Biochimica et Biophysica Acta 1833 (2013) 1924–1935

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Curcumin affects proprotein convertase activity: Elucidation of the molecular and subcellular mechanism

Jingjing Zhu [a], Geert Bultynck [b], Tomas Luyten [b], Jan B. Parys [b], John W.M. Creemers [c], Wim J.M. Van de Ven [a,d], Alphons J.M. Vermorken [a,*]

[a] Laboratory for Molecular Oncology, Department for Human Genetics, KU Leuven, Herestraat 49, bus 602, B-3000 Leuven, Belgium
[b] Laboratory of Molecular and Cellular Signaling, KU Leuven, O&N I, Herestraat 49, bus 802, 3000 Leuven, Belgium
[c] Laboratory of Biochemical Neuroendocrinology, Department for Human Genetics, KU Leuven, Herestraat 49, bus 602, B-3000 Leuven, Belgium
[d] Charles E. Schmidt College of Science, Florida Atlantic University, 777, Glades Road, SC 216, Boca Raton FL 33431, USA

ARTICLE INFO

Article history:
Received 1 December 2012
Received in revised form 2 March 2013
Accepted 2 April 2013
Available online 10 April 2013

Keywords:
Curcumin
Proprotein convertases
SERCA inhibitors
Tumor cell growth
IGF-1R
Akt signaling

ABSTRACT

Proprotein convertases (PCs) form a group of serine endoproteases that are essential for the activation of proproteins into their active form. Some PCs have been proposed to be potential therapeutic targets for cancer intervention because elevated PC activity has been observed in many different cancer types and because many of the PC substrates, such as pro-IGF-1R, pro-TGF-beta, pro-VEGF, are involved in signaling pathways related to tumor development. Curcumin, reported to possess anticancer activity, also affects many of these pathways. We therefore investigated the effect of curcumin on PC activity. Our results show that curcumin inhibits PC activity in a cell lysate-based assay but not in vitro. PC zymogen maturation in the endoplasmic reticulum appears to be inhibited by curcumin. Treating cells with thapsigargin or cyclopiazonic acid, two structurally unrelated inhibitors of the sarco- and endoplasmic reticulum Ca^{2+}ATPase (SERCA), also hampered both the PC zymogen maturation and the PC activity. Importantly, curcumin, like the SERCA inhibitors, impaired ATP-driven $^{45}Ca^{2+}$ uptake in the endoplasmic reticulum. These results indicate that curcumin likely restrains PC activity by inhibiting SERCA-mediated Ca^{2+}-uptake activity. Experiments in three colon cancer cell lines confirm that curcumin inhibits both the $^{45}Ca^{2+}$ uptake and PC activity, notably the processing of pro-IGF-1R. Both curcumin and thapsigargin inhibit the anchorage-independent growth of these three colon carcinoma cell lines. In conclusion, our findings indicate that curcumin inhibits PC zymogen maturation and consequently PC activity and that its inhibitory effect on Ca^{2+} uptake into the ER allows and is sufficient to explain this phenomenon.

1. Introduction

Proprotein convertases (PCs) form a family of seven closely related subtilisin-like serine endoproteases. The family members include furin, PC1/3, PC2, PC4, PC5/6, PACE4 and PC7 [1]. The proprotein convertases, depending of their compartments of action, have different optima for pH and calcium (Ca^{2+}) concentration. The post-translational processing of their inactive precursor molecules – zymogens – through endoproteolytic cleavage, is a prerequisite for the formation of biologically active proprotein convertases. There are also differences in pH optima and Ca^{2+} requirements for these maturation processes [2,3]. PCs cleave and thereby activate a large variety of proproteins, such as growth factors, receptors, enzymes and cell adhesion molecules. As a consequence these enzymes play important roles in maintaining homeostasis [4].

Furin and its family members have similar substrate specificities and preferentially cleave C-terminally of paired basic amino acids. Knockout mouse models have been generated to investigate the roles of PCs in various pathologies [5] and the possible redundancies of various PCs [6]. Embryos lacking furin die in the second week of pregnancy. They show multiple tissue abnormalities including severe ventral closure defects and the failure of the heart tube to fuse and undergo looping morphogenesis [7]. In contrast to the indispensable role of furin during embryogenesis, its function in adult life appears to be partially redundant, as shown by conditional furin inactivation in mice in specific organs [8–12].

Abbreviations: ACF, aberrant crypt foci; AMC, 7-amino-4-methyl coumarin; Akt1, v-akt murine thymoma viral oncogene homolog 1; ATCC, American Type Culture Collection; CCL, culture clone; CHO, Chinese hamster ovary cells; CPA, cyclopiazonic acid; D9R, nona-D-arginine; DMEM/F12, Dulbecco's modified Eagle's medium/nutrient mixture F12; DMSO, dimethylsulfoxide; (−)EGC, (−)epigallocatechin; ER, endoplasmic reticulum; EDTA, ethylenediaminetetraacetic acid; FAP, familial adenomatous polyposis; FLAG, polypeptide N-DYKDDDDK-C (1012 Da); GPC3, glypican 3; GPC4, glypican 4; IGF-1R, insulin-like growth factor 1 receptor; IGF-1, insulin-like growth factor 1; PAGE, polyacrylamide gel electrophoresis; PC, proprotein convertase; Pyr, pyroglutamic acid; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; TG, thapsigargin; TPCK, tosyl phenylalanyl chloromethyl ketone; α1-PDX, 1-antitrypsin Portland inhibitor; GAPDH, glyceraldehydephosphate dehydrogenase

* Corresponding author. Tel.: +32 16 346076; fax: +32 16 346073.
E-mail address: fons.vermorken@med.kuleuven.be (A.J.M. Vermorken).

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http://dx.doi.org/10.1016/j.bbamcr.2013.04.002

PCs have also been implicated in several pathological processes. Firstly, the expression of some PCs is increased in several cancer types, such as breast, non-small-cell lung cancers and head and neck cancers [13]. Moreover, for some PCs, for instance furin, expression and tumor aggressiveness are highly correlated [13,14]. A number of PC substrates, once processed, are involved in various signaling pathways which play very essential roles in tumor development. Examples are: transforming growth factor beta (TGF-beta) [15], vascular endothelial growth factor (VEGF) [16], glypicans (GPCs) [17], insulin-like growth factor 1 and its receptor (IGF-1 and IGF-1R) [18,19].

Curcumin, a polyphenol and a main component of the Indian spice turmeric, has been reported to possess anti-cancer effect in several cancer types [20]. Curcumin affects several signaling pathways in which PC substrates are playing an important role. In fact the signaling by most of the matured forms of the PC substrates mentioned above, as *e.g.* TGF-beta [21,22], VEGF [23,24] and IGF-1R [25], is also influenced by curcumin. For this reason it seemed opportune to investigate whether curcumin had impact on PC activity and therefore on downstream targets in different signaling pathways.

While fully aware that such potential PC modulating activity would have impact on many pathways, we focused our study on the impact of curcumin on IGF-1R processing in the context of a potential anti-cancer effect in the intestine.

Curcumin has been shown to inhibit the IGF-1 signaling pathway in breast carcinoma cells [26]. In colon cancer cells both pro-IGF-1 and pro-IGF-1R are processed by PCs [18,19]. It has been shown to have chemopreventive activity in a mouse model of familial adenomatous polyposis (FAP) [27]. Moreover, curcumin showed some efficacy in a small clinical trial of FAP chemoprevention [28]. Aberrant crypt foci (ACF), clusters of abnormal tube-like glands in the lining of the colon and rectum have been claimed to represent the earliest histologically identifiable changes in the colon of patients with FAP [29]. A recent Phase IIa clinical trial of curcumin for the prevention of colorectal neoplasia showed a significant reduction in the number of ACF at a daily dose of 4 g of curcumin [30]. With respect to the neoplastic progression of colorectal polyps towards cancer, a significant correlation between the expression of the IGF-1R and neoplastic progression from normal mucosa to adenomatous polyps and finally to colorectal cancer has been demonstrated [31]. In light of the latter, IGF-1R is being considered as a promising therapeutic target in colorectal cancer [32].

As a consequence, it is plausible that curcumin could act on the level of the IGF-1R which is synthesized as a proprotein that needs PC activity for its activation. We therefore investigated whether curcumin acted as an inhibitor of pro-IGF-1R processing because such an inhibitory activity could also explain its action on several other signaling pathways. Furthermore, we have investigated the molecular basis of curcumin-induced PC inhibition.

2. Materials and methods

2.1. Materials and cell lines

A purified, biologically active, soluble, recombinant human furin fragment prepared as previously described [33] was used for in vitro enzyme assays. This human furin fragment was isolated from CHO-K1 cells stably transfected with a DNA construct expressing a truncated human pro-furin fragment consisting of amino acids 1–573. This pro-furin fragment is processed into a soluble, enzymatically active furin fragment, which consists of amino acids 108–573 and has been designated hfurin^{573} in this manuscript.

Curcumin was obtained from Sigma-Aldrich and a 10 mM stock solution for use in experiments was made in ethanol. Thapsigargin (TG) and cyclopiazonic acid (CPA) were purchased from Sigma-Aldrich and 1 mM stock solutions were prepared in ethanol and dimethylsulfoxide (DMSO), respectively. As fluorogenic peptide substrate Pyr-RTKR-AMC (Pyr = pyroglutamic acid, AMC = 7-Amino-4-methylcoumarin) was used (Bachem). Uncleaved pro-diphtheria toxin was obtained from Merck and the proprotein convertase inhibitor nona-D-arginine (D9R) from Pepscan, Prestio.

Cell lines included CHO (ATCC CCL-61), RPE.40 [34], HT-29 (ATCC CCL-218), SW480 (ATCC CCL-228) and Caco-2 (ATCC HTB-37).

2.2. Cell culture and incubation of cells with curcumin, TG or CPA

RPE.40, CHO, HT-29, SW480 and Caco-2 cells were grown in DMEM/F12 (1:1) (Invitrogen™) supplemented with 10% fetal calf serum (Hyclone) and cultured at 37 °C in a humidified 5% CO_2 atmosphere. For in vitro experiments, a stock solution of 10 mM curcumin (prepared in ethanol) was diluted with cell culture medium to obtain concentrations of 2–20 μM curcumin for cell treatments. Cells were treated with curcumin for 24 h. In experiments with transfected cells, cells were after transfection first grown in culture medium without curcumin during 24 h and, subsequently, the cells were cultured for 24 h in medium containing various concentrations of curcumin. During curcumin application, light exposure was reduced as much as possible. In control experiments, the effect of 0.2% ethanol was monitored which is the highest concentration of the solvent used. In experiments with the SERCA inhibitors TG or CPA, cells were treated according to a similar protocol as used in experiments with curcumin.

2.3. DNA constructs and transfection

To test the effect of curcumin on the processing of pro-protein substrates by endogenous PCs in cells, full length cDNA encoding pro-IGF-1R, HA-tagged pro-GPC3, or HA-tagged pro-GPC4, which constitute established PC substrates, were transfected into CHO cells or cells of the furin-deficient cell line RPE.40. Full length human *IGF-IR* cDNA (*hIGF-IR*) (kind gift of Dr. Kooijman, VUB, Belgium; [35]), full length human *GPC3* cDNA (kind gift of Dr. G. David; [36]) or full length human *GPC4* cDNA (kind gift of Dr. G. David; [36]) in the pcDNA3 expression vector were used. In similar cotransfection experiments, the effect of curcumin on processing of the afore-mentioned proproteins by exogenously expressed human furin, PC5/6 or PC7 was studied in RPE.40 cells. Furthermore, to test the effect of curcumin on the maturation of PCs, expression constructs encoding human furin, PC1/3 or PC7 were transfected into CHO cells. For all constructs expressing one of these members of the mammalian family of PCs, expression vector pcDNA3 was used. For expression of wild-type human furin, the 4.1 EcoR1 *FUR* cDNA [37] was used. Human PC1/3 was expressed as a FLAG-tagged PC1/3 fusion protein [38] and expression constructs for human PC5/6 [39] and PC7 [40] have been described before. Expression of a human PC1/3 mutant construct encoding pro-PC1/3$_{mut}$, which contains an inactivating mutation in the middle domain (G593R) causing ER retention of its pro-form [38], was used in control experiments. Transfections were performed using Fugene™ 6 Transfection Reagent (Roche) according to the manufacturer's protocol. About 24 h after transfection, the PC zymogens, the proprotein substrates and their corresponding processing products were studied by Western blotting analysis using specific antibodies. Similar experimental conditions as used to study the effects of curcumin on pro-furin maturation were selected to evaluate the effects of TG or CPA on the maturation of pro-furin as well as the effects of curcumin and TG on the maturation of pro-PC1/3 and pro-PC7.

2.4. Western blotting analysis

Cells were lysed about 24 h after transfection in SDS-PAGE sample buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, and 4% β-mercaptoethanol) and after boiling of the solution for 10 min, samples were loaded on a 10% SDS-polyacrylamide gel. After separation on the basis of molecular weight, proteins were transferred electrophoretically onto Protran® nitrocellulose transfer membranes (Whatmann GmbH) and subjected to Western blotting analysis.