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Mini-Review

Regulation and role of calcium in cellular senescence

Nadine Martin*, Kexin Zhu, Joanna Czarnecka-Herok, Mathieu Vernier and David Bernard*

Centre de Recherche en Cancérologie de Lyon, Inserm U1052, CNRS UMR 5286, Centre Léon Bérard, Université de Lyon, Equipe Labellisée la Ligue Contre le Cancer, Lyon, France

*Correspondence to: nadine.martin@lyon.unicancer.fr and david.bernard@lyon.unicancer.fr

Short title: Calcium and senescence

ABSTRACT

Cellular senescence is a state of stable cell proliferation arrest accompanied by a distinct secretory program impacting the senescent cell microenvironment. This phenotype can be induced by many stresses, including telomere shortening, oncogene activation, oxidative or genotoxic stress. Cellular senescence plays a key role in the organism throughout life, with beneficial effects at a young age for instance in embryonic development and wound healing, and deleterious effects during aging and in aging-related diseases. In the last decade calcium and calcium signaling have been established as critical factors in the implementation and regulation of cellular senescence. In this review we will present and discuss the main discoveries in this field, from the observation of an increased intracellular calcium concentration in senescent cells to the identification of calcium-binding proteins, calcium channels (TRP, ITPR, ...) and MERCs (mitochondria-endoplasmic reticulum contact sites) as key players in this context.

KEYWORDS

Calcium, signaling, senescence, cancer, aging, mitochondria-ER contacts

ABBREVIATIONS

ATF3	activating transcription factor 3
ARF	alternative reading frame
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CDK	cyclin-dependent kinase
CDKI	CDK inhibitor
CDKN1A/2A	cyclin-dependent kinase inhibitor 1A/2A
cGAS	cyclic GMP-AMP synthase
CEBP- β	CCAAT/enhancer-binding protein beta
hMEC	human mammary epithelial cells
hMESC	human endometrium-derived mesenchymal stem cells
ER	endoplasmic reticulum
IL	interleukin
IP3	inositol-1,4,5-trisphosphate
ITPR	inositol-1,4,5-trisphosphate receptor
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
NFAT	nuclear factor of activated T-cells

NF κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
MAM	mitochondria-associated endoplasmic reticulum membranes
MCU	mitochondrial calcium uniporter
MERC	mitochondria-ER contacts
PLC	phospholipase C
PKB	protein kinase B
PML	promyelocytic leukemia protein
PPAR γ	peroxisome proliferator-activated receptor gamma
PTEN	phosphatase and tensin homolog
RB	retinoblastoma protein
ROS	reactive oxygen species
RXR	retinoid X receptor
SAHF	senescence-associated heterochromatin foci
SASP	senescence-associated secretory phenotype
STING	stimulator of interferon genes
TR	thyroid hormone receptor
TRPC	transient receptor potential channel
UV	ultraviolet

1. Cellular senescence

Cellular senescence was first described in 1961 by Leonard Hayflick and Paul Moorhead, who reported that normal human fibroblasts in culture stop proliferating after serial passages [1]. This cell cycle arrest is due to telomere shortening. It was later observed that a similar phenotype than this replicative senescence could be induced prematurely by many different stimuli, including stresses such as oncogene activation, oxidative stress and genotoxic stress. More recently, cellular senescence was also shown to be induced in a programmed manner during embryonic development, as well as by paracrine signaling of neighboring senescent cells (Fig. 1). Cellular senescence has been reported to occur in most human cell types and in a wide variety of species [2].

Senescent cells are in a state of stable cell cycle arrest, which is implemented by a complex network of factors in which p53, inhibitors of cyclin-dependent kinases (CDKIs) and the retinoblastoma protein (RB) play a key role. Activation of p53 by senescence-inducing stimuli leads to the induction of the expression of the p53 target gene CDKN1A, which encodes CDKI p21. Expression of p15 and p16, two other CDKIs encoded by the INK4-ARF locus, is also activated in response to senescence-inducing stimuli. These three CDKIs inhibit cyclin/CDK complex formation, impeding, among others, the phosphorylation of RB. RB represses the expression of E2F target genes promoting cell cycle progression [2].

Senescent cells also secrete multiple soluble factors, including pro-inflammatory cytokines and chemokines, growth factors and metalloproteases, which are collectively referred to as the senescence-associated secretory phenotype (SASP). This secretome, the composition of which is heterogeneous and dynamic, is regulated at multiple levels. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) and CCAAT/enhancer-binding protein beta (CEBP- β)

transcription factors critically induce the expression of SASP pro-inflammatory factors. NOTCH also regulates the expression of some SASP components. In addition, pathways such as the mammalian target of rapamycin (mTOR) pathway control the SASP at the translational level. The inflammasome and the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) signaling pathway are also key regulators of the SASP. The SASP can reinforce senescence in an autocrine manner and has many paracrine functions, being able to trigger senescent cell elimination by activating the immune system and to induce senescence in neighboring cells, as well as epithelial-mesenchymal transition, migration and invasion in cancer cells [3].

The stable cell cycle arrest and the SASP are two critical hallmarks of cellular senescence. However, senescent cells also exhibit a number of other features (Fig. 1). Cell entry into senescence is accompanied by a profound change in cell morphology, adopting a flat and enlarged shape *in vitro*, and an increase in nuclear size. Senescent cells display lysosomal senescence-associated β -galactosidase activity, which is used as a biomarker to identify these cells. Significant modifications of cell physiology and metabolism can also be observed in senescent cells, especially resistance to apoptosis and altered mitochondrial activity that often leads to the production of reactive oxygen species (ROS). Finally, in the nucleus, senescent cells accumulate unrepaired DNA damage and display profound changes in chromatin, including epigenetic modifications and formation of senescence-associated heterochromatin foci (SAHFs), and in gene expression [2].

Owing to their two important characteristics (stable cell cycle arrest and SASP), senescent cells play a key role in many different pathophysiological contexts throughout life (Fig. 1). Cellular senescence has a pleiotropic effect on the organism [4]. Senescence is associated with beneficial

effects early in life, contributing to normal morphogenesis during embryonic development. Moreover, when induced in a timely- and spatially-controlled manner, senescence promotes wound healing, cell reprogramming and stemness, and acts as a critical tumor suppressive barrier in the first steps of tumorigenesis. However, exposure to chronic stresses, persistent damage and decay of immune function and clearance during life leads to the accumulation of senescent cells in the organism, which is detrimental as it impacts cell proliferation, disrupts tissue architecture and function and establishes a pro-inflammatory and pro-fibrotic microenvironment [4;5]. In the last decade, genetic mouse models engineered to allow senescent cell elimination showed that accumulation of senescent cells promotes aging and many aging-associated disorders such as type II diabetes, osteoporosis, atherosclerosis, lung degenerative diseases, neurodegenerative disorders, as well as cancer relapse and progression [6]. Huge efforts have been made since to discover senomorphic or senolytic drugs, able to modulate senescence (especially the SASP) or kill senescent cells, respectively. Several of these compounds have proven their efficiency to improve age-related alterations in mouse pre-clinical models and some are currently tested in clinical trials [7].

Understanding how senescence is controlled at the molecular and cellular level is critical to identify potential druggable targets to modulate it. In the last decade accumulating evidence has established calcium (Ca^{2+}) and calcium signaling as key regulators of cellular senescence. Calcium is a pivotal second messenger controlling a broad range of molecular processes and cellular functions, including cell proliferation, secretion, migration and death. Its effects are achieved through a fine-tuned regulation of its intracellular concentration and fluxes, in which many proteins, including channels, transporters, pumps and calcium-binding proteins, are involved. In this review we will present the current knowledge on the role of calcium and calcium

signaling in cellular senescence and discuss potential ways to target senescence by modulating them.

2. Evolution and impact of intracellular calcium concentration in cellular senescence

Intracellular Ca^{2+} concentration increases in senescent cells. This has been observed in the cytosol and mitochondria. Accumulated calcium in the cytosol may come from the extracellular space through plasma membrane channels, from ER calcium release but might also be a consequence of mitochondrial calcium accumulation and mitochondrial dysfunction [8]. Increased intracellular calcium accumulation was documented in several cell types and in response to many different senescence-inducing stimuli (Table 1) [9-19]. Calcium chelation with BAPTA was shown to prevent senescence in several contexts [12;13], by attenuating DNA damage response and p53/p21/RB pathway activation [13] and abolishing RB-dependent mitotic gene repression [12], indicating that elevation of intracellular calcium concentration triggers cellular senescence.

Interestingly, expression of calbindin 1 (CALB1), a calcium-binding protein known to buffer cytosolic calcium, was found to be upregulated in response to many senescence-inducing stresses in hMECs. The calcium-dependent calmodulin/calcineurin/NFAT pathway participates in CALB1 induction in this context [11]. The calcium sensor protein calmodulin is activated by an increase in cytosolic calcium. Calcium-bound calmodulin then activates calcineurin phosphatase, which catalyzes the dephosphorylation of the NFAT (nuclear factor of activated T-cells) transcription factor and thus induces its translocation to the nucleus where it regulates the expression of its target genes. Overexpression of CALB1 was reported to buffer the rise in intracellular calcium concentration in senescent hMECs [11], and its overexpression could inhibit

senescence in cancer cells, albeit it remains unknown whether Ca^{2+} buffering is involved in this process [20]. Furthermore, upregulated expression of calbindin 2 (CALB2) was observed upon senescence in other cell types [11], suggesting that the calcium buffering system may play a general role in modulating intracellular Ca^{2+} levels in senescence, which will need to be further investigated.

Depending on the context, the rise in intracellular Ca^{2+} concentration observed in senescent cells can be triggered either by calcium influx through plasma membrane calcium channels or/and by calcium release from the intracellular Ca^{2+} stock, for instance from the endoplasmic reticulum (ER) which is one of the main intracellular calcium stores. These two events will be further described below.

3. Role of plasma membrane calcium channels in cellular senescence

Growing evidence advocates for a key role for plasma membrane calcium channels in cellular senescence. Isradipine, a L-type Ca^{2+} channel blocker, was shown to impair the rise in intracellular calcium concentration and the subsequent PI3K/Akt- and p53/p21-dependent senescence induced by rotenone in human neuroblastoma SH-SY5Y cells [9]. Expression of Piezo1, a plasma membrane mechanosensitive ion channel with calcium affinity, was reported to be induced in senescent chondrocytes as well as in aged cartilage tissue. Piezo1 activation is responsible for intracellular calcium accumulation in senescent chondrocytes and triggers senescence through this activity [17].

Several recent reports also indicate a key role for TRPC (Transient Receptor Potential Cation Channel Subfamily C) channels in cellular senescence. TRPC plasma membrane cation channels constitute a family of seven members (TRPC1-7) that play important roles in physiology as well

as in pathologies, including cancer and age-associated diseases. TRPC7 was shown to contribute to UVB-induced Ca^{2+} influx and increased ROS, likely leading to increased cellular senescence, skin aging and skin tumorigenesis [21].

TRPC3 expression is downregulated in many senescence contexts [14;19] and its re-expression allows cells to escape oncogene-induced senescence [19]. Moreover, senescence induced by TRPC3 knockdown was shown to lead to a pro-inflammatory and pro-tumorigenic secretome, which promotes cancer cell proliferation and tumor growth *in vivo* [19]. Surprisingly senescence induced by TRPC3 knockdown was correlated with increased cytosolic and mitochondrial Ca^{2+} levels. TRPC3 acts by inhibiting Inositol 1,4,5-Trisphosphate Receptors 3 (ITPR3)-based ER calcium release via direct protein-protein interaction [19] (see below – chapter 4 for detailed action of ITPRs on senescence). Although TRPC channels have been mainly described in the plasma membrane, TRPC3 was shown to localize both in the plasma membrane and in the ER. As ITPR3 can also be detected in these two sites, where the TRPC3-ITPR3 interaction takes place remains to be determined.

4. Role of ITPR ER calcium release channels in cellular senescence

Having discussed above the rise in intracellular calcium concentration from the extracellular space through plasma membrane calcium channels, we will now focus on its release from the ER intracellular stock. The three ITPRs (ITPR1-3) are ER calcium release channels that play a critical role in cellular senescence. Oncogene activation in hMECs and telomere shortening in normal human fibroblasts, which both induce senescence, were shown to trigger Ca^{2+} release from the ER through the phospholipase C (PLC)-inositol triphosphate (IP3) pathway and subsequent activation of ITPRs. Knockdown of ITPRs allows escape from oncogene-induced

senescence in HMECs and a delay in replicative senescence in fibroblasts [22]. The PLC-IP3-ITPR pathway and ER calcium release were also shown to induce the prompt rise in cytosolic calcium concentration observed in response to H₂O₂ treatment in hMESC [13]. As mentioned in the previous paragraph, ITPR3 activity can also be increased in senescent fibroblasts and contribute to the senescent phenotype. This increased activity could result from decreased expression of TRPC3, releasing ITPR3 from its interaction with TRPC3 and promoting ER Ca²⁺ release and senescence [19].

Recent evidence demonstrated a key role for ITPR2 in cellular senescence *in vivo*. Indeed, *Itp2* knockout (KO) mice had an extended lifespan and less age-associated alterations, such as less immunosenescence, a better response to metabolic stress and lower liver steatosis and fibrosis. These mice, as well as mouse embryonic fibroblasts derived from them, displayed less cellular senescence, which is well-known to promote these alterations [23].

Calcium release activity of ITPRs is directly regulated through interaction with several oncogenes and tumor suppressors that are involved in cellular senescence [24]. For example, by phosphorylating ITPR3 [25], the PKB/AKT pro-survival serine/threonine kinase inhibits its ER calcium release activity [26]. The promyelocytic leukemia protein PML, a tumor suppressor known to induce cellular senescence [27], interacts with ITPR3 and PKB/AKT, recruits PP2A phosphatase to counteract ITPR3 inhibition by PKB/AKT and thus promotes ER calcium release [28]. Whether this impact on calcium signaling contributes to the effect of these oncogenes and tumor suppressors on cellular senescence remains to be determined.

ITPRs are also regulated at the transcriptional level, and modulation of ITPR gene expression was shown to be involved in the regulation of cellular senescence. Indeed, the retinoid X receptor alpha (RXR α), a transcription factor from the nuclear receptor family, was reported to counteract

senescence by directly repressing ITPR2 expression [29]. RXR α also inhibits ITPR1 expression [30]. This ability to regulate ITPR expression and cellular senescence is shared by a close relative of RXR α , namely RXR β , and several type II nuclear receptors, including PPAR γ (Peroxisome Proliferator Activated Receptor Gamma) and TR α (Thyroid Hormone Receptor Alpha), which heterodimerize with RXR receptors [30].

5. Role of ER-mitochondria contacts and of mitochondrial calcium in cellular senescence

ER calcium release often leads to calcium uptake in mitochondria. Mitochondrial calcium is necessary to maintain proper mitochondrial respiration and cell bioenergetics and is thus required for cell viability [31]. However, calcium accumulation in mitochondria is a key driver of senescence, as previously documented [19;22;29]. Knockdown of the mitochondrial calcium uniporter MCU was shown to allow senescence escape [22;29]. The rise in mitochondrial calcium concentration causes a drop in mitochondrial membrane potential and triggers ROS production, which induce cellular senescence [22;29]. Of note, massive calcium release from the ER and uptake in the mitochondria trigger cell death. How the same phenomena lead to different outcomes still need to be understood. The cell fate triggered might depend on the dynamics of the calcium flux (calcium concentration, timing, ...), which regulation is thus crucial.

ER and mitochondria can be in close vicinity and physically and functionally linked through contact sites called mitochondria-ER contacts (MERCs) or mitochondria-associated ER membranes (MAMs). The distance between the ER and mitochondria is between 10 nm and 50 nm within these contacts [32]. MERCs encompass hundreds of proteins, among which tethering proteins, ion channels, transport binding proteins and enzymes, that form hotspots for calcium and lipid transfer and important signaling platforms. A critical role for MERCs in the regulation

of cellular senescence was recently discovered, as forcing contacts between ER and mitochondria through the use of a synthetic linker was shown to trigger cellular senescence in human embryonic lung fibroblasts [23]. Inside MERCs, ITPR2 could have 2 complementary roles: a more classical role as Ca^{2+} channel to increase the release of Ca^{2+} from the ER, and a structural role to maintain MERCs, thus orienting the transfer of released Ca^{2+} to the mitochondria [22;33]. The possible levels of interplay between MERCs and cellular senescence have been thoroughly reviewed in [34].

6. Role of cytosolic calcium-regulated pathways in cellular senescence

Aside from the ER-mitochondria axis described above, pathways regulated by calcium in the cytosol are functionally involved in cellular senescence. For instance, as mentioned above, the calcium-dependent NFAT transcription factor is a critical player in cellular senescence as it regulates CALB1 expression. NFATc1 is also known to activate ITPR2 expression [35] and modulation of ER calcium release could thus be one of the ways for the NFAT pathway to regulate senescence. Seemingly opposite effects of this factor on senescence have been reported depending on the context. The calcium/calmodulin/calcineurin/NFAT pathway, through its repression of the NFATc1 target gene ATF3, a negative regulator of p53, was shown to be required for p53-dependent cancer cell senescence and protection against skin squamous cancer development [36]. However, NFATc1 activation was reported to be pro-oncogenic in the context of prostate tumorigenesis, promoting prostate adenocarcinoma and overcoming PTEN loss-induced cellular senescence by inhibiting p21 activation [37]. Besides this effect of the calmodulin/calcineurin/NFAT pathway, p53 can be regulated by calcium through its interaction with calcium-binding proteins. p53 binds to S100 calcium-binding proteins in a calcium-

dependent manner. In melanoma, p53 interaction with S100B leads to a decrease in p53 protein level and activity [38]. RB, another key inducer of senescence-associated cell cycle arrest, can also be regulated by calcium, as calmodulin increases the activity of CDK1 and CDK2 which phosphorylate and thus inhibit RB [39;40]. However, how calcium regulates p53 and RB has not been much explored yet in senescent cells.

Calcium can also regulate the SASP. Calcium controls the processing of interleukin 1 alpha (IL1 α), a pro-inflammatory cytokine with a known critical role in the induction of other SASP cytokines, such as IL6 and IL8. In response to senescence-inducing stimuli, the rise in intracellular calcium concentration activates calcium-dependent calpain protease, promotes proteolytic cleavage of IL1 α precursor by this enzyme and thus upregulates the expression of downstream SASP cytokines [16]. In addition, SASP components and regulators may be modulated by calmodulin, although this still needs to be demonstrated in the context of senescence. Calcium-mediated interaction of calmodulin with IL1 β precursors promotes IL1 β processing and secretion [41]. Calcium-activated calmodulin can regulate the NF κ B pathway, inhibiting c-REL and triggering RELA translocation to the nucleus [42-44]. Calmodulin is also able to associate with mTORC1, one of the complexes of the mTOR pathway, and stimulate its kinase activity [45].

In summary, calcium and its downstream signaling act at multiple levels to regulate cellular senescence (Fig. 2). We mainly focused here on evidence demonstrating their direct role in senescence, but there are many other ways in which they can control senescence-associated features, as reviewed in [46] and [47].

7. Concluding remarks

Cellular senescence is a key cellular response to a broad spectrum of stimuli, with profound impacts on health and longevity. Understanding how senescence is implemented and controlled is the essential first step towards possible strategies to target it. Based on the discoveries made over the past decade, calcium is now established as a key effector and regulator of cellular senescence. Several players of calcium signaling have been shown to be involved in it, including calcium channels, calcium-binding proteins and calcium-regulated enzymes and transcription factors. Furthermore, the observation that ER-mitochondria contacts and calcium transfer play a key role in cellular senescence has shed light on the importance of calcium fluxes between different subcellular compartments. However, a thorough assessment of the functional contribution and regulation of each of the factors involved in calcium signaling is still needed to fully understand its role in cellular senescence.

The observed impact of NFAT and ITPR2 on cellular senescence in mouse models provided proof-of-concept that calcium is a critical regulator of senescence *in vivo* [23;36;37]. Studying the effect of other members of calcium signaling on cellular senescence and associated pathophysiological contexts *in vivo* may reinforce and broaden this idea. Calcium is already known to play a key role in several processes in which senescence is involved, such as wound healing, cell migration and invasion as well as insulin sensitivity (as reviewed in [47]). This suggests that calcium and senescence may be functionally linked in many contexts.

Senotherapeutic approaches hold great promise for medical applications [7]. Exploiting calcium could be considered as a potential novel avenue to design innovative therapeutic strategies targeting senescence. Spatio-temporal regulation of calcium concentration may determine cell fate, cells being driven towards proliferation, senescence or death depending on changes in calcium homeostasis. For instance, it is known that an acute massive rise in mitochondrial

calcium level precedes and induces apoptosis [48]. CALB1 was shown to protect cells from death by buffering calcium [49] but also by interacting with caspase 3, a key apoptotic effector, in a calcium-dependent manner and inhibiting it [50;51]. One hypothesis could be that the expression of calbindins is induced in cellular senescence to buffer the rise in intracellular calcium concentration and prevent the cytotoxic effects of excessive calcium levels. This regulatory loop is achieved by the calmodulin/calcineurin/NFAT pathway which acts as a calcium sensor. In this context it will be interesting to explore if inhibiting calbindins has a senolytic effect. Apart from calcium-binding proteins, calcium channels and druggable candidates of calcium signaling pathways could be good targets to impact senescence by manipulating calcium levels.

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TABLE

CELL TYPE	SENESCENCE INDUCER	REF
human neuroblastoma SH-SY5Y cells	rotenone	9
human lung adenocarcinoma A549 cells	bleomycin	11
human mammary epithelial cells (hMECs)	MEK and RAF oncogenes	11
	bleomycin	11
	nutlin	11
	KCl-induced plasma membrane depolarization	11
		12
human endometrium-derived mesenchymal stem cells (hMESC)	H ₂ O ₂	13
human lung embryonic fibroblasts	telomere shortening due to serial passages	14, 16
	oxidative stress	16
	RAS oncogene	15, 19
human prostate fibroblasts	docetaxel	19
rat chondrocytes	telomere shortening due to serial passages	17
mice skeletal muscle fibers	aging	18

Table 1. Senescence contexts where an increase in intracellular calcium concentration has been documented.

FIGURE LEGENDS

Figure 1. Inducers, hallmarks and pathophysiological effects of cellular senescence.

Many different stimuli can induce cellular senescence. Senescent cells are characterized by an array of features among which the stable cell cycle arrest and the senescence-associated secretory phenotype (SASP) are the most important ones for senescence pathophysiological effects. The main effectors playing a role in the implementation of these two hallmarks are indicated below them. Acute and localized cellular senescence has beneficial effects. However, exposure to chronic stresses, persistent damage and decline of the immune system over time leads to the accumulation of senescent cells in the organism, which promotes aging and aging-associated disorders. This figure has been created with BioRender.com.

Figure 2. Current knowledge on the regulation and role of calcium in cellular senescence.

Senescence-inducing stimuli trigger a rise in intracellular calcium levels. Intracellular calcium fluxes and calcium signaling promote senescence by regulating key senescence effector pathways. This figure has been created with BioRender.com.