Purinergic Signalling
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BRIEF COMMUNICATION

Loss of P2X7 receptor function dampens whole body energy expenditure and fatty acid oxidation

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Abstract
The established role of ATP-responsive P2X7 receptor in inflammatory, neurodegenerative, and immune diseases is now expanding to include several aspects of metabolic dysregulation. Indeed, P2X7 receptors are involved in β cell function, insulin secretion, and liability to diabetes, and loss of P2X7 function may increase the risk of hepatic steatosis and disrupt adipogenesis. Recently, body weight gain, abnormal lipid accumulation, adipocyte hyperplasia, increased fat mass, and ectopic fat distribution have been found in P2X7 KO mice. Here, we hypothesized that such clinical picture of dysregulated lipid metabolism might be the result of altered in vivo energy metabolism. By indirect calorimetry, we assessed 24 h of energy expenditure (EE) and respiratory exchange ratio (RER) as quotient of carbohydrate to fat oxidation in P2X7 KO mice. Moreover, we assessed the same parameters in aged-matched WT counterparts that underwent a 7-day treatment with the P2X7 antagonist A804598. We found that loss of P2X7 function elicits a severe decrease of EE that was less pronounced in A804598-treated mice. In parallel, P2X7KO mice show a drastic increase of RER, thus indicating the occurrence of a greater ratio of carbohydrate to fat oxidation. Decreased EE and fat oxidation is predictive of body weight gain, which was here confirmed. Taken together, our data provide evidence that P2X7 loss of function produces defective energy homeostasis that, together with disrupted adipogenesis, might help to explain accumulation of adipose tissue and contribute to disclose the potential role of P2X7 in metabolic diseases.

Keywords Purinergic signaling · P2X7 receptor · Energy metabolism · Energy expenditure · Fat acids oxidation · Obesity

Introduction

The purinergic signaling system encompasses a complex network of membrane receptors among which the P2X7 receptor (P2X7) subtype, a member of the ionotropic family of ligand-gated cation channels [1]. Endogenously activated by high concentrations of extracellular ATP, the P2X7 enables the entry of Ca^{2+} and Na^{+}, and the outward flow of K^{+} across the plasma membrane [1] and, upon repeated stimulation, the opening of a larger membrane pore that impacts on cell homeostasis [1]. Since broadly distributed in immune cells, the P2X7 has been associated with immune responses, inflammatory processes, and cancer [2]. Being involved in neuroinflammation [2], the blockade of P2X7 has been exploited in several neurodegenerative and neuropsychiatric conditions, such as Alzheimer's [3] and Parkinson's diseases [4]. Recently, we highlighted the role of P2X7 in multiple sclerosis [5] and amyotrophic lateral sclerosis [6]. Moreover, the emergent role of P2X7 in β cell function and insulin secretion [7], as well as in type 1 diabetes [8] and diabetes-induced co-morbidities [9], further expands the clinical impact of P2X7-mediated signaling. Indeed, impairment of glucose and lipid metabolism is of clinical significance not only for diabetes, but also for neurodegenerative diseases and cognitive decline [10]. P2X7 null mice (P2X7KO) are hyperglycemic, and show glucose intolerance after chronic exposure to high fat/high sucrose diet [11]. Hyperglycemia and concomitant increase of serum insulin levels supports the idea of possible insulin resistance in P2X7KO mice, in which it was also observed hepatic steatosis and facilitated glucose delivery into the bloodstream [12]. The critical involvement of P2X7 in regulating energy metabolism has been further demonstrated in P2X7KO mice showing

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higher body weight and increased epididymal fat mass with adipocyte hyperplasia and ectopic lipid accumulation in organs, such as kidney and pancreas [13]. Considering the importance of adiposity, dyslipidemia, and diabetes for obesity burden and metabolic diseases, in this work, we investigated whether loss of P2X7 function alters whole-body energy metabolism, as measured by energy expenditure (EE) and respiratory exchange ratio (RER). We analyzed these parameters in vivo in P2X7KO mice as well as in WT mice administered with the selective P2X7 antagonist A804598.

Materials and methods

Animals

Adult C57BL/6J (WT) and B6.129P2-P2rx7tm1Gab/J mice (P2X7KO), originally obtained from Jackson Laboratories (Bar Harbor, ME, USA), were backcrossed for at least ten generations and experiments were performed with F10 and following generations. Animals were bred in the indoor animal facility in groups of 4–5 mice/cage in standard conditions with free access to food and water, at constant temperature (22 ± 1 °C) and relative humidity (50%), with a regular 12-h light cycle (light 7 AM–7 PM). All animal procedures have been performed following European Guidelines for the use of animals in research (86/609/CEE) and requirements of Italian laws (D.L. 26/2014). The ethical procedure has been approved by the Animal Welfare Office, Department of Public Health and Veterinary, Nutrition and Food Safety, General Management of Animal Care, and Veterinary Drugs of Italian Ministry of Health. All efforts were made to minimize animal suffering and the number of animals necessary for reliable results. To overcome the issue of sex-mixed results and low reproducibility due to the limited number of animals enrolled, in the present work, we used female mice because of the gender differences reported in P2X7KO mice [14].

Western blotting

Protein lysates of mice lung and gut were obtained in homogenization buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 1% Triton X-100, 10 mM EDTA) added with protease inhibitor cocktail (Sigma-Aldrich). After centrifugation at ×14000*g* (20 min at 4 °C) supernatants were collected and assayed for protein content by a Bradford detection kit (Bio-Rad Laboratories, Hercules, USA). Protein analysis was performed by Mini-PROTEAN® TGX™ Gels (Bio-Rad, USA) and transferred onto nitrocellulose membranes. After saturation with 5% non-fat dry milk, blots were probed with rabbit anti-P2X7 (1:500, Alomone Labs, Israel) at 4 °C, and incubated with HRP-conjugated secondary antibody for 1 h. Detections were performed on X-ray film (Aurogene, USA), using an ECL Advance detection kit (Amersham Biosciences, USA) and signal intensity visualized by a Kodak Image Station analysis software. Values were normalized with mouse anti-β-actin (1:2500, Sigma-Aldrich, Italy).

Pharmacological P2X7 receptor blockade

Adult WT female mice were randomly grouped into vehicle-treated (10% DMSO in saline) or P2X7 antagonist A804598-treated mice (Tocris Bioscience, Bristol, UK) [15] and daily intraperitoneally (i.p.) administered with A804598 (90 mg/kg) or vehicle for 7 days.

Energy metabolism

EE, oxygen consumption (VO2), and RER were measured by an indirect calorimeter (IC) system (TSE PhenoMaster/LabMaster System®) with a constant air flow of 0.35 L/min, as described [16]. Twenty-month-old P2X7KO mice, age-matched WT, and A804598-treated WT mice were adapted for 24 h to the metabolic chamber prior recording, and VO2 and VCO2 were measured every 20 min, for total 24 h (12-h dark-light phase comparison). Room temperature was kept constant (22° ± 1 °C).

Statistical analysis

Data are expressed as mean ± standard error of the mean. Depending on the data, statistical analysis was performed either by one-way analysis of variance (ANOVA) or two-way ANOVA for repeated measures followed by Tukey post-hoc test. The level of significance was set as $p < 0.05$.

Results and discussion

The P2X7 engages different functions in distinct cell populations, or even within the same cell type, thus orchestrating complex roles, as for instance in the immune and nervous systems [17]. Recently, a novel role has been established for P2X7 in adipogenesis and lipid metabolism, uncovering abnormal fat distribution in P2X7KO mice [13]. Our results now show that loss of P2X7 function has a major impact on energy metabolism homeostasis. We firstly verified in P2X7KO mice the absence of P2X7 protein from representative peripheral organs. As shown in Fig. 1a, the ~ 80 KDa band corresponding to P2X7 protein was absent in P2X7KO mice lung and gut, as compared to WT mice.

The in vivo IC analysis of energy expenditure (EE) and nutrient substrate oxidation (respiratory exchange ratio, RER) indeed showed a significant decrease of metabolic rate (Kcal/h/kg) in P2X7KO and A804598-treated WT mice

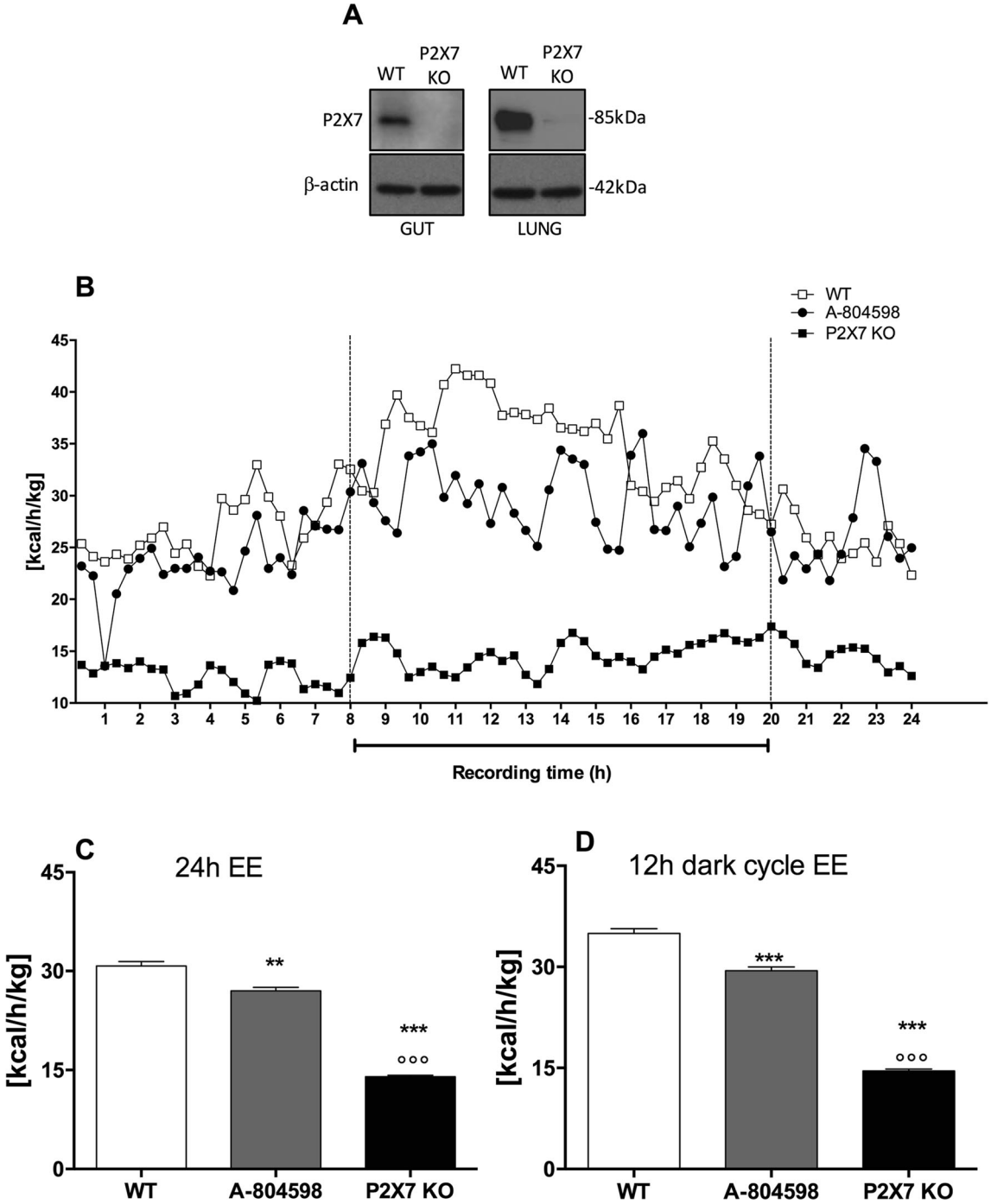

Fig. 1 **a** Equal amounts of gut and lung total lysates from WT and P2X7KO adult mice ($n = 3$) were subjected to western blotting and immunoreactions with anti-P2X7 antibody. Anti-β-actin was used for protein normalization. **b** Mean of pooled data portraying continuous 24-h recording of energy expenditure (EE) in WT ($n = 6$), A804598-treated ($n = 4$) and P2X7KO ($n = 4$) mice via the IC. EE is expressed as Kcal emitted per hour (h)/Kg. Black line below the recording time (*X*-axis) denotes the nocturnal phase of the light/dark cycle. **c** Mean of 24-h whole-body EE (Kcal/k/kg), and **d** mean of 12-h whole-body EE during the dark cycle (**$P < 0.001$ and ***$P < 0.0001$ vs. WT; °°°$P < 0.0001$ vs. A804598)

(Fig. 1b), which was evident on circadian base (Fig. 1c) and more pronounced during the nocturnal phase (Fig. 1d). Notably, the decrease of EE was not attributable to significant changes of food intake and, consequently, to the reduction of food-induced thermogenesis. Moreover, the decrease of EE induced by the subchronic blockade of P2X7 was lower than observed in P2X7KO mice with respect to untreated WT mice (Fig. 1b). Because of the physiological increase of EE during the dark cycle, the reduction of EE observed in A804598-treated mice was more pronounced (Fig. 1d) in this phase with

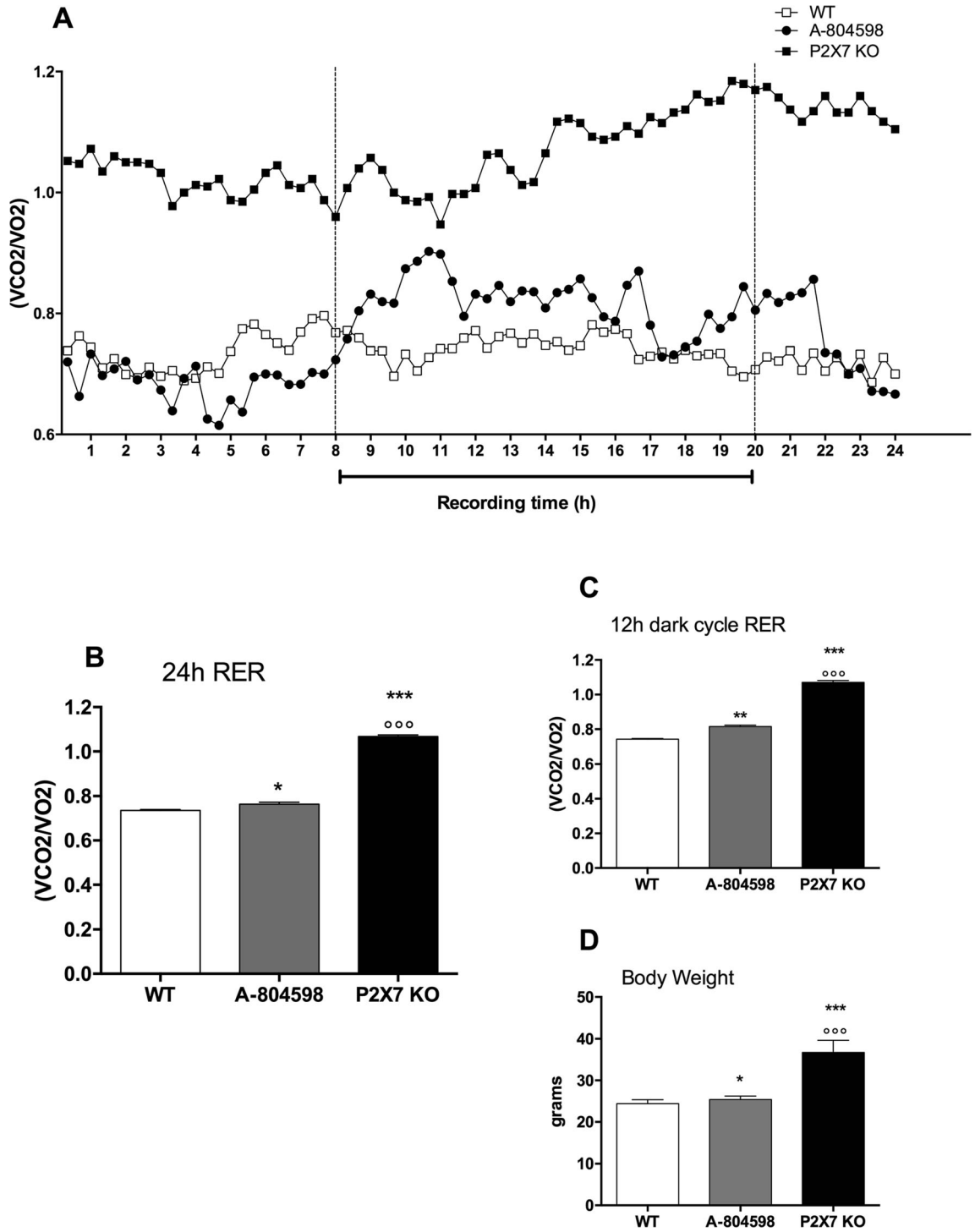

Fig. 2 **a** Mean of pooled data portraying continuous 24-h recording of respiratory exchange ratio (RER) expressed as VCO2/VO2 ratio. **b** Mean of 24-h whole-body RER (VCO2/VO2). **c** Mean of 12-h whole-body RER (VCO2/VO2) during the dark cycle. **d** Mean body weight (BW) collected before and immediately after the end of 24 h IC analysis (*$P < 0.01$, **$P < 0.001$, and ***$P < 0.0001$ vs. WT; °°°$P < 0.0001$ vs. A804598)

respect to the entire circadian phase (Fig. 1c). The RER was significantly elevated by the loss of P2X7 function (Fig. 2a, b) and resulted enhanced, although to a lesser degree, also in A804598-treated mice during the entire circadian period (Fig. 2b, c). The increase of RER denoted a significant shift towards a prevalent increase of carbohydrate oxidation and relative sparing of fatty acids storage in P2X7KO animals. Simultaneous decrease of EE (Fig. 1b–d) and fat oxidation (Fig. 2a–c) produced a significant body weight gain in P2X7KO and, to a lesser extent, in A804598-treated mice

(Fig. 2d). These findings support the recent evidence that loss of P2X7 function generates lipid accumulation, adipocyte hyperplasia, increased fat mass, and ectopic fat distribution and, ultimately, weight gain [13]. We believe that the lack of P2X7 yielding a dramatic decline of EE, and a lower ratio of fat to carbohydrate oxidation, can thus account for both adiposity and lipid accumulation, as previously demonstrated [19]. Thus, our data prove that loss of P2X7 function leads to excessive increase of EE and corroborate what posited by Beaucage and co-workers that establish abnormal fat distribution in P2X7KO mice [13]. Interestingly, the stimulation of P2X7 inhibits adipocytes differentiation [18], thus supporting the dramatic remodeling of fat cells towards the accretion and expansion of white adipose tissue (WAT) depots in mice lacking P2X7 [13]. Cellular and whole-body energy metabolism contribute to several developmental processes, such as osteogenesis and bone remodeling [19]. In keeping with the role of P2X7 in bone formation [14], the activation of P2X7 was shown to produce a sustained increase of cell metabolism (i.e., proton efflux and metabolic acid production) in osteoblasts [20]. In recent years, a variety of adipose tissue factors, such as low-density lipoprotein receptor relative, hypoxia-inducible factor-1α, and inhibitor of differentiation 1 (Id1) have been shown to interfere with brown adipose tissue-mediated non-shivering thermogenesis, suppressing energy expenditure and eliciting obesity [21–23]. It is therefore conceivable that loss of P2X7 may have disinhibit adipocytes differentiation promoting WAT enlargement and ectopic distribution of white cells at the expenses of brown (or beige) adipocytes and thermogenic capacity. The peroxisome proliferator-activated receptor γ (PPARγ) is a well-known master regulator of white and brown adipocytes differentiation, and PPARγ agonists are insulin-sensitizers drugs that can also elicit adipocytes remodeling in WAT, increase mitochondria density, and browning of white adipocytes [24, 25]. Of note, there is evidence that part of the channel opening activity of the P2X7 is regulated by PPARγ [26], for instance in astrocytes. Therefore, the possibility that loss of P2X7 may have a drastic impact on PPARγ-mediated functional activity also in WAT to control adipocyte remodeling should also be considered.

It is known that human adipocytes express functionally active P2X7, whose activation triggers multiple inflammatory responses and increased release of cytokines such as interleukin (IL)-6 [27]. Despite being associated with chronic inflammatory conditions [28], the pathogenetic role of IL-6 is highly controversial [29]. Indeed, several lines of evidence demonstrate that IL-6 deficiency may elicit late-onset obesity [30], hepatic inflammation, and insulin resistance [31], whereas IL-6 signaling facilitates insulin sensitivity, glucose homeostasis, suppression of hepatic inflammation, uncoupling protein 1 expression, and fat oxidation [32–35]. Skeletal muscle is a key organ to account for whole-body EE and fuel utilization [36], and the decrease of muscle pyruvate dehydrogenase activity induced by IL-6 treatment [37] demonstrate that IL-6 signaling can also be responsible for decreased carbohydrate metabolism. Notably, the metabolic phenotype of IL-6 KO mice showing decreased EE and increased RER [38] largely overlaps with our results of P2X7KO mice. The positive correlation between P2X7 expression and IL-6 raises the possibility that reduced IL-6 signaling in P2X7KO mice may contribute to explain reduced EE, increased carbohydrate oxidation, and body weight gain. This will be the object of future investigation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All animal procedures have been performed following European Guidelines for the use of animals in research (86/609/CEE) and requirements of Italian laws (D.L. 26/2014).

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