Patulin inhibits LPS-induced nitric oxide production by suppressing MAPKs signaling pathway

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\textbf{ABSTRACT}

Patulin (PAT) is a natural product isolated from several species of fungi. Here, we evaluated the effect of PAT (62.5–4,000 ng/ml) in lipopolysaccharide (LPS)-activated murine peritoneal macrophages. Cell viability assay showed that PAT at concentrations up to 250 ng/ml did not affect macrophage viability. PAT (250 ng/ml) significantly reduced LPS-induced nitric oxide production (by 98.4%), inducible nitric oxide synthase (iNOS) expression (by 83.5%), and iNOS messenger ribonucleic acid expression (by 100.0%). Moreover, PAT significantly reduced LPS-induced interleukin-1β (by 80.6%), cluster of differentiation (CD) 69 (by 63.1%), and Toll-like receptor (TLR) 4 (by 91.9%) protein expression. Finally, PAT significantly reduced LPS-triggered phosphorylation of all mitogen-activated protein kinases (MAPK) assessed: extracellular signal-regulated kinase (ERK; by 89.5%), c-Jun N-terminal kinase (JNK; by 77.5%), and p38 (by 72.3%). Taken together, these data suggest that PAT downregulates acute inflammatory response, inhibiting nitric oxide production by suppressing CD69-TLR4/ERK-JNK-p38 MAPKs/iNOS signaling pathway.

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1. Introduction

Natural products are compounds enriched, isolated, and synthesized from or based on natural sources. The major sources of natural products are plants, animals, and microorganisms (Bills and Gloer 2016). Patulin (PAT) is a fungi-derived natural product isolated from 60-plus species of fungi encompassing over 30 genera (Moake et al. 2005). This compound was originally described by Birkinshaw et al. (1943) and Raistrick (1943) as an antibiotic isolated from *Penicillium patulum*. It has been shown that PAT has immunosuppressive properties (Bourdiol et al. 1990; Oh et al. 2012; Tsai et al. 2016). However, the signaling pathways involved in the effect of PAT remain undefined.

Macrophages have well-established roles during acute inflammation (MacMicking et al. 1997). First, damage- and pathogen-associated molecular patterns are recognized by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs). In the presence of lipopolysaccharide (LPS), TLR4 activates intracellular signaling pathways, such as mitogen-activated protein kinase (MAPK), which in turn activates transcription factors that induce the expression of inflammation-related genes, such as *Nos2* (Ramachandran et al. 2018). *Nos2* encodes inducible nitric oxide synthase (iNOS), responsible for the production of high concentrations of nitric oxide during the acute inflammation (MacMicking et al. 1997). Furthermore, crosstalk and synergy have been reported between nitric oxide pathway and other components, such as cluster of differentiation (CD) 69 (Marzio et al. 1997) and cytokines (Geller and Billiar 1998). Thus, this work aimed to evaluate the effect of PAT in the acute inflammatory process using the LPS-activated murine peritoneal macrophage model.

2. Results and discussion

MTT assay demonstrated that PAT, at concentrations up to 250 ng/ml, did not affect primary cultured murine peritoneal macrophages (*Figure S1 A*); the half-maximal inhibitory concentration was 963.2 ng/ml. Moreover, as shown in *Figure S1 B*, the non-cytotoxic PAT concentrations (62.5, 125, and 250 ng/ml) did not increase LPS-induced cell dead, being chosen for the next assays. This result is in agreement with those
obtained by Bourdiol et al. (1990) using resident murine peritoneal macrophages and Tsai et al. (2016) using RAW264.7 murine macrophages. On the other hand, bovine macrophages appear to be more sensitive to PAT, as shown by Oh et al. (2012). These different effects of PAT on cell viability could be associated with biochemical, morphological, and functional changes that can occur during cell immortalization (Tommasi et al. 2013).

Macrophages perform several functions in the acute inflammation phase, such as nitric oxide production (MacMicking et al. 1997). Thus, to investigate the effect of PAT in the acute inflammatory process, nitric oxide production following LPS stimulation was carried out using macrophages. As a result, PAT (62.5, 125, and 250 ng/ml) significantly reduced LPS-triggered nitric oxide production (by 41.1, 77.3, and 98.4%, respectively; Figure S2), being PAT 250 ng/ml chosen for the next assays. This result is consistent with those obtained by Tsai et al. (2016).

Nitric oxide is synthesized by nitric oxide synthase (NOS), a family of enzymes that converts L-arginine and molecular oxygen to \( N^\alpha \)-hydroxy-L-arginine and further to citrulline and nitric oxide. In mammals, there are three isoforms of NOS. Notably, although all known NOS isoforms catalyze the same reaction, iNOS is the major isoform engaged in an inflammatory setting (Kleinert et al. 2003). It was evaluated whether PAT can decrease iNOS expression using LPS-activated macrophages. Flow cytometry analysis showed that PAT (250 ng/ml) could significantly reduce LPS-dependent iNOS expression (by 83.5%; Figure S3 A and C). This result agrees with the inhibition of nitric oxide production, observed in the present work.

Once iNOS was expressed, it continuously produces nitric oxide until the enzyme is degraded. Therefore, iNOS activity is primarily regulated by transcriptional and post-transcriptional mechanisms (Kleinert et al. 2003). For this reason, we also quantified iNOS messenger ribonucleic acid (mRNA) expression. As a result, PAT (250 ng/ml) completely inhibited LPS-induced iNOS mRNA expression (by 100.0%; Figure S4 C). For the first time, the effect of PAT on the nitric oxide pathway was expanded to the mRNA level.

Besides nitric oxide, many mediators coordinate the initial events of acute inflammation, such as anti- and pro-inflammatory cytokines. Moreover, it has been described that these cytokines can modulate nitric oxide production. Generally, anti-inflammatory cytokines downregulate the nitric oxide pathway, whereas pro-inflammatory cytokines upregulate it (Geller and Billiar 1998). In this regard, the release of anti- and pro-inflammatory cytokines in macrophages was evaluated. It was seen that PAT (250 ng/ml) significantly reduced interleukin (IL)-1\( \beta \) release (Figure S5 A, B, C, and D), although IL-1\( \beta \) mRNA expression was upregulated in combination with LPS (Figure S4 B). The IL-1\( \beta \) cytokine is synthesized as a precursor (pro-IL-1\( \beta \)) and then cleaved to generate a mature form (IL-1\( \beta \)). The IL-1\( \beta \) cleavage is controlled by inflammasome-dependent proteolytic maturation. Murine inflammasomes require a two-step activation. The first one (priming) activates the pro-IL-1\( \beta \) and caspase-1 expression, and the second signal (driving) promotes pro-IL-1\( \beta \) cleavage by the cysteine protease caspase-1 (Hughes and O’Neill 2018). Thus, IL-1\( \beta \) reduction release upon PAT treatment could be related to the impairment of the inflammasome/caspase-1-dependent maturation. This hypothesis is supported by evidence from Tsai et al. (2016), who showed that
PAT blocked the caspase-1 and NLRP3 inflammasome expression, as well as IL-1β release, in J774.1 macrophage. Furthermore, the PAT specificity for IL-1β could be partly explained by the distinct regulatory mechanisms involved.

It has also been reported that CD69 stimulation induces a strong nitric oxide production in murine macrophages (Marzio et al. 1997). CD69 is a membrane-bound II C-lectin receptor readily upregulated upon macrophage activation (Sancho et al. 2005; Cibrian and Sanchez-Madrid 2017). The effect of PAT on CD69 protein and mRNA expression upon LPS stimulation were studied by our group. Flow cytometry analysis showed that PAT significantly reduced LPS-dependent CD69 expression (Figure S3 A and B), but mRNA expression was not affected (Figure S4 A). Thus, the reduced availability of CD69 on the cell surface could maximize suppression of the nitric oxide pathway.

Besides CD69, TLR4 is another membrane-bound receptor that triggers a robust nitric oxide response when stimulated. TLR4 is a member of the TLR family that is activated by LPS. It has been well known that TLR4 transmits the agonist-recognition signal to stimulate nitric oxide production via intracellular signaling pathways, such as MAPK, which consist of three serine/threonine kinases in several isoforms: extracellular signal-regulated kinase (ERK) 1–8, c-Jun N-terminal kinase (JNK) 1–3, and p38 α/β/γ/δ (Ramachandran et al. 2018). Flow cytometry analysis showed that PAT significantly reduced expression of TLR4 protein (Figure S3 A and D) but not mRNA (data not shown) in the presence of LPS. Also, PAT reduced LPS-induced phosphorylation of all MAPK assessed (ERK, JNK, and p38; Figure S6 A, B, C, and D). Therefore, the reduction of nitric oxide production could be a consequence of reduced TLR4 and MAPK phosphorylation. Furthermore, it is possible to suggest the following signaling pathway involved in the inhibition of LPS-induced nitric oxide production in murine peritoneal macrophages upon PAT treatment: CD69-TLR4/ERK-JNK-p38 MAPKs/Nos2/iNOS.

3. Conclusions

In conclusion, the present work demonstrated that PAT could suppress acute inflammation response by inhibiting nitric oxide production. Moreover, the impairment of nitric oxide production by PAT most likely involves CD69-TLR4/ERK-JNK-p38 MAPKs/Nos2/iNOS signaling pathway, suggesting a new mode of action for this substance.

Disclosure statement

No potential competing interest was reported by the authors.

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