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Catherine Baugé, Nicolas Girard, Sylvain Leclercq, Philippe Galéra, Karim Boumédiene. Regulatory mechanism of transforming growth factor beta receptor type II degradation by interleukin-1 in primary chondrocytes. *BBA - Biochimica et Biophysica Acta*, 2012, 1823 (5), pp.983-6. 10.1016/j.bbamcr.2012.02.017 . hal-01149560

HAL Id: hal-01149560

<https://hal.science/hal-01149560>

Submitted on 29 Aug 2019

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Regulatory mechanism of transforming growth factor beta receptor type II degradation by interleukin-1 in primary chondrocytes

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ARTICLE INFO

Article history:

Received 23 July 2011

Received in revised form 27 February 2012

Accepted 28 February 2012

Available online 8 March 2012

Keywords:

TGF β receptor

Interleukin-1

Turnover regulation

Caveolin-1

Proteasome

Osteoarthritis

ABSTRACT

Interleukin-1 β (IL-1 β), a key-cytokine in osteoarthritis, impairs TGF β signaling through T β RII down-regulation by increasing its degradation. Here, we investigated the molecular mechanism that controls T β RII fate in IL-1 β treated cells.

Chondrocytes were treated with IL-1 β in the presence of different inhibitors. T β RII and Cav-1 expression were assayed by Western blot and RT-PCR.

We showed that IL-1 β -induced degradation of T β RII is dependent on proteasome and on its internalization in caveolae. In addition, IL-1 β enhances Cav-1 expression, a major constituent of lipid raft.

In conclusion, we enlighten a new mechanism by which IL-1 β antagonizes TGF β pathway and propose a model of T β RII turnover regulation upon IL-1 β treatment.

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1. Introduction

Articular cartilage has a very limited capacity of repair, resulting in progressive and irreversible degradation of this tissue, and subsequently osteoarthritis. This tissue is composed of single cell type, named chondrocytes, which synthesize an abundant extracellular matrix, containing a network of collagen fibers (mainly composed of type II collagen), and proteoglycans (aggrecan). In osteoarthritis cartilage, chondrocytes undergo modifications of their phenotype. In particular, they change their expression profile, notably of transforming growth factor β receptors and collagens. Interleukin-1 β , a pro-inflammatory cytokine which is increased during osteoarthritis, is known to contribute to these processes [1].

Transforming growth factor β 1 (TGF β 1) is a major cytokine that regulates the anabolic process of articular cartilage. It induces its signal through activation of type I (T β RI) and type II (T β RII) receptors. These receptors play a major role in osteoarthritis (OA) [2,3]. Their expression as well as their membrane compartmentalization regulates TGF β 1 signaling [4–6]. However, while regulation of their expression is well documented, little is known about mechanisms which govern their localization and degradation.

They reside in both lipid raft and non-raft membrane domains [4]. Their internalization by classical clathrin-dependent pathway or raft-

caveolin route facilitates Smad activation or mediates receptor degradation, respectively [4]. Caveolae are vesicular invaginations of the plasma membrane formed from a local accumulation of cholesterol, glycosphingolipids and caveolin-1 (Cav-1). Cav-1 is found in hyaline cartilage, and more abundantly in OA tissues. Its knock-out leads to abnormal hyaline cartilage morphology and abnormal chondrocyte physiology [7]. Besides, Cav-1 suppresses TGF β -mediated transcriptional activation by interacting with T β RI [8].

Recent reports have highlighted cross-talk between TGF β and IL-1 β in chondrocytes, with IL-1 β playing a role in the negative regulation of TGF β signaling. In particular, IL-1 β reduces T β RII expression by enhancing its degradation rate [9]. However, the molecular mechanism that controls T β RII decay in IL-1 β treated cells is totally unknown. The aim of this study was to examine the role of T β RII partitioning and ubiquitin/proteasome pathway on the regulation of T β RII expression by IL-1 β .

2. Materials and methods

2.1. Reagents

Reagents were provided by Invitrogen (Cergy Pontoise, France) unless otherwise noted. Human recombinant IL-1 β (Sigma-Aldrich Co., St. Quentin Fallavier, France) was dissolved in PBS-BSA. Cycloheximide (Sigma), nystatin (Calbiochem-VWR, Fontenay sous Bois, France.), bortezomib (PS-341), and leupeptin (Roche, Meylan, France) were resuspended in PBS or DMSO. Antibodies were obtained from Santa Cruz Biotechnology (TeBU-bio SA, Le Perray en Yvelines, France).

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2.2. Cell culture and treatments

Human cartilage was obtained from the femoral heads of 15 patients who underwent hip replacement (ages between 52 and 82 years with median age of 75). Chondrocytes were released and cultured as previously described [9]. They were amplified in 10%FCS-DMEM. Then, cells were kept for 24 h in 2%FCS-containing medium before treatment, which was performed in 2%FCS-medium.

2.3. Protein extraction and Western blot

Proteins (cytoplasmic, membrane and nuclear) were extracted using RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0), and Western blot experiments were performed as previously described [9].

Densitometric analyses were performed with ImageJ software, and histograms represent ratio between TβRII and βactin.

2.4. RNA isolation and real-time RT-PCR

Total RNA was extracted using Trizol reagent and their integrity was checked by migration on agarose gel. Reverse transcription and real-time PCR were performed as previously described [9]. The relative expression was calculated with the $2^{-\Delta\Delta CT}$ method.

2.5. Statistical analysis

All experiments were repeated at least three times, each with different donors. Data are presented as means ± SD. Statistical significance was determined by Student's *t*-test. Differences were

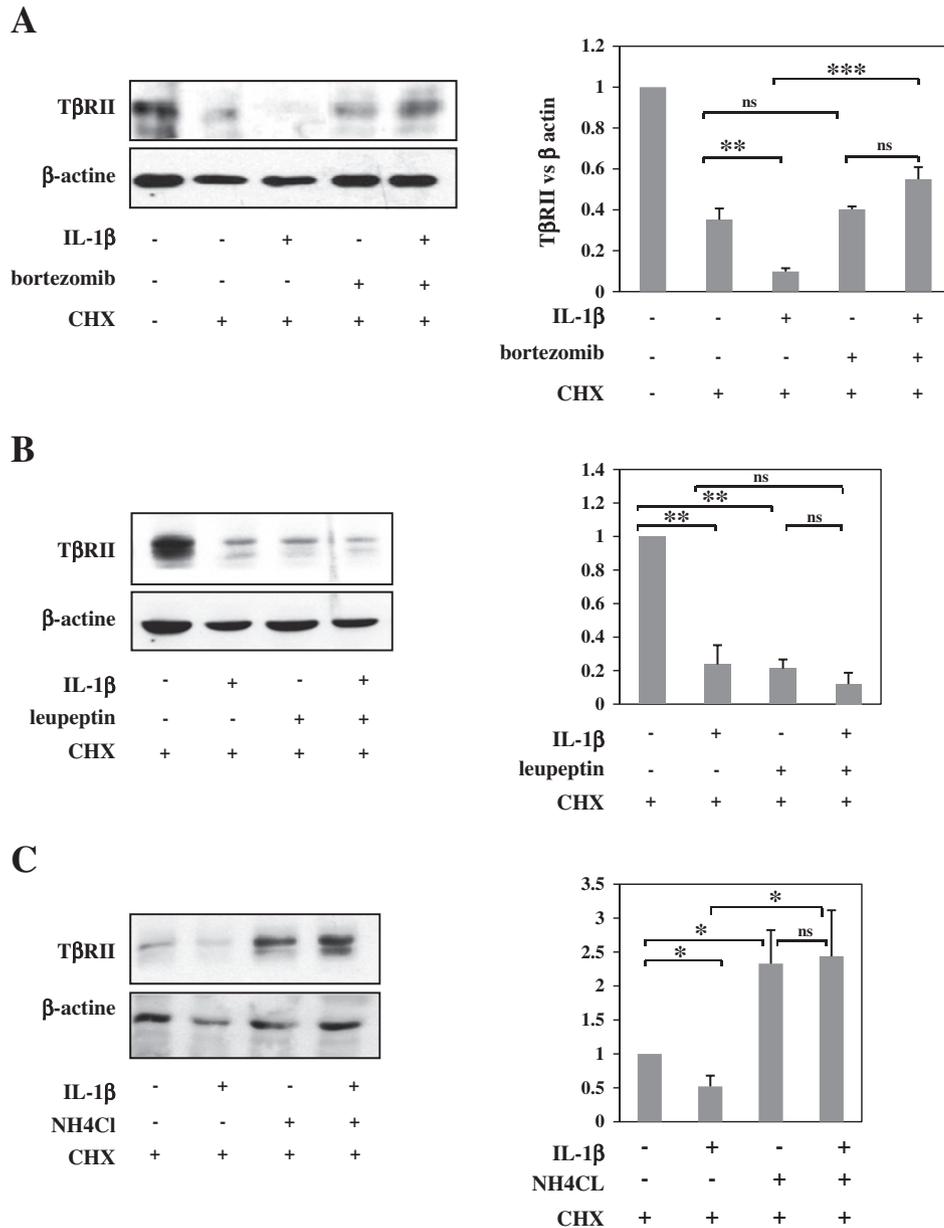


Fig. 1. Bortezomib counteracts the increase of TβRII turnover induced by IL-1β. HACs were cultured for 5–6 days in 10% FCS-containing DMEM. Then, they were incubated in DMEM + 2% FCS for 24 h. Thereafter, they were treated with IL-1β (1 ng/ml) together with cycloheximide (10 μg/ml) for 7 h in the presence of bortezomib (10 nM) (A), leupeptin (100 μg/ml) (B) or ammonia chloride (20 mM) (C). Then, proteins were extracted and TβRII expression was analyzed by Western blot and quantified by densitometry. Histograms represent the mean of values relative to control, and SD of three experiments. ****P*<0.001; ***P*<0.01; **P*<0.05 versus controls.

considered to be statistically significant at $P < 0.05$. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ versus controls; NS: no significant difference.

3. Results

3.1. IL-1 β -induced degradation of T β RII is reduced by proteasome and lysosome inhibitors

Proteolysis of membrane receptor, such as TGF β receptors, is mediated by both the proteasome and lysosome. To test whether the enhanced degradation of T β RII upon IL-1 β treatment occurred through proteasome pathway, we assessed T β RII turnover in the presence of bortezomib, a proteasome inhibitor. T β RII levels were very similar with and without proteasome inhibitor, but in the presence of IL-1 β , bortezomib led a significant difference. This treatment totally counteracted the increase of T β RII turnover induced by IL-1 β (Fig. 1A), indicating that IL-1 β induced degradation of T β RII is dependent on proteasome pathway.

Similarly, T β RII degradation induced by IL-1 β was evaluated in cells treated with leupeptin or ammonia chloride, two inhibitors of lysosomal degradation. Surprisingly, under cycloheximide, leupeptin induced T β RII degradation whereas ammonium chloride treatments reduced it. Interestingly, both treatments reduced the IL-1 β induced degradation (Fig. 1B and C). These experiments indicate that T β RII degradation induced by IL-1 β is dependent on proteasome and impaired by lysosome inhibitors.

3.2. IL-1 β -induced degradation of T β RII is dependent on caveola trafficking

To evaluate the importance of the trafficking of TGF β receptors via the lipid raft in decay of the receptor upon IL-1 β treatment, we followed the degradation of T β RII in cells incubated with nystatin, which induces depletion of membrane-associated cholesterol and consequently disruption of both lipid rafts and caveolae whereas it does not interfere with clathrin-dependent endocytosis [4]. Nystatin, by itself, did not affect T β RII degradation (lanes 2, 4, and 6). However, in the presence of IL-1 β , it counteracted T β RII degradation (lanes 3–5). This effect was dependent on chelation of cholesterol, as the addition of cholesterol to nystatin-treated cells restored receptor turnover in the presence of IL-1 β (lanes 6–7) (Fig. 2).

3.3. IL-1 β increases caveolin-1 expression

Since receptor trafficking may be modulated by caveolin-1 expression, we investigated Cav1 expression in IL-1 β -treated chondrocytes. Fig. 3 shows that caveolin-1 mRNA level is increased by IL-1 β . This effect appeared as soon as 1 h of incubation and persisted for at least 72 h. Therefore, we investigated Cav1 protein level by Western Blot. Again, as at mRNA level, we found that IL-1 β increases Cav1 protein expression.

4. Discussion

This paper enlightens a new mechanism by which IL-1 β downregulates T β RII expression in human chondrocytes. We show that IL-1 β increases T β RII decay through receptor-mediated endocytosis by raft lipid and proteasomal pathway. Interestingly, intra-articular injections of MG132, a proteasome inhibitor, significantly reduces OA lesions in animal OA model [10]. So, our study may explain part of the protective effect of MG132.

Since degradation of proteins by the proteasome pathway is dependent on their ubiquitination, the regulation of T β RII degradation might involve specific E3 ubiquitin ligases. Among them, Smurf2 is interesting. It opposes to TGF β signal in inducing mediators

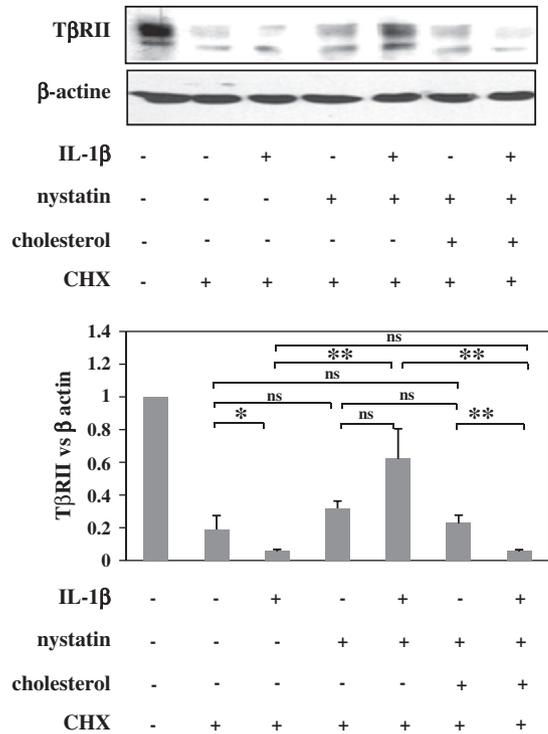


Fig. 2. Nystatin counteracts the IL-1 β -induced degradation of T β RII. HACs were cultured as Fig. 1. Then, they were treated with IL-1 β (1 ng/ml) together with cycloheximide (10 μ g/ml) for 7 h in the presence of nystatin (50 μ g/ml) and cholesterol (25 μ g/ml). Then, proteins were extracted and T β RII expression was analyzed by Western blot and quantified by densitometry. Histograms represent the mean of values relative to control, and SD of three experiments. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ versus controls. NS: no significant difference.

degradation through ubiquitin/proteasome pathway [11,12]. In particular, Smurf2 is able to interact with Smad7 which functions as an adaptor protein that recruits Smurf2 to the activated TGF β receptor complex to promote its degradation [12]. It also negatively regulates TGF- β signaling by attenuating the activity of Smad3 [13]. Additionally, Smurf2 is up-regulated during OA, and Smurf2-transgenic mice spontaneously develop an OA-like phenotype, which is similar to transgenic mice that express a dominant-negative mutant of T β RII [11,3].

Another candidate is NEDD4 family, which is known to negatively regulate TGF-beta superfamily signaling [14]. Of interest,

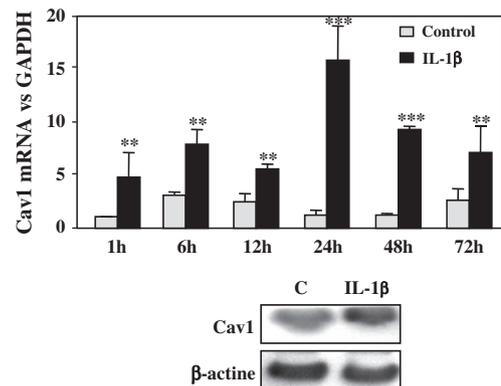


Fig. 3. IL-1 β increases Cav-1 expression. HACs were incubated in DMEM + 2% FCS for 24 h, before addition of IL-1 β (1 ng/ml) or vehicle (control) for indicated times. mRNA levels of caveolin-1 gene were assayed by real-time RT-PCR. Results are shown as histograms of relative expression versus GAPDH signal (A). Similarly, HACs were treated with IL-1 β (1 ng/ml) for 48 h. Then, proteins were extracted and Cav1 expression was analyzed by Western blot (B).

Nedd4 is abundant in lipid rafts [15] and abundantly expressed in proliferating and prehypertrophic chondrocytes [16]. So, since T β R2 degradation depends on traffic in lipid rafts, we suggest that Nedd4 family members could be responsible of this phenomena. Further experiments will be required to determine which E3 ubiquitin ligase is involved.

Concerning the putative role of lysosomal degradation, our experiments cannot permit to definitively conclude. Indeed, in the presence of leupeptin and ammonium chloride, two lysosomotropic agents, we observed a reduction of the IL-1 β induced degradation of T β R2 induced by IL-1 β . However, we cannot exclude an indirect action of these lysosomal inhibitors on proteasome. Indeed, inhibition of lysosomal functions reduces proteasomal activity [17]. In addition, we found a differential effect of these lysosomotropic agents. NH₄Cl reduced T β R2 degradation, whereas leupeptin increased it. This differential effect on receptor degradation (without IL-1 β treatments) may be due to differential functions of these inhibitors. Indeed, NH₄Cl affected the endosomal and lysosomal compartments, whereas leupeptin had a preferential effect on lysosomes [18]. Further experiments will be required to better characterize the role of endosome/lysosome pathways. Moreover, in this study, we used protein extracts obtained from cell lysis with Ripa buffer. Consequently, we analyzed the global level of T β R2 (present in cytoplasmic, nuclear and membrane fractions) and not the membrane fraction of the receptor. Using membrane extracts, and subcellular fractionation techniques may be useful to improve our understanding of the mechanisms of T β R2 internalization and degradation.

In agreement with observations of Veluthakal and colleagues, who indicate a significant translocation of Cav-1 into Cav-1 rich compartments from isolated cells after brief exposure to IL-1 β [19], we established that IL-1 β enhances Cav-1 expression. This induction of Cav-1 could promote T β R2 traffic in lipid raft and subsequently increase the turnover of this receptor upon IL-1 β treatment. In this regard, similarly to T β R1, T β R2 may physically interact with Cav-1. Indeed, T β R2 cofractionates with caveolin-1 in caveola enriched-microdomains [7]. This Cav1/T β R2 interaction could presumably occur via the T β R2 serine/threonine kinase domain, because functional caveolin-binding motifs have been deduced in this domain [20]. Furthermore, consistent with our hypothesis, expression ectopic of caveolin-1 reduces TGF β receptors half-life by cooperating with Smad7-Smurf2 [4].

Together, our data permits us to propose a model of control T β R2 decay by IL-1 β . We suggest that IL-1 β increases caveolin-1 expression, leading the internalization of T β R2 in lipid raft and subsequently its degradation by the proteasome. Additionally, internalization in lipid raft could be promoted by a physically interaction between IL-1 receptor and T β R2 in complex named “receptosome” which was described by Stark’s group [21]. Association of the two types of receptors might trigger cross-talk in two signaling pathways. A similar mechanism by which turnover is regulated by interaction between distinct receptor populations on the cell surface (CD44 and TGF β receptors) has been previously described [5].

In conclusion, we enlightened a new mechanism by which IL-1 β downregulates T β R2 expression. We bring evidence that IL-1 β is able to reduce TGF β function through T β R2 protein destabilization occurring through receptor-mediated endocytosis by raft lipid and proteasomal pathway.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgement

We thank Brigitte Sola (Laboratoire de Biologie Moléculaire et Cellulaire de la Signalisation, EA 3919, Université de Caen, France) for providing us bortezomib.

This work was support by French Ministry of Research and partially by Lions Club of Normandy (France).

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