Evidences of hydroxytyrosol as an anti-inflammatory agent in Parkinson's disease: insights into the mechanisms of action

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Neuroinflammation in neurodegenerative diseases: Inflammatory processes play a critical role in neurodegenerative diseases, such as Parkinson's disease (PD). Thus, neuroinflammation is involved in the progression and development of these diseases, becoming an important pathological hallmark. Microglial cells, the "macrophages" from central nervous system, are the initiating cells of the innate immune response against different stimuli in the brain. Even though they have a major role in brain homeostasis maintenance, their uncontrolled activation results in the secretion of pro-inflammatory cytokines and production of reactive oxygen species (ROS), leading to neuronal damage and death. Microglial cells present different phenotypes, ranging from pro-inflammatory/neurotoxic to anti-inflammatory/neuroprotective phenotype, depending on the stimulants involved (Shen et al., 2018). For this reason, novel therapeutic strategies are being addressed in order to shift the microglia polarization. Among them it is worth mentioning the search for dietary bioactive compounds, which may prevent or delay the progression of neuroinflammation and, in consequence, reduce the neuronal damage caused by microglia overactivation.

Mediterranean diet (MD) as a source of anti-inflammatory bioactive compounds. The case of hydroxytyrosol (HT): Supporting evidence has associated the adherence to MD with a significant decrease in the risk of developing PD. Actually, a recent systematic review based on 32 cohort studies concluded that MD has positive effects on cognitive health linking this effect to the high consumption of fruits, vegetables and virgin olive oil, characteristic of this diet pattern (Chen et al., 2019). All of these food sources are rich in polyphenolic compounds, a family of naturally occurring bioactive compounds (more than 8000) with proven overwhelming biological properties. Among them, it is worth mentioning 3,4-dihydroxyphenylethanol, also known as HT. HT, one of the major compounds present in the phenolic fraction of virgin olive oil (together with tyrosol and their secoiridoids derivatives), is considered nowadays a potent bioactive molecule for several reasons. Its concentration is high in virgin olive oil and olives, but significant quantities can be also found in beverages such as wine and beer. Moreover, HT is originated from the hydrolysis of olive oil secoiridoids, such as oleuropein. Indeed, after absorption of oleuropein in the gastrointestinal tract, its hydrolysis and metabolic transformation gives rise to HT. In addition, HT can be formed endogenously in humans from dopamine metabolism through the reduction of 3,4-dihydroxyphenylacetaldehyde to HT (Hashimoto et al., 2004). Thus, circulating HT not only depends on the dietary uptake but also on this endogenous formation. In this sense, higher HT plasma concentration should be expected in comparison with other polyphenols. Moreover, HT is able to cross the blood brain barrier (Serra et al., 2012), an important requirement if we want to use it as a neuroprotective agent.

First evidences of the potential role of HT as an anti-inflammatory agent in brain: Very recently, *in vivo* and *in vitro* studies carried out by us and other authors have firstly demonstrated that HT could be used as anti-inflammatory agent to combat diseases such as PD, modulating microglial response (Gallardo-Fernández et al., 2019; Zhang et al., 2020). Until now, data about the anti-inflammatory properties of HT have been restricted to macrophages peripheral stimulated by lipopolysaccharide (LPS) (Yonezawa et al., 2018), which highlights the importance of these findings. Thus, HT has demonstrated to be able to reduce the inflammatory response in microglia of mice intraperitoneally injected with LPS (Zhang et al., 2020) and *in vitro* using primary microglia and BV2 cells upon two different stimuli: LPS (pro-inflammatory agent that triggers a very strong neuroinflammatory response) (Gallardo-Fernández et al., 2019; Zhang et al., 2020) and α-synuclein (α-syn) fibrils (main component of Lewy bodies, and associated with dopaminergic cell death) (Gallardo-Fernández et al., 2019). It should be also noted that this effect was found at concentrations compatible with a traditional MD (Gallardo-Fernández et al., 2019; Zhang et al., 2020). These authors suggest that the following molecular pathways could be implicated in the anti-inflammatory effect of HT (**Figure 1**).

Inflammatory mediators: Inflammatory cytokines and mediators are elevated in both serum and brain of PD patients (Tansey et al., 2007). The results of these studies indicated that HT significantly decreased mRNA and protein expression levels of pro-inflammatory mediators after LPS treatment in BV2 and primary microglial cells (Gallardo-Fernández et al., 2019; Zhang et al., 2020). Besides, a decrease in the expression of pro-inflammatory genes and microglia activation was observed in the mouse brain (Zhang et al., 2020), which supports and confirms the results obtained *in vitro* (Gallardo-Fernández et al., 2019; Zhang et al., 2020). It has been also shown a decrease and increase in the expression of CD86 (M1 marker) and CD206 (M2 marker) *in vitro*, respectively. This indicates that the anti-inflammatory effect of HT could be associated with modulation of microglia polarization (Zhang et al., 2020). On the other hand, BV2 microglial cells were also stimulated by α-syn. In this case, HT also decreased the expression levels of pro-inflammatory markers (Gallardo-Fernández et al., 2019). As in the LPS case, HT may suppress inflammation via modulation of microglia polarization.

Nuclear factor kappa B (NF-κB) pathway: One of the main mechanisms that leads to expression of inflammatory cytokines in microglia cells is the activation of Toll-like receptors (TLRs), through several transduction pathways such as NF-κB and mitogen-activated protein kinases (MAPKs) pathway, among others (Kawai and Akira, 2007). LPS and α-syn are recognized by TLR4 (Kawai and Akira, 2007; Shao et al., 2019), so the anti-inflammatory effect of HT could be mediated through these pathways. NF-κB is a transcription factor normally located in the cytoplasm. When an inflammatory insult occurs (i.e., LPS), it is translocated to the nucleus regulating the expression of numerous target genes (*TNF-α*, *iNOS*, *IL-1β*, and *IL-6*, among others) (Kawai and Akira, 2007). In order to know if this pathway was involved in the down-regulation of the above mentioned pro-inflammatory markers, the translocation to the nucleus of NF-κBp65 after stimulation of BV2 cells with LPS and α-syn was studied. As it is well-known, LPS induced the nuclear translocation of NFκBp65 which was prevented by HT (Gallardo-Fernández et al., 2019). Similar results were found by Zhang et al. (2020), supporting that this pathway could be involved in the anti-inflammatory effect of HT. However, the nuclear translocation induced by α-syn was not reverted by HT (Gallardo-Fernández et al., 2019). These results suggest that the anti-inflammatory effect of HT when stimulating cells with α-syn may be mediated by another mechanism not including the regulation of NF-κB translocation to the nucleus.

MAPKs pathway: MAPKs pathway is another critical axis in the induction of inflammatory response by LPS through TLR4 activation, leading to the expression of co-stimulatory molecules, chemokines, and cytokines (Kawai and Akira, 2007). HT treatment resulted in a decrease of JNK1/2 and p38 phosphorylated forms, in the case of LPS and α-syn-stimulated BV2 cells, respectively (Gallardo-Fernández et al., 2019). On the other hand, a decrease in pERK1/2 form has been found in BV2 cells treated with LPS and HT (Zhang et al; 2020). The differences observed in both studies make this hypothesis less likely to explain the anti-inflammatory effect of HT.

NLRP3 inflammasome: Finally, another route involved in the transcriptional regulation of inflammatory molecules is mediated by the activation of NLRP3 inflammasome. The activation of this molecular machinery requires the induction of TLR4/NF-κB signaling pathway and produces cleavage and release of pro-inflammatory cytokines such as interleukin (IL)-1β, Caspase-1 dependent (Shen et al., 2018). Since TLR4 signaling seems to be involved in the anti-inflammatory effect of HT (Gallardo-Fernández et al., 2019; Zhang et al., 2020), another hypothesis is that the activation of NLRP3 could be regulated by HT. Interestingly, data showed that, in the case of LPS, although an increase in the expression of NLRP3 mRNA was reduced by HT, this was not accompanied by a significant reduction in the mature IL-1β production (Gallardo-Fernández et al., 2019). For α-syn, HT treatment was not able to reduce the induced assembly of this molecular platform and any effect was observed regarding IL-1β protein levels (Gallardo-Fernández et al., 2019). These results suggest that there should be a different mechanism of action to explain the anti-inflammatory effect of HT.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and ROS production: Another common feature seen in PD and similar disorders is the induction of oxidative stress. In microglial cells, NADPH oxidase is the main source of ROS. This enzyme is a complex consisting of several subunits. When a pathogenic stimulus occurs, the different subunits of the NADPH oxidase are associated, leading to its activation (Belarbi et al., 2017). Thus, the expression of different subunits ($p47^{phox,}$ $p22^{phox}$ and $gp91^{phox}$) and ROS production were also measured in order to determine if both processes would be inhibited by HT. Results show how the activation of the enzyme was decreased when the treatment with LPS and α-syn was accompanied by HT. Regarding ROS production induced by both stimuli, although a downward trend can be

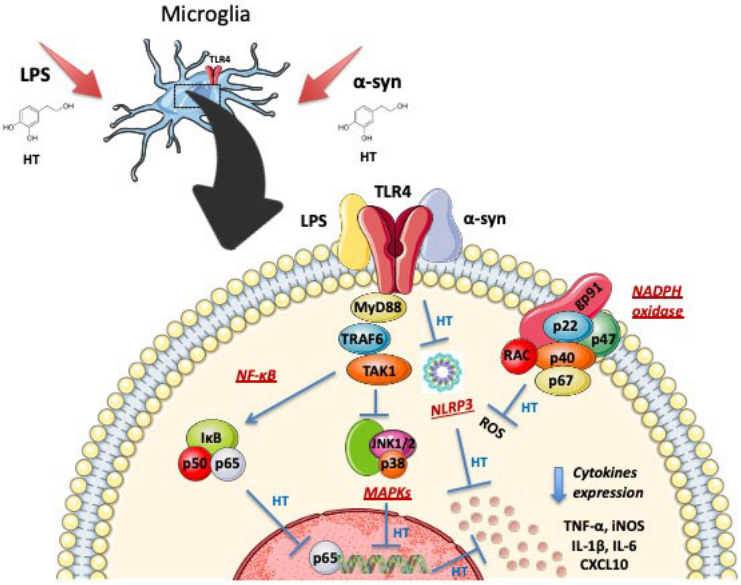

Figure 1 | **Schematic representation of the possible mechanisms of action for the anti-inflammatory effect of HT in microglia cells stimulated by LPS and α-syn.**
Both lipopolysaccharide (LPS) and α-synuclein (α-syn) interact with toll-like receptor 4 (TLR4) in microglial cells triggering the activation of different molecular pathways that involve the secretion of pro-inflammatory cytokines and production of ROS, leading to damage and neuronal death. According to the discussed results nuclear factor kappa B (NF-κB), mitogen-activated protein kinases (MAPKs), NLRP3 inflammasome pathways and reactive oxygen species (ROS) production could be target of hydroxytyrosol (HT), leading to a decrease in the neuroinflammation. CXCL10: chemokine (C-X-C motif) ligand 10; IL: interleukin; iNOS: inducible nitric oxide synthase; NADPH: nicotinamide adenine dinucleotide phosphate; TNF-α: tumour necrosis factor-α.

observed in the case of LPS, this production was only significantly reduced by HT when cells were treated with α-syn. Thus, HT could also protect the brain from oxidative stress via inhibition of NADPH oxidase activity (Gallardo-Fernández et al., 2019).

In summary, HT was able to prevent the immune-associated alterations induced by the different stimuli. However, some differences in the mechanisms of action have been found depending on whether the stimulant agent was LPS or α-syn protein. In fact, in the case of LPS-stimulated cells the most likely pathway through which HT would exert its anti-inflammatory action would be the one mediated by TLR4-NF-κB (Gallardo-Fernández et al., 2019; Zhang et al., 2020). By contrast, the antioxidant potential of HT could be the most promising via when microglial cells are stimulated with α-syn, since inhibition of NADPH oxidase activation and ROS production were observed (Gallardo-Fernández et al., 2019). Furthermore, HT has also showed the capacity to prevent α-syn aggregation counteracting thus the toxicity induced by this protein (Hornedo-Ortega et al., 2018). Regardless of the pathway/s involved in each case, these data suggest that the adherence to MD, which contains great quantities of HT, could lead to a decrease in PD incidence and/or progression by decreasing the inflammatory environment of the brain. These results become more relevant after *in vivo* validation of the effects seen *in vitro* (Zhang et al., 2020).

Perspectives: Understanding the mechanism of action of dietary bioactive compounds is a challenging task since the scientific community demands the development of more specific dietary recommendations to decrease or even tackle neurodegenerative diseases. Since suppression of inflammation mediated by microglia can be considered as an important strategy in the prevention of neurodegenerative diseases, these data could support the use of HT as a therapeutic strategy for diseases where neuroinflammation plays a crucial role. In conclusion, these results open an interesting research line, practically unexplored, about the preventive role of HT on neuroinflammation, besides suggesting possible mechanisms of action. Most likely these protective effects are not conducted by a single molecule, so more research is still necessary. For example, to test the combination of different food bioactive compounds would represent a situation more similar to reality. Likewise, prospective human interventions must be performed in order to take into account the complexity and physiology of the human body. Another consideration that must be addressed when working with dietary compounds is to test actual doses in the brain. In fact, a great number of published papers generally use high doses without reflecting important processes such as absorption and metabolism, the role of gut microbiota or even other dietary factors as calories, fatty acids, protein, sugar or alcohol intake. In addition, stressors including environmental perturbations as pharmacological challenges (drugs or toxin exposure) should be also considered.

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