barrier and enables the organisms thrive in harsh environmental conditions (Snyder and McIntosh, 2000; Qiao et al., 2014; Rios et al., 2016). Lipopolysaccharide is a virulence factor that binds to a specific target (especially cells of the innate immune system) and the resulting complex activates signal transduction pathways, transcription factors and induces gene expression (Guha et al., 2001). The events cause cell activation and production of endogenous mediators including pro-inflammatory cytokines, adhesion molecules, acute proteins, vasoactive amines, nitric oxide, prostaglandins which could be chemotactic (Zhang and Ghosh, 2000; Guha et al., 2001; Triantafillou et al., 2002). The current work investigates the protective effect of rutin against lipopolysaccharide-mediated cytotoxicity, production of reactive oxygen species and activation of the inflammatory response.

**Materials and Methods**

**Cell culture and toxicity**

The cell line U937 was grown in complete RPMI medium as reported by Okoko and Oruambo (2009). Briefly, cells were grown in a CO$_2$ (5%) incubator at 37 °C with RPMI supplemented with heat inoculated fetal calf serum, L-glutamine (0.02M) and penicillin-streptomycin. Cell numbers were maintained at 5 x 10$^4$ cells/mL before exposure to either rutin or lipopolysaccharide. For toxicity study, cells were incubated with or without rutin (50 - 200 µM) for 24 h before exposure to lipopolysaccharide (5 µg/mL) at 37 °C for 1 h. Cell viability was analysed via the MTT reduction assay according to Zhou et al. (2006) as modified (Okoko and Ndoni, 2021). The production of reactive oxygen species (ROS) was also assessed according to the method of Koga and Meydani (2001) as modified (Okoko, 2020).

**Cytokine production**

Cell line U937 was subjected to PMA (phorbol-12, myristate-13.acetate)-induced differentiation as described (Okoko and Oruambo, 2009). Media were removed and replaced with or without rutin and incubated for 24 h at 37 °C. Cells (now differentiated to macrophages) were subsequently incubated with or without lipopolysaccharide (5 µg/mL) and production of TNF-α and IL-6 analysed via cytokine capture ELISA as described (Okoko and Oruambo, 2009).

**Quantitative RT-PCR**

The expression of the transcription factors inducible nitric oxide synthase (iNOS), nuclear factor kappa B (NF-xB), and the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) was performed as reported by Okoko and Ndoni (2021). Briefly, total RNA was extracted from cells followed by cDNA synthesis. Sequences specific to iNOS, NF-xB, SOD and CAT were amplified with primer pairs listed in Table 1. Real-Time PCR data were analyzed and presented as fold change in expression to the GAPDH housekeeping gene of same sample.

**Data analysis**

Values are expressed as mean ± SEM from six replicates. Comparisons were done by subjecting raw data to analysis of variance followed by Duncan’s multiple range tests. Significance was set at p < 0.05.
Table 1. Primers pairs for RT-PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>FP: GTGCCACCTCCAGTCCAG</td>
</tr>
<tr>
<td></td>
<td>RP: GCTGCCACAGTTTTTGATCC</td>
</tr>
<tr>
<td>NF-κB</td>
<td>FP: GCCCTTGCATCTAGCCACAGAG</td>
</tr>
<tr>
<td></td>
<td>RP: GATGTCAGCACCCAGCCTTCAG</td>
</tr>
<tr>
<td>SOD</td>
<td>FP: GACTGAAGGCTGCATGGATTC</td>
</tr>
<tr>
<td></td>
<td>RP: CACATCGGCAACACCTTTTG</td>
</tr>
<tr>
<td>CAT</td>
<td>FP: CTTCGACCAGCAACATGC</td>
</tr>
<tr>
<td></td>
<td>RP: GATAATTGGGTCCCAGGCGATG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>FP: GTCGGAGTCAAGGATTTTGTC</td>
</tr>
<tr>
<td></td>
<td>RP: CTTCCGTCTCAGGCTTGAC</td>
</tr>
</tbody>
</table>

Results

Cell toxicity and production of ROS

Cell viability was determined via the MTT reduction assay and presented in Figure 1. It revealed that LPS caused significant cytotoxicity when compared to controls. Pre-incubating cells with rutin prior to LPS exposure enhanced cell viability closer to control value. Viable cells following pre-incubation with 100 µM rutin was significantly higher than treatment with 50 µM rutin (p < 0.05). However, there was no significant difference in viable cells between treatment with 100 µM and 200 µM rutin (p > 0.05). As shown in Figure 2, incubating the cells with LPS alone resulted in significant production of ROS when compared with control (p < 0.05). However, pre-incubating cells with rutin (at different concentrations) reduced production of ROS which was concentration-dependent (p < 0.05).

![Figure 1](image_url)

Figure 1. Effect of rutin on lipopolysaccharide-induced cell death in U937 cells
LPS, cells treated with 5 µg/mL lipopolysaccharide only; 50µM RUT, cells supplemented with 50 µM RUT before exposure to lipopolysaccharide (5 µg/mL); 100 µM RUT, cells supplemented with 100 µM RUT before exposure to lipopolysaccharide (5 µg/mL); 200 µM RUT, cells supplemented with 200 µM RUT before exposure to lipopolysaccharide (1 µg/mL). Each bar represents mean ± S.E.M of six replicates expressed as % viability in comparison to control. *Significantly different from control; **Significantly different from LPS and ***Significantly different from 50 µM RUT. p < 0.05
Figure 2. Effect of rutin on lipopolysaccharide-induced production of ROS in U937 cells
LPS, cells treated with 5 µg/mL lipopolysaccharide only; 50 µM RUT, cells supplemented with 50 µM RUT before exposure to lipopolysaccharide (5 µg/mL); 100 µM RUT, cells supplemented with 100 µM RUT before exposure to lipopolysaccharide (5 µg/mL); 200 µM RUT, cells supplemented with 200 µM RUT before exposure to lipopolysaccharide (1 µg/mL). Each bar represents mean ± S.E.M of six replicates expressed as % viability in comparison to control. *Significantly different from control; † significantly different from LPS and ‡ significantly different from 50 µM RUT; § significantly different from 100 µM RUT. p < 0.05

Excretion of TNF-α and IL-6
The effect of rutin on LPS-induced production of pro-inflammatory cytokines in U937-derived macrophages is presented in Figure 3. Lipopolysaccharide caused significant production of TNF-α and IL-6 which was reduced following pre-incubation with rutin (at different concentrations). There was no significant difference in the reduction of cytokine production following pre-treatment of macrophages with 50 µM and 100 µM rutin. However, pre-treatment of cells with 200 µM rutin significantly reduced cytokine production when compared with pre-incubation with other rutin concentrations (p < 0.05).

Quantitative RT-PCR
The effect of rutin on the expression of transcription factors and antioxidant enzymes is shown in Figure 4. Lipopolysaccharide significantly stimulated the expression of the transcription factors iNOS and NF-κB in U937-derived macrophages when compared to cells not treated with LPS (p < 0.05). Rutin significantly reduced the LPS-mediated inductions which was not concentration-dependent. Incubating the macrophages with LPS alone caused significant reduction in the expression of SOD and CAT. However, pre-incubating the macrophages with rutin prior to LPS exposure significantly enhanced the expression of both SOD and CAT closer to control values. However, the variations among pre-treatments of cells with different concentrations of rutin were not significant (p < 0.05).
**Figure 3.** Production of TNF-α (left) and IL-6 (right) in U937-derived macrophages
LPS, cells treated with 5 µg/mL lipopolysaccharide only; 50 µM RUT, cells supplemented with 50 µM RUT before exposure to lipopolysaccharide (5 µg/mL); 100 µM RUT, cells supplemented with 100 µM RUT before exposure to lipopolysaccharide (5 µg/mL); 200 µM RUT, cells supplemented with 200 µM RUT before exposure to lipopolysaccharide (1 µg/mL). Each bar represents mean ± S.E.M of six replicates expressed as % viability in comparison to control. *Significantly different from control; **significantly different from LPS and ***significantly different from 50 µM RUT; significantly difference from 100 µM RUT. *p < 0.05

**Figure 4.** Expression of (A) iNOS; (B) NF-κB; (C) SOD; and (D) CAT in U937-derived macrophages
LPS, cells treated with 5 µg/mL lipopolysaccharide only; 50 µM RUT, cells supplemented with 50 µM RUT before exposure to lipopolysaccharide (5 µg/mL); 100 µM RUT, cells supplemented with 100 µM RUT before exposure to lipopolysaccharide (5 µg/mL); 200 µM RUT, cells supplemented with 200 µM RUT before exposure to lipopolysaccharide (1 µg/mL). Each bar represents mean ± S.E.M of six replicates expressed as % viability in comparison to control. *Significantly different from control; **significantly different from LPS. *p < 0.05
Discussion

Lipopolysaccharide (LPS) is a major virulence factor of bacteria due to its powerful role in host pathogen relationship thus used as a model to induce infection and inflammation (Maldonado et al., 2016; Zhang et al., 2018). Though inflammation is a process employed by immune cells to fight infection, persistence is cytotoxic thus it is essential that a balance exists between immune activation and deactivation (to maintain immune homeostasis) (Gerber and Mosser, 2001). The dysregulation of the inflammatory response has been implicated in several pathological conditions such as septic shock, rheumatoid arthritis, diabetes, cancer, organ failure and even death and other chronic inflammatory diseases (Nicolls et al., 2007; Chen et al., 2018; Muller et al., 2019; Nakamura et al., 2020).

In this experiment, LPS induced significant toxicity and production of reactive oxygen species (ROS) in raw U937 cells which was reduced by rutin. It has been reported that LPS elicits either apoptotic or necrotic cell death which in some cases, is a consequence of the production of ROS (Meßmer et al., 1999; Simon and Fernández, 2009; Ozal et al., 2018). Flavonoids (including rutin) are well known antioxidants which inhibit the production of reactive oxygen species either directly or indirectly. They protect cells by inducing the expression of phase II detoxification proteins such as glutathione, γ-glutamylcystein ligase, glutathione S-transferase and NAD(P)H:quinone oxidoreductase in different cell systems (Maher and Hanneken, 2005; Angeloni et al., 2007). Though the cell has robust mechanisms to maintain oxidation/antioxidation balance, excessive production of ROS could trigger a shift in this homeostasis and rutin could be an important flavonoid that protects cells from the effect of these highly reactive species.

The inflammatory process (most times triggered by non-self-signals) is the mechanism the immune system employs to remove pathogens, toxic compounds etc to initiate the healing process (Chen et al., 2018). The process activates various signaling pathways that modulate the production of cytokines, vasoactive amines, nitric oxide, etc (Dinarello, 1997; Zhang and An, 2007; Chen et al., 2018). Lipopolysaccharide induces the secretion of the pro-inflammatory cytokines TNF-α and IL-6 from U937-derived macrophages in this current experiment which could be attributable to many regulatory factors. This has been reported as a major mechanism of lung inflammation from exposure to some gram-negative bacteria (Park et al., 2017; Shi et al., 2018; Liu et al., 2018). However, in this current work, pre-incubation of the macrophages with rutin significantly reduced cytokine production which could be concentration-dependent.

The data also revealed the expression of iNOS and NF-κB was upregulated by LPS. The production of nitric oxide (NO) is catalyzed by iNOS thus up-regulation of its expression increases NO levels. Nitric oxide is an inorganic signal molecule that activates a dysregulated immune response (Kim et al., 2017). The signal molecule also reacts with superoxide radical to produce the highly reactive peroxynitrite which reacts freely with important macromolecules to cause tissue damage (Radi, 2018; Ahmad et al., 2019). This reveals that improper upregulation of iNOS production could alter redox balance (Somasundaram et al., 2019). The RT-PCR data revealed that rutin reduced LPS-mediated expression of iNOS and NF-κB. Nuclear factor kappa B (NF-κB) is a transcriptional factor that induces the production of pro-inflammatory cytokines and iNOS thus it is regarded as one of the most important factors during the inflammatory process and a potential drug target (Ferraz et al., 2020). Flavonoids inhibit the production of inflammatory mediators such as ROS, nitric oxide, iNOS, cytokines and the expression of NF-κB (Leyva et al., 2016; Salaritabar et al., 2017; Ferraz et al., 2020).

It has been reported that rutin decreases the activation of NF-κB expression in human embryonic cell line which correlates with the reduced production of IL-6, TNF-α and NO (Choy et al., 2019).

In order to further investigate the antioxidant potential of rutin, its effect on the elaboration of the enzymes catalase and superoxide dismutase was analyzed. These are important enzymes that convert highly reactive oxidants to water thus help maintain oxidation/antioxidation balance. The results revealed that pretreatment of transformed U937 cells (i.e. U937-derived macrophages) with rutin prior to LPS exposure
significantly promoted the activities of the enzymes at the transcription level. The mechanism via which rutin reversed the LPS-mediated alterations is a subject of further investigation.

Conclusions

Lipopolysaccharide at the concentration of 5 µg/mL caused significant cytotoxicity which could be ascribed to the production of ROS. The glycolipid also enhanced the production of pro-inflammatory cytokines which could be mediated via inhibition of key regulatory and antioxidant genes. But the supplementation of rutin significantly reversed the LPS-induced alteration close to control levels which could be concentration-dependent. In summary, rutin significantly reduced LPS-mediated cytotoxicity and production of ROS via the alteration of the inflammatory response.

Authors’ Contributions

Both authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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