Curcumin and Ethanol Effects in Trembler-J Schwann

Subjects: Biochemistry & Molecular Biology

Contributor: Lucia Vázquez Alberdi , Gonzalo Rosso , , Joaquina Farias , Miguel Calero , Alejandra Elizabeth Kun Gonzalez

Charcot-Marie-Tooth (CMT) syndrome is the most common progressive human motor and sensory peripheral neuropathy. CMT type 1E is a demyelinating neuropathy affecting Schwann cells due to peripheral-myelin-protein-22 (PMP22) mutations, modelized by Trembler-J mice. Curcumin, a natural polyphenol compound obtained from turmeric (*Curcuma longa*), exhibits dose- and time-varying antitumor, antioxidant and neuroprotective properties, however, the neurotherapeutic actions of curcumin remain elusive. Here, the researchers propose curcumin as a possible natural treatment capable of enhancing cellular detoxification mechanisms, resulting in an improvement of the neurodegenerative Trembler-J phenotype.

CMT1E Trembler-J curcumin ethanol Hsps autophagy

1. Introduction

The group of human hereditary peripheral neuropathies, known as Charcot-Marie-Tooth disease (CMT), has a prevalence of 1/2500 [1]. Within the CMT, demyelinating neuropathies (CMT1) have mutations that alter the structural integrity of myelin [2].

The mutations affecting the *pmp22* gene, play a central pathognomonic role in the CMT disease, representing between 60% and 70% of total myelinopathies [3]. PMP22 is highest expressed in the Schwann cells as a 160-amino-acid myelin glycoprotein, of 22 kDa, with four transmembrane domains, representing approximately 5% of the total compact myelin proteins [4]. However, central expression of PMP22 has also been signaled [5][6][7][8][9].

However, PMP22 is ubiquitously expressed in various tissues and organs in addition to the nervous system. PMP22 has been reported to play a role in adhesion and proliferation regulation [10], and described in epithelial cells, where it localizes with tight junctions and forms complexes with integrins and P2X7 channels [11][12][13]. It has been suggested that the regulation of PMP22 expression may also be increased by the action of steroid hormones [3][14], which has contributed to the exploration of hormonal therapies in the treatment of the CMT1A phenotype [14][15][16].

The expression of human PMP22 has been also reported during the proliferative and secretory phase of the menstrual cycle, and PMP22 has been shown to colocalize with alpha-6-integrins both in vitro and in human tissue samples. Thus, PMP22 appears to be associated in the endometrium with both cell adhesion and endometrial

differentiation [17]. Up to 5% of all CMT1s integrate the group of CMT1E, myelinopathies caused by different point mutations in the *pmp22* [18].

The Trembler J (TrJ/+) mouse is an animal model of CMT1E [19][20][21][22], carrying the same spontaneous mutation in *pmp22* as that found in a human family [23]. Under normal conditions, only 20% of PMP22 is inserted into the membrane, with chaperone assistance, at the cost of high energy expenditure (by the synthesis of unused PMP22 and for the maintenance of the proteasome machinery in charge of eliminating the protein surplus) [24][25]. In disease, the percentage of myelin that is inserted is even lower, so it is common to find intracellular PMP22 aggregates in the Schwann cells (SCs) of TrJ/+ mice [26], interfering with the regular protein transport. Thus, the peripheral nerve fibers from the neurodegenerative phenotype in TrJ/+ show altered autophagic-lysosomal pathways, PMP22 cytoplasmic aggregation, increased ribosome and translational activity [21][27][28][29][30][31].

One of the mechanisms underlying cellular stress situations is the response by Heat Shock Proteins (Hsps) [32][33][34][35][36]. Heat Shock Factor 1 (HSF1), the main regulator of the Hsps, is activated by mTOR under stress [34][37] and its inhibition prevents autophagosome formation [38]. Hsp27, recognized for its dual role in normal situations and tumor processes [39][40][41][42], has also been pointed out as a possible target of action for neurodegenerative diseases [43]. In stressful situations, such as the accumulation of intracellular proteins, Hsp27 activates and modulates serine/threonine protein kinase B (PKB/AKT) action, mTOR main activator [44][45][46]. It has also been reported that Hsp70, another member of the Hsps family, assists in the processing of PMP22 aggregates in TrJ/+ through the Golgi apparatus and their release into Rab7-positive vesicles to the lysosome [47]. It has been observed, both in vivo and in vitro, that there is co-localization of Hsps with PMP22 aggregates [21][47]. In addition, beneficial effects of autophagy, promoted by chaperones and preventing the accumulation of misfolded PMP22, have been reported in TrJ/+ [48]. In CMT2, histone deacetylase 6 (HDAC6) has also been signaled as a potential therapeutic target for the amelioration of the neurodegenerative phenotype, reversing motor and sensory deficits induced by Hsp27 activation [49].

A possible cellular and molecular perspective on the therapeutics of these until now incurable hereditary conditions may focus on cellular drainage or detoxification promoted on the autophagic-lysosomal and UPS-chaperone pathways, together with inhibition or reduction of the mTOR pathway. Furthermore, decreased energy availability is a common key player for the modulation of these pathways. For this reason, caloric restriction (CR) at the neuromotor level has been proposed as a valid therapeutic approach for the alleviation of neurodegenerative conditions (including peripheral neuropathies) [50][51][52][53]. The researchers' group demonstrated that dietary CR activates canonical autophagic pathways by decreasing the levels of aggregated PMP22 and increasing ribophagy in TrJ/+ and in wild-type (+/+) nerves (manuscript in preparation).

CR can be emulated, under certain conditions, by effector molecules of the aforementioned pathways [54][55][56][57][58]. Among them, curcumin, a polyphenol extracted from *Curcuma longa* (Linnaeus, Species Plantarum 1:2. 1753), has shown promising results [59][60]. This compound is used as an antitumor, anti-inflammatory, antioxidant, among other beneficial effects. This wide spectrum of curcumin applications depends mainly on the dosage used and the time of application of the treatments. For example, at high concentrations, which in culture range from 25 µM to

160 µM, curcumin is used as a potent anti-tumoral [61][62]. At low concentrations, it decreases reactive oxygen species (ROS) (in myoblast cell cultures 4 µM curcumin and SC from 0.001 to 1 µM curcumin) [63][64], show anti-inflammatory effects at decreasing signal transducer and activator of transcription 3 (STAT3) activation (in human multipotent adipose tissue-derived stem, 10 µM curcumin) [65], and promotes autophagy by inhibiting acetyltransferases (glioblastoma multiforme cell line, 10 µM curcumin) [59][66] and cell regeneration (primary myoblast culture, 1 µM curcumin) [67][68]. Interestingly, in TrJ mice, there is evidence that curcumin treatment can improve the neurodegenerative phenotype [69]. On the other hand, CR activates the autophagy process, prevents the formation, and promotes the elimination of PMP22 aggregates in cultured SCs [70]. However, little is known about the effect of curcumin and ethanol (EtOH) used as curcumin vehicle in the modulation of PMP22 aggregates, and whether this effect could activate effector molecules in SCs ameliorating the neurodegenerative condition of TrJ mice.

2. Current Insights

PMP22 expression has been studied both in vivo, using different animal models [19][25][48][71][72][73][74], and in vitro in SC cultures [24][63][75], respectively. These approaches denote different but complementary physiological conditions. While in vivo approaches allow understanding how and where the main expression of this protein is located in SCs arrested in G0, the in vitro studies allow the evaluation of the expression in those cells that are in a proliferative state. One of the contributions of the researchers' work lies in the evaluation of the basal culture conditions for the expression of PMP22 in TrJ/+ SCs, compared with that of +/+, discriminating the nuclear and cytoplasmic compartments. Overall, in both cellular domains, PMP22 expression was higher in TrJ/+ SCs compared to +/+ SCs. This result is in line, not only with works reporting the existence of cytoplasmic aggregates of PMP22 in TrJ/+ nerves [29][69][73][74] but also in agreement with previous work from the researchers' group, which determines the PMP22 expression in +/+ and TrJ/+ SCs inside the nucleus [8][9].

The low dose of curcumin treatment was applied as a possible strategy to stimulate cellular detoxification pathways, and thus alleviate the neurodegenerative phenotype. The researchers' curcumin treatment had an additional effect caused by the EtOH vehicle (used to let cur-cumin solubilization for ulterior cell entry), in both +/+ and TrJ/+ SCs. This collateral effect, observed after six days of treatment, led the researchers to inquire about the use and, more importantly, about the validation of the vehicle so that it could be used without specific effects. In this sense, the recommended vehicles for solubilization of curcumin by the manufacturer are ethanol and dimethyl sulfoxide (DMSO). Although in many papers the effects of the sol-vents on culture viability are not shown [52][64][65][76] (or are not discussed [62][77][78]), if they do not present cytotoxic effects, the reality is that both vehicles produce effects that vary widely depending on the cell type and duration of treatment [77][79][80][81]. The pre-established idea of the absence of the effects of these vehicles is recurrent in the literature. However, the contribution of the vehicle prevents the researchers from clearly discriminating the real effect and pharmacological potential of curcumin. For this reason, it must be settled as this constitutes a central point, to circumscribe the results only to the applied treatment. The researchers have tested the impact of DMSO as a vehicle of curcumin on fibroblast from +/+ sciatic nerves and the researchers' preliminary results seem to indicate an equivalent effect com-pared to that of EtOH. In

the researchers' primary results, different concentrations of DMSO were test-ed and in all cases, after five days of DMSO exposure, the viability and proliferation were significant differences compared with the untreated control. In future work, the researchers will seek to determine other strategies that allow solubilizing and targeting curcumin, evaluating at each step the cell viability to corroborate a negligible effect of the vehicle.

The impact of curcumin on the studied pathways in +/+ SC could not be analyzed in the TrJ/+ genotype, because all markers showed no significant difference between EtOH control and curcumin treatment. The results highlight the lability of the TrJ/+ genotype, expressed in its poor capacity to recover from the impact of the vehicle. In the literature, ethanol has been reported to increase ROS and mitochondrial dysfunction [82][83][84]. In zebrafish, at concentrations of 1% ethanol [82], a differential effect in mitochondrial function, with acute and chronic treatment, has been described. However, different performances showed that the mechanisms triggered are also dependent on administration protocols. Since the MTT assay is based on the conversion of tetrazolium to formazan by mitochondrial dehydrogenases, the researchers do not rule out the possibility that ethanol also affects the mitochondria in Schwann cells. Therefore, the increase in the ethanol control relative to the negative control could be due to dehydrogenases' functionality changes in response to the vehicle, rather than the normal increase in viability. In this sense, the fact that both genotypes showed equivalent responses in ethanol control to the negative control, supports this hypothesis.

In addition, the results found by the researchers' group show mitochondrial differences in the nerves of +/+ and TrJ/+ mice. From the analysis of electron microscopy images, the researchers obtained the number of mitochondria per fiber in the axonal and SC domains. These results show that there are differences in the number of mitochondria when comparing SCs of +/+ vs. TrJ/+ fibers. The morphological analysis considering the largest and smallest diameters of mitochondria, also showed apparent differences between +/+ and TrJ/+. Although there is a correlation between both parameters for the two genotypes, the equations representing the linear correlation are different. Furthermore, when looking at the genome expression, a qPCR analysis of the cytochrome b gene transcript level shows a higher amount of the transcript in +/+ compared to TrJ/+.

Thus, the study of the vehicle takes on particular relevance for in vitro approaches to the autophagy/mTOR and chaperone pathways in the TrJ/+ neurodegenerative genotype.

In the present work, the effect of ethanol was visualized equally between +/+ and TrJ/+ SCs, and the percentage of viability was calculated taking the ethanol control as 100%. The latter allowed the researchers to obtain the effect of curcumin to analyze the data. Despite the side effect of ethanol, the researchers were able to determine a 0.25 µM curcumin as the lowest concentration that showed no difference in viability to the ethanol control.

From the study of the heat-stress markers' response pathway, the researchers were able to establish in +/+ SC the HSF1 and Hsp27 expression concordant with that reported in the literature. The HSF1 functions as a transcription factor, which under stress situations is activated and translocated to the nucleus, inducing the expression of the pathway's effectors such as Hsp27 [85][86][87][88] Furthermore, curcumin treatment allowed the researchers to observe an increase in HSF1 at the nuclear level and a decrease at the cytoplasmic level, together with an

increase in Hsp27, indicating a possible activation of this pathway. In addition, HDAC6 expression increased after treatment with curcumin. This increase of the protein, a member of basal autophagy in-volved in the selective elimination of aberrant protein aggregates [89][90] suggests that in the wild-type genotype, curcumin treatment may be stimulating this degradation pathway. Conversely, the increase in ribosomal expression under mTOR regulation [91][92] supports the idea of a favorable nutritional and energetic context in +/+ SC after curcumin treatment.

The impact of curcumin on the studied pathways in +/+ SC could not be analyzed in the TrJ/+ genotype because all of the markers showed no significant difference between the EtOH control and curcumin treatment. These results indicate a TrJ/+ genotype lability that prevents SCs from recovering from the ethanol shock. Thus, the study of the vehicle takes on particular relevance for in vitro approaches to the autophagy/mTOR and chaperone pathways in the TrJ/+ neurodegenerative genotype.

On the horizon of the TrJ/+ in vitro approaches, and its response to the action of different neuroprotective and anti-inflammatory agents, such as curcumin, the analysis of the effects of the vehicle itself is an essential, inescapable, and conditional step for the fine-tuning of the experimental strategy to be applied.

3. Conclusions

The researchers' work established a new experimental strategy for obtaining enriched cultures of SC from +/+ and TrJ/+ mice. The researchers were able to determine a curcumin concentration with no effect on viability in both genotypes for an extended period of time, which allowed the researchers to study the expression of key autophagic-pathway markers in the accumulation of PMP22 protein in SCs. The researchers found an intrinsic ethanol effect in +/+ and TrJ/+ SC that was reversed by curcumin treatment in +/+, but not in TrJ/+ SC. These in vitro cultures allow pre-clinical investigations of promising therapeutic strategies or pharmacological compounds such as curcumin, for the alleviation of human-related peripheral neuropathies.

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