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Oenothera biennis cell culture produce lignans activating Piezo1 triggering the Myosin Light Chain Kinase depending pathways

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ABSTRACT

Piezo1 and Piezo2 are mechanoreceptors involved in sensing both internal and external mechanical forces converting them in electrical signals to the brain.

Piezo1 is mainly expressed in the endothelial system and in epidermis sensing shear stress and light touch. The internal traction forces generated by Myosin Light Chain Kinase (MYLK) activate Piezo1, regulating cell contraction.

We observed *Oenothera biennis* cell culture hydro-soluble extract (ObHEx) activated MYLK regulating cell contraction ability. The aim of this work was to test the hypothesis that ObHEx activates Piezo1 through MYLK pathway using CHO cell overexpressing Piezo1, HUVEC and SHSY5Y cells endogenously expressing high levels of Piezo1. Results showed that ObHEx extracts were able to activate Piezo1 and the effect is due to Liriodendrin and Salvadoraside, the two most abundant lignans produced by the cell culture. The effect is lost in presence of MYLK specific inhibitors confirming the key role of this pathway and providing indication about the mechanism of action in Piezo1 activation by lignans.

In summary, these results confirmed the connection between Piezo1 and MYLK, opening the possibility of using lignans-containing natural extracts to activate Piezo1.

1. Introduction

The perception of mechanical stimuli, which is ubiquitous in all cell systems, needed a rapid signal transduction. The phenomenon is named mechanotransduction (MT) and it is mediated by mechanosensitive (MS) ion channels, preserved during evolution, to feel sensations and to respond to the surrounding environment [1]. Among MS pathways, a pivotal role is played by Piezo receptors belonging to a family of evolutionally maintained ion channels, able to translate mechanical force into electrical signals (gating model) [2].

Piezo1 and Piezo2 are assembled as a helicoidal core built by homotrimers with a central pore that allows the transport of calcium cations, an extracellular cap and a helical ring at the C-terminal portion [3]. Many studies have confirmed the prominent role of Piezo channels in numerous mammalian organs and systems [4–7], as for example in endothelial cells in which Piezo1 regulates arterial tone, blood pressure and response to shear stress [8].

Investigation of Piezo receptors in skin physiology is still in an early stage, the skin is involved in the transduction of tactile information to the central nervous system. The expression of Piezo2 is limited to the Merkel cells in which it is required for touch sensation [9].

Piezo1 is expressed in epidermis, and it is related to keratinocyte mechanical sensitivity [10].

It was demonstrated that mice knock out for PIEZO1 lose the firing rate of sensory nerve fibers in response to mechanical stimulation of the skin and blunts behavioral responses to both innocuous and noxious mechanical stimuli *in vivo*. Epidermal Piezo1 is critical for the physiological touch sensation [11] and the role of Piezo1 in skin barrier integrity and skin sensory perception was recently reported [12].

Two models have been proposed to understand the gating mechanism of MS channels: the membrane tension model and the tether model [13,14]. The identification of two polycyclic aromatic molecules, named

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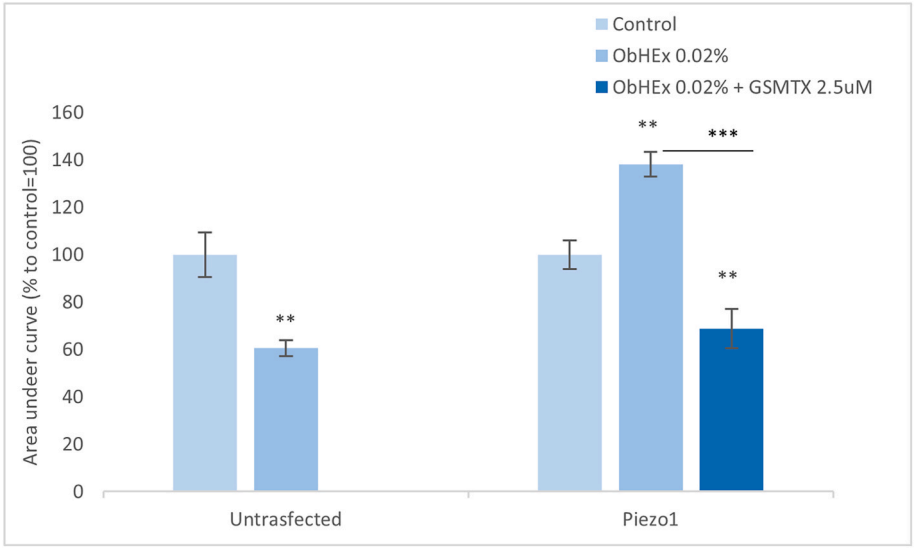

Fig. 1. Calcium influx detection in HsPiezo1 overexpressing CHO cells. Intracellular Ca^{2+} measurements in CHO cells transfected with HsPiezo1 encoding vector and exposed to ObHEx alone or in presence of Gsmtx 2.5 μM. Control samples were treated with HBSS medium alone.

Yoda1 and Jedi 1/2, as specific agonists of Piezo1 channels, sheds new light on the possibility of an exogenous modulation of these channels, independently from mechanical stimuli [15,16]. Jedi 1/2 activates Piezo1 through its extracellular side instead of the C-terminal extracellular domain of the pore, indicating a long-range allosteric modulation to gate opening. Ellefsen and co-workers revealed that Piezo1-dependent calcium signals were prompted by the activated cytoskeletal Myosin II, previously phosphorylated by Myosin Light Chain Kinase (MYLK) [17]. They found the activation of Piezo1 by an intracellular force generated by Myosin II motors, for the first time.

MYLK phosphorylates a specific Myosin II domain able to promote, among the other functions, the cell contraction [18]. A low MYLK activity is related to a reduction of matrix collagen contraction and actin polymerization [19]. This down-regulation decreases the production of collagen and of other extra-cellular matrix (ECM) proteins, leading to loss of dermal mass and skin fragility. Finally, MYLK synthesis decreased significantly with aging. We recently reported hydrophilic extract of *Oenothera biennis* cell cultures (ObHEx) to promote matrix collagen contraction, actin polymerization, and the production of essential ECM proteins such as phospho-Myosin by increasing MYLK gene expression [20]. This prompted us to investigate the hypothesis that the action of ObHEx could involve Piezo1 modulation.

ObHEx has been chemically characterized by advanced mass spectrometric-based approaches, assisted by bioinformatic tools [20]. ObHEx extract contains bioactive compounds belonging to several classes of secondary metabolites, mainly to lignans as Salvadoraside (8 μg/g) and Liriodendrin (50 μg/g) and to triterpenes as Myrianthic acid, Arjunolic acid, Asiatic acid, and Hederagenin.

The aim of this work is to test the effect of ObHEx and of purified lignans to regulate Piezo 1 receptor activity using a mammalian cell lines screening platform. Piezo1 overexpressing mammalian cell lines were used to measure Piezo1-mediated Ca^{2+} entry in absence of mechanical stimulation. Specific inhibitors of Piezo activation and MYLK pathways were used to confirm the mechanism of action and to disclose the ObHEx ability to modulate the actomyosin-based traction forces.

2. Materials and methods

2.1. Cell culture

Human Umbilical Vein Endothelial Cells (HUVEC) were grown in Endothelial Growth Medium (EBM), and Chinese Hamster Ovary cells (CHO) were grown in DMEM/F12 plus 10% FBS. Human neuroblastoma cells (SHSY5Y) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS. All cell cultures were maintained at 37 °C in 5% CO_2 humidified air.

2.2. Transient transfection and calcium influx assay

1×10^4 CHO cells were seeded in a 96 well plate and transfected with 50 ng of human Halo Piezo1 plasmid (Promega) using the reagent X-tremeGENE™ HP DNA Transfection Reagent (Roche). 48 h after transfection, the cells were screened for calcium influx assay. The cells were incubated with 2,5 μM Fluo3/AM for 45' at 37 °C. Cells were then washed with HBSS and agonists/antagonists were added. Fluorescence was immediately read using the VICTOR Nivo microplate reader (PerkinElmer) (excitation 480/30 nm, emission 530/30 nm) every second for 1 min. Results were analyzed by calculating the area under the curve (AUC). The same protocol was used for HUVEC and neuroblastoma cell lines.

2.3. Statistical analysis

All experiments were performed at least three times in triplicate. Graphs and statistical analysis were generated using Excel (Microsoft). The bars represent the standard deviations while asterisks indicate significant variations (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$), according to Student's t-test.

3. Results

A screening platform to monitor Piezo1 activation was set up by overexpressing human Piezo1 in CHO cells and monitoring intracellular calcium in response to molecule stimulation [21]. The system is sensitive and specific: Yoda stimulation caused calcium response reduced in presence of Gsmtx4, a non-specific mechanoreceptor inhibitor as observed by other authors [22].

3.1. Effect of ObHEx and its components on calcium influx in Piezo 1 overexpressing cells

As reported in Fig. 1, ObHEx increased the entrance of Ca^{2+} by 40% when used at 0.02% while control cells, transfected with an empty vector, were completely insensitive. The ObHEx effect was reversed by

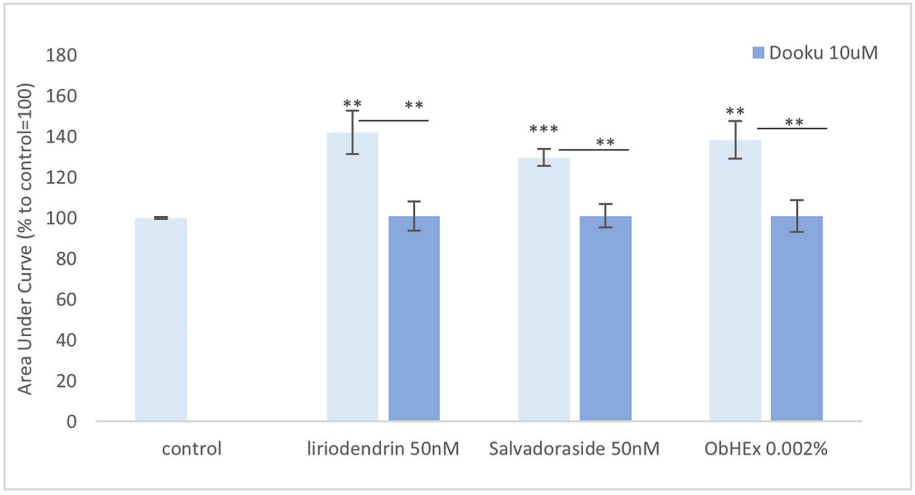

Fig. 2. Calcium influx detection in HsPiezo1 overexpressing CHO cells. Intracellular Ca^{2+} measurements in CHO cells transfected with HsPiezo1 encoding vector and exposed to Liriodendrin 50 nM, Salvadoraside 50 nM, ObHEx alone or in presence of Dooku1 10 μM. Control samples were treated with HBSS medium alone.

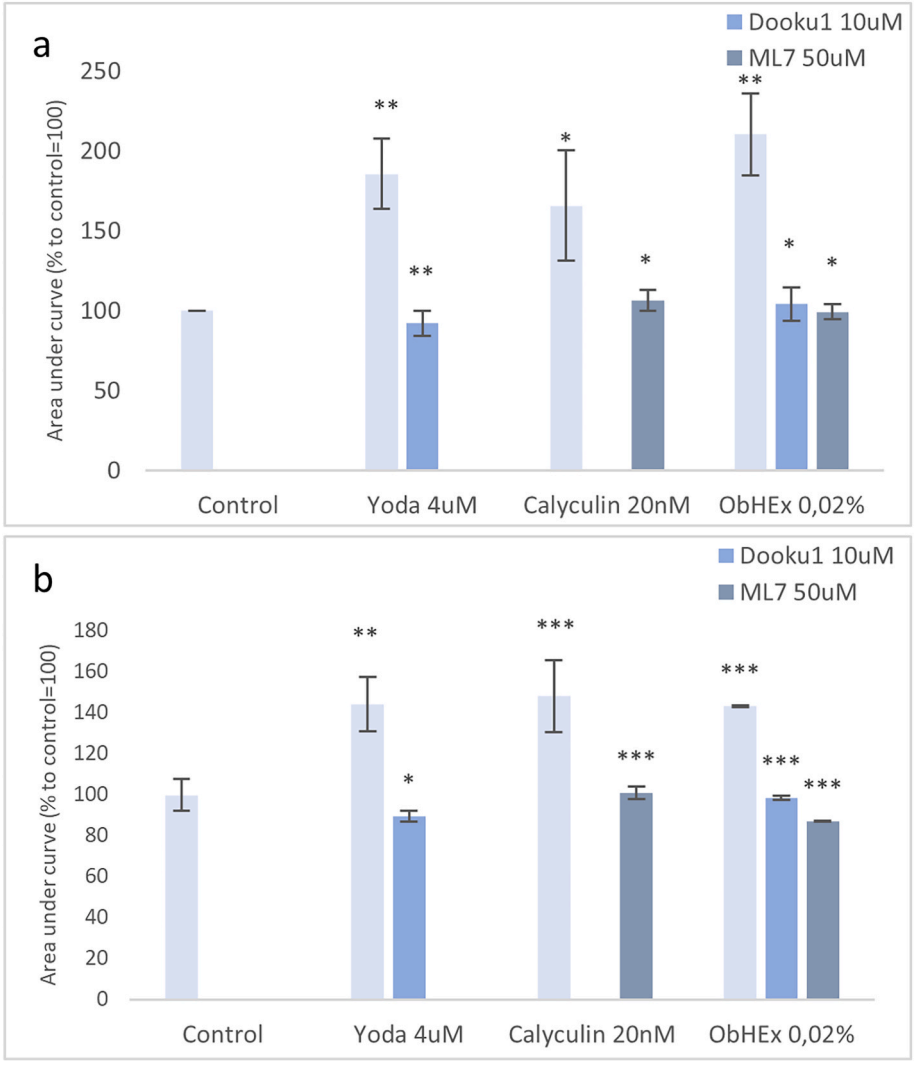

Fig. 3. Calcium influx detection in HUVEC and SHSY5Y cells. Intracellular Ca^{2+} measurements in cells endogenously expressing Piezo1 channels treated with Yoda1 4 μM, Calyculin A 20 nM or ObHEx 0,02% alone or in presence of specific inhibitors. Control samples were treated with HBSS medium alone. 3a HUVEC, 3b SHSY5Y.

adding 2.5 μM Gsmtx4.

Liriodendrin and Salvadoraside, the main lignans in ObHEx, are able to stimulate the calcium influx in Piezo1 overexpressing CHO cells. As reported in Fig. 2, the addition of 50 nM of both compounds increased calcium influx at the same level of ObHEx, around 38%. The stimulation is completely abolished in presence of 10 μM of Dooku1, an antagonist of YODA1 activation of Piezo1. This indicated lignans have a Yoda-like mechanism in the activation of Piezo1.

3.2. Calcium influx activation in Piezo1 endogenously expressing cells

To elucidate the mechanism leading to Ca^{2+} influx inside the cells, a calcium influx assay in HUVEC and SHSY5Y, which are known to express Piezo1 [23,24], was performed. In addition to Yoda and ObHEx, we also tested calyculin A, a known MYLK activator [25]. The data of these experiments were reported in Fig. 3a and b. In both cell lines, 4 μM of Yoda1 significantly increased calcium influx and the same effect was obtained by 20 nM of calyculin A. This evidence clearly pointed out the key role of MYLK in Piezo activation. To confirm this connection, we used two selective inhibitors, Dooku1 (10 μM) for Yoda1 activated Piezo1 and ML7 (50 μM) for MYLK pathway: in both cases the effect was specifically reverted [26,27]. Interestingly, the specificity of the inhibitors was maintained also when the induction of Ca^{2+} influx was triggered by ObHEx extract: both Dooku1 and ML7 were able to completely revert the Piezo1 activation.

4. Discussion

Piezo1 and Piezo2 are mechanically gated, non-selective cation channels that share approximately 42% amino acid similarity and are widely expressed in tissues that respond to mechanical force as lung, skin, bladder, and vascular tissue, among others. Piezo1 is mainly expressed in epidermis and in endothelial system having a prominent role in sensing touch and blood pressure.

There is no evidence of natural products able to regulate the function of Piezo1, thus far. We previously found *Oenothera biennis* cell culture hydrosoluble extract (ObHEx) is able to activate MYLK and regulate skin cell contraction [20]. We hypothesized this effect also involving Piezo1 and the above showed results confirmed ObHEx is a modulator of the Piezo1 channel. We also demonstrated that this effect is due to Liriodendrin and Salvadoraside, the two most abundant lignans present in ObHEx. They were able to increase calcium influx mediated by Piezo1: the effect was abolished in presence of Dooku1, a selective antagonist of the endogenous Piezo1 channel suggesting ObHEx has a Yoda1-like mode of action. To better understand the mechanism of action, we looked at the ObHEx effect in cells endogenously expressing high levels of Piezo1, namely HUVEC and SHSY5Y cells. Data showed ObHEx treatment in both endothelial and neuronal cells significantly increased intracellular flux of Ca^{2+} ions and that this effect was abolished when cells were co-treated with GsMTx4 or Dooku1, two specific mechanoreceptor inhibitors. MYLK mediated Myosin II phosphorylation was involved in triggering Piezo1 gating and so we speculated that ObHEx could activate Piezo1 through MYLK [17]. The involvement of MYLK pathway in Piezo1 activation was confirmed by treating cells with calyculin, a known activator of MYLK, induced calcium influx mediated by Piezo1. Our hypothesis was fully confirmed showing that MYLK pathway activation by ObHEx was inhibited by ML7, a selective MYLK pathway inhibitor.

In conclusion, our data demonstrated ObHex effectively regulates Piezo1 activation through traction forces generated by Myosin II phosphorylation by MYLK. The action is mainly due to the lignan compounds present in the ObHex extracts, namely Liriodendrin and Salvadoraside. Ellefsen et al., already in 2019 [17] hypothesized the presence of a feedback loop in which traction forces activating Piezo1 become stronger as a result of this Piezo1-dependent calcium signaling. We now discovered that lignans are involved in this feedback loop. Their ability of activating Piezo1 calcium signal is abolished in presence of both Piezo1 and MYLK inhibitor indicating that ObHEx could regulate Piezo1 following MYLK but also directly Piezo1 generating MYLK activation.

Lignans can be used as natural regulator of Piezo1 channels and actomyosin-based traction forces as we showed ObHEx modulates upstream signaling mechanisms involved both in the phosphorylation of Myosin II by MYLK and the activation of Piezo1 channels.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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