IP$_3$, IP$_3$ receptor, and cellular senescence

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Huang, Ming-Shyan, Olugbenga A. Adebanjo, Emmanuel Awumey, Gopa Biswas, Antoliy Koval, Bali R. Sodam, Li Sun, Balijit S. Moonga, Joshua Epstein, Samuel Goldstein, F. Anthony Lai, David Lipschitz, and Mone Zaidi. IP$_3$, IP$_3$ receptor, and cellular senescence. Am J Physiol Renal Physiol 278: F576–F584, 2000.—Herein we demonstrate that replicative cellular senescence in vitro results in sharply reduced inositol 1,4,5-trisphosphate (IP$_3$) receptor levels, reduced mitogen-evoked IP$_3$ formation and Ca$^{2+}$ release, and Ca$^{2+}$ store depletion. Human diploid fibroblasts (HDFs) underwent either 30 mean population doublings [mean population doublings (MPDs) thymidine labeling index (TI)] >92% ("young") or between 53 and 58 MPDs (TI < 28%; "senescent"). We found that the cytosolic Ca$^{2+}$ release triggered by either ionomycin or by several IP$_3$-generating mitogens, namely bradykinin, thrombin, platelet-derived growth factor (PDGF), and epidermal growth factor (EGF), was attenuated markedly in senescent HDFs. Notably, the triggered cytosolic Ca$^{2+}$ transients were of a smaller magnitude in senescent HDFs. However, the response latency seen with both PDGF and EGF was greater for senescent cells. Finally, a smaller proportion of senescent HDFs showed oscillations. In parallel, IP$_3$ formation in response to bradykinin or EGF was also attenuated in senescent HDFs. Furthermore, senescent HDFs displayed a sharply diminished Ca$^{2+}$ release response to intracellularly applied IP$_3$. Finally, to compare IP$_3$ receptor protein levels directly in young and senescent HDFs, their microsomal membranes were probed in Western blots with a highly specific anti-IP$_3$ receptor antiserum, Ab$^{260}$, a ~260-kDa band corresponding to the IP$_3$ receptor protein was noted; its intensity was reduced by ~50% in senescent cells. Thus, we suggest that reduced IP$_3$ receptor expression, lowered IP$_3$ formation, and Ca$^{2+}$ release, as well as Ca$^{2+}$ store depletion, all contribute to the deficient Ca$^{2+}$ signaling seen in HDFs undergoing replicative senescence.

The cytoplasmic release of Ca$^{2+}$ from intracellular stores is critical for the survival, function and propagation of eukaryotic cells. One mechanism through which Ca$^{2+}$ is released into the cytosol involves activation of microsomal membrane Ca$^{2+}$ channels gated by the inositol trisphosphate (IP$_3$) receptor (3). That intracellular Ca$^{2+}$ release becomes defective during cellular senescence is now well established. Namely, senescent human and murine T lymphocytes (12, 28), fibroblasts and neutrophils from elderly humans (19, 29), and parotid cells isolated from aging mice (16) all display attenuated cytosolic Ca$^{2+}$ signals in response to hormonal and mitogenic stimulation. Paradoxically, fibroblasts harvested from patients with neurodegenerative diseases, such as Alzheimer’s disease, exhibit greater agonist-induced Ca$^{2+}$ release (10, 26). The elevated cellular Ca$^{2+}$ is thought to contribute to the characteristic neuronal degeneration (10, 26). Despite the somewhat common occurrence of senescence-associated defects in Ca$^{2+}$ release, we have little insights into their mechanism.

Pathways of cellular Ca$^{2+}$ influx are also known to be defective in senescent cells, such as the human diploid fibroblast (HDF) cell line WI-38 (20, 23). Most notably, one of our groups observed markedly suppressed Ca$^{2+}$ currents in HDFs that were induced into replicative senescence through the forced expression of a Ca$^{2+}$-binding protein specific for Werner’s syndrome (20). Senescent WI-38 HDFs also did not show the usual cell cycle-dependent changes in calmodulin expression (4, 22). The latter remained elevated throughout G$\text{1}$, possibly buffering cytosolic Ca$^{2+}$ to lower levels. In contrast, however, the expression of a different Ca$^{2+}$-binding protein, calbindin, becomes low in certain neurodegenerative diseases (15), possibly accounting for elevated cytosolic Ca$^{2+}$ levels.

In the present study, we have used the HDF in vitro aging model to study the molecular mechanism(s) underlying the deficient cytosolic Ca$^{2+}$ release seen in senescent cells. HDFs have a finite lifespan, and on passage in vitro, undergo replicative senescence. Al-
though the number of mean population doublings (MPDs) accrued before senescence correlates inversely with the donor’s age, this concept has recently been revisited (6). In general, however, cells from a given donor can be made to age in vitro and harvested at defined MPDs for studies into their biological characteristics (11, 24, 31).

The HDF cell culture system has been thoroughly validated as an in vitro model of “replicative senescence” (6–8). Notably, these cells exhibit a limited proliferative lifespan. Although HDFs may be at different stages of proliferation/differentiation initially, they ultimately reach a stage on subculturing where they are no longer able to proliferate in response to mitogenic stimuli; this itself is a hallmark of replicative senescence (6–8). In addition to their failure to proliferate, a host of morphological and physiological changes, including the appearance of several molecular markers, characterize HDF senescence (reviewed in Refs. 5–7).

We demonstrate that as HDFs undergo senescence in vitro, their IP3 receptor content and responsiveness to intracellularly applied IP3 falls dramatically. This decline is associated with an attenuated IP3 production and cytotoxic Ca2+ release in response to mitogens. As the IP3 pathway is fundamental to intracellular Ca2+ release, its reduction in an aging cell may underlie the cell’s defective function and proliferative potential.

MATERIALS AND METHODS

Materials. The Ca2+-sensitive fluorochromes, indo 1 and indo 1-AM, were purchased from Molecular Probes (Eugene, OR). Bradykinin, thrombin, EGTA, and HEPES were purchased from Sigma Chemical (St. Louis, MO). Recombinant human EGF and platelet-derived growth factor (PDGF; BB homodimer) were purchased from R&D Systems (Minneapolis, MN). Ionomycin was obtained from Calbiochem (San Diego, CA).

Cell culture. HDFs were derived from the anterior forearm skin of a normal 9-yr-old female, A25 (11). The cells were cultured in MEM supplemented with fetal bovine serum (15%, vol/vol) and maintained in humidified 5% CO2. The A25 cells have a replicative lifespan of 58 MPDs. “Young” HDFs are classified as those having a vigorous growth curve, being within the first half of their maximum MPD, i.e., accruing ≤30 MPDs with a ³Hthymidine-labeling index (TLI) >92%. “Senescent” HDFs are late-passage cells with a reduced growth potential positioned within the last 10% of their replicative lifespan, i.e., having accrued between 53 and 58 MPDs (TLI ≤ 28%) (11, 19). Both young and senescent HDFs were subcultured in four chamber slides. They were harvested while proliferating (day 1) for immunoblotting, or on confluence (day 3) for functional studies. In separate experiments, cells were synchronized in different phases of the cell cycle by incubation with known agents, namely rapamycin (for G0), KH-93 (for G1), colcemid (for G2) or methotrexate (for S).

Cellular morphology and microspectrofluorimetry. We used an ACAS 570 interactive laser cytomter (Meridian Instruments, Okemos, MI) for single-cell morphology and microspectrofluorimetry. First, the outline of up to 60 HDFs was traced into a digitizing tablet, and their spread area was computed (in µm²). Next, cytosolic Ca2+ measurements were made in single HDFs by using indo 1, a Ca2+-sensitive fluorochrome (for details, see Ref. 33). Both young and senescent HDFs were incubated with 5 µM indo 1AM at 37°C for 60 min in Hanks’ balanced salt solution (HBSS) containing HEPES (HEPES-HBSS, 10 mM) and BSA (recrystallized, 0.1% wt/vol). The cells were then washed three times with HEPES-HBSS, incubated for a further 30 min, and positioned on the microspectrofluorimeter stage. The cells were subjected to excitation by a 5-W pulsed argon laser at λ of 357 nm (range, 351–363 nm), and the emission was monitored every 30 s in the image-scanning mode at 405 and 485 nm. The intensities of the captured fluorescent images at the two wavelengths, I405 and I485, were then transformed to yield the ratio, I405/I485.

Basal cytosolic Ca2+ levels were recorded for periods up to 2 min, during which the cells were exposed to prewarmed HEPES-HBSS (Ca2+ = 1.25 mM) containing one of several agonists: ionomycin (5 µM), bradykinin (5 µg/l), thrombin (5 kU/l), PDGF (5, 10, or 20 µg/l), or EGF (100, 400, or 800 µg/l). Experiments with ionomycin, bradykinin, and thrombin were repeated by using the same protocol, but in the Ca2+-free medium (i.e., in 1 mM EGTA).

For experiments with IP3, the plasma membrane was permeabilized gently by exposing HDFs to saponin (60 mg/l, wt/vol; Sigma Chemical), for 5 min at 37°C, in “intracellular buffer” containing (in mM) 110 KCl, 10 NaCl, 2 MgCl2, 5 K2HPO4, 20 phosphocreatine, 10 antimycin A, 3 ATP, and 20 HEPES-KOH, as well as 20 kU/l creatine kinase and 10 µg/l oligomycin (pH = 7.2). Note that we used the intracellular buffer because the cells were permeabilized. Note also that this applied protocol was considered optimal after our multiple trials using different incubation times versus different saponin concentrations. Permeabilized HDFs were then loaded with 5 µM indo 1 (free acid) in intracellular buffer for 10 minutes at 37°C. The cells were next allowed to reseal for 60 min in HEPES-HBSS (Ca2+ = 1.25 mM), during which time dye leakage was monitored in samples of cells. Although, as with other nonpermeabilized cells, dye leakage did indeed occur over the experimental periods, the signal-to-noise ratio (an indicator of dye retention) was found to be within acceptable limits. The latter was assessed by monitoring photon counts at I485. Finally, chambers containing permeabilized HDFs were mounted on the microscope stage and exposed to prewarmed solutions of IP3 (6 µM).

Indo 1 was calibrated by perfusing the chamber with its free acid solutions (0.5 µM) in 140 mM KCl, 10 mM HEPES, 10 mM EGTA, and different concentrations of CaCl2 (0.02, 1, 2, 3, 4, 5, 7, 8, or 9.0 mM, pH = 6.85 ± 0.01). The Kd for Ca2+ and EGTA (20°C, ionic strength 0.1 M, pH = 6.85) is 747 nM. By using the equation calcium ion concentration ([Ca2+]) = (Kd ÷ [free EGTA])/([Ca2+ ] - [EGTA]), these solutions corresponded to [Ca2+] values of 1.5, 83.2, 187, 321, 499, 748, 6.85) is 747 nM. By using the equation calcium ion concentration ([Ca2+]) = (Kd ÷ [free EGTA])/([Ca2+ ] - [EGTA]), these solutions corresponded to [Ca2+] values of 1.5, 83.2, 187, 321, 499, 748, 747 nM. By using the equation calcium ion concentration ([Ca2+]) = (Kd ÷ [free EGTA])/([Ca2+ ] - [EGTA]), these solutions corresponded to [Ca2+] values of 1.5, 83.2, 187, 321, 499, 748, 747 nM.

Membrane isolation, SDS-PAGE, and immunoblot analysis. For the isolation of endoplasmic reticular membrane (ER), old and young HDFs were homogenized in sucrose-mannitol buffer ([in mM] 20 HEPES, 70 sucrose, 220 mannitol, 2 EDTA, 0.1 phenylmethylsulfonyl fluoride, as well as 1.25 µg/ml, each, of antipain, chymostatin, leupeptin, and pepstatin). The homogenate was centrifuged (15,000 g, 20 min) to remove mitochondrial and nuclear membrane fractions. The supernatant was recentrifuged (10,000 g, 1 h; 4°C) to obtain the endoplasmic reticular membrane fraction. The latter was suspended in homogenization buffer and repelleted (100,000
CA²⁺ CHANNEL EXPRESSION AND AGING

RESULTS

Senescent HDFs had a significantly increased spread area (means ± SE, 2,450 ± 174 μm²) compared with that of young cells (1,718 ± 167 μm²; Student's paired t-test, P = 0.002, n = 60 cells/group) (24). Cytosolic Ca²⁺ measurements were made in up to 100 young or senescent HDFs that were either proliferating asynchronously or arrested at a given cell cycle phase (4, 22). We found cytosolic Ca²⁺ levels were significantly lower in senescent HDFs compared with young cells in every cell cycle phase, except during G₂, when the difference was reversed (Table 1). Note that cytosolic Ca²⁺ levels followed a virtually overlapping Gaussian distribution in both young and senescent HDFs (not shown).

Table 2 shows mean peak cytosolic Ca²⁺ levels in young and senescent HDFs in response to 5 µM ionomycin either in the presence or absence of extracellular Ca²⁺. In both instances, the mean peak cytosolic Ca²⁺ level of young HDFs was significantly higher (P = 0.005 and 0.001, respectively) than that of senescent cells. In Ca²⁺-free, EGTA-containing medium, ionomycin will release Ca²⁺ from intracellular stores without permitting Ca²⁺ influx. The rate of such release should thus be proportional to the store-cytosol Ca²⁺ gradient and, provided the cytosolic Ca²⁺ level is constant, to the fullness of the Ca²⁺ store. In this situation, the rate of Ca²⁺ release should thus reflect the fullness of Ca²⁺ stores. Figure 1A shows the best-fit curves for the effect of ionomycin in Ca²⁺-free, EGTA-containing medium for young and senescent HDFs. The rate of Ca²⁺ release

Table 1. Resting cytosolic Ca²⁺ levels in young and senescent human diploid fibroblasts either proliferating asynchronously or synchronized in a given phase of the cell cycle

<table>
<thead>
<tr>
<th>Cell Cycle</th>
<th>Young</th>
<th>Senescent</th>
</tr>
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<tbody>
<tr>
<td>Asynchronized</td>
<td>80.1 ± 1.42 (100)</td>
<td>76.2 ± 0.74 (100)*</td>
</tr>
<tr>
<td>Synchronized</td>
<td>81.2 ± 0.99 (64)</td>
<td>72.0 ± 0.87 (64)*</td>
</tr>
<tr>
<td>G0</td>
<td>67.5 ± 0.73 (48)</td>
<td>62.7 ± 1.01 (39)*</td>
</tr>
<tr>
<td>G1</td>
<td>69.1 ± 0.83 (40)</td>
<td>72.6 ± 1.51 (41)*</td>
</tr>
<tr>
<td>G2</td>
<td>70.3 ± 0.81 (65)</td>
<td>63.9 ± 0.87 (37)*</td>
</tr>
</tbody>
</table>

Values are means ± SE of cytosolic Ca²⁺ level (in nM). Nos. in parentheses, no. of cells. *P = 0.05 by ANOVA for cytosolic Ca²⁺ levels of young vs. senescent HDFs.

Table 2. Peak cytosolic Ca²⁺ levels in young and senescent human diploid fibroblasts exposed to either ionomycin, bradykinin, or thrombin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[Ca²⁺]₀</th>
<th>Young</th>
<th>Senescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionomycin (5 µM)</td>
<td>1.25</td>
<td>812 ± 46.5 (20)</td>
<td>594 ± 26.7 (20)*</td>
</tr>
<tr>
<td>Bradykinin (5 µg/l)</td>
<td>1.25</td>
<td>443 ± 26.4 (15)</td>
<td>261 ± 16.8 (15)*</td>
</tr>
<tr>
<td>Thrombin (5 kIU/l)</td>
<td>0</td>
<td>371 ± 15.2 (24)</td>
<td>257 ± 19.8 (24)*</td>
</tr>
</tbody>
</table>

Values are means peak cytosolic Ca²⁺ level (in nM). Nos. in parentheses, no. of cells. The experiments were carried out either at an extracellular Ca²⁺ concentration ([Ca²⁺]₀) of 1.25 mM (shown as 1.25) or in Ca²⁺-free, EGTA-containing medium ([Ca²⁺]₀ < 5 nM; shown as 0; see MATERIALS AND METHODS for details). *P < 0.005; †P < 0.005; ‡P < 0.01 by ANOVA for cytosolic Ca²⁺ levels of young vs. senescent HDFs.

...
was estimated from these plots assuming a near equal, albeit statistically different, basal cytosolic Ca\(^{2+}\) level (median \(\sim 5\) nM). Although Ca\(^{2+}\) was released at a rate \(\tau\) of 4.69 nm/s in young cells, this was reduced dramatically to 0.67 nm/s in senescent HDFs.

Finally, we used thapsigargin, a microsomal membrane Ca\(^{2+}\)-ATPase inhibitor. By preventing active store refilling, thapsigargin is known to deplete intracellular Ca\(^{2+}\) stores. The magnitude of the transient increases of cytosolic Ca\(^{2+}\) elicited by thapsigargin is thus indicative of the fullness of intracellular Ca\(^{2+}\) stores. Figure 1B shows that the magnitude of thapsigargin-induced Ca\(^{2+}\) release in young cells was significantly greater than in senescent cells.

Our results with ionomycin and thapsigargin suggested that the store content of Ca\(^{2+}\) was low in senescent HDFs. We therefore chose to deplete specifically the IP\(_3\)-sensitive stores by using several mitogenic agonists, namely bradykinin, thrombin, PDGF, and EGF (5, 14, 29, 32). Application of bradykinin (5 µg/l) in either the presence or absence of extracellular Ca\(^{2+}\) triggered a rise in cytosolic Ca\(^{2+}\) in both young and senescent HDFs (Fig. 2A, Table 2). The mean peak cytosolic Ca\(^{2+}\) level of young cells was, however, significantly (\(P < 0.01\)) higher than that of senescent HDFs (Table 2). Furthermore, although cytosolic Ca\(^{2+}\) declined to basal within 100 s of agonist application in young HDFs, this decay was much slower in senescent cells (Fig. 2A). In contrast, the application of thrombin (5 kIU/l) to HDFs in Ca\(^{2+}\)-free, EGTA-containing medium resulted in a more moderately enhanced cytosolic Ca\(^{2+}\) level in young cells, with virtually no increase in senescent cells (Fig. 2B). Again, this resulted in a significant \(P < 0.001\) difference between the mean peak cytosolic Ca\(^{2+}\) in young and senescent HDFs (Table 2).

The growth factors PDGF or EGF also triggered cytosolic Ca\(^{2+}\) signals in both young and senescent HDFs. Figure 3 shows that, in both cell populations, PDGF (10 µg/l) elicited either a transient rise in cytosolic Ca\(^{2+}\), an oscillatory response, or a biphasic elevation, consisting of a sharp initial rise followed by a plateau. The cytosolic Ca\(^{2+}\) signals triggered by EGF were similar to those elicited by PDGF, except that 1) a smaller overall number of HDFs responded to EGF; 2) sustained responses were by far never observed with EGF; and 3) oscillatory responses were observed in only 14% of EGF-treated senescent HDFs (Fig. 4).
Table 3 summarizes key data with PDGF and EGF. First, with either agonist, the number of responding cells was somewhat higher for young compared with senescent HDFs. Second, although oscillatory changes were observed more frequently in young cells, single responses were more common in senescent HDFs. Third, the peak signal amplitude was significantly greater in young compared with senescent HDFs. Finally, response latency, defined as the interval between agonist application and the first response, was markedly greater in senescent cells after either agonist. It often took as long as 10 min after EGF application before cytosolic Ca$^{2+}$ transients were elicited (Fig. 4).

We next measured cytosolic Ca$^{2+}$ levels in response to IP$_3$. Figure 5 shows the dramatically reduced cytosolic Ca$^{2+}$ signal triggered by intracellularly applied IP$_3$ to senescent HDFs. Note that the plasma membrane of both groups of cells was permeabilized in an identical protocol by using 60 mg/l saponin (for 5 min) to allow for the introduction of IP$_3$ into the cytoplasm. Note also that, as would be expected, basal cytosolic Ca$^{2+}$ levels of permeabilized HDFs are higher (~400 nM) than those
of nonpermeabilized cells (~100 nM). As indicated in the MATERIALS AND METHODS, after saponization and fluorochrome loading, the cells were bathed in HEPES-HBSS (cytosolic Ca\(^{2+}\) = 1.25 mM). We expect that this would allow for some re-sealing of the membrane, but because of the partially permeable membrane, intracellular Ca\(^{2+}\) should equilibrate to a new steady-state level; hence the high basal cytosolic Ca\(^{2+}\) levels. During this period, we also measured dye leakage by monitoring photon counts at \(\lambda_{485}\). Although the fluorochrome did leak over time, the signal-to-noise ratio remained stable, hence allowing valid comparisons. It is also important to note that the overall cytosolic Ca\(^{2+}\) response to applied IP\(_3\) was less marked than, for example, that due to bradykinin (cf. Fig. 2). This could be due to several reasons: suboptimal IP\(_3\) permeation, altered Ca\(^{2+}\) stores in permeabilized cells, or different (or additional) signaling pathways used by bradykinin.

We next compared the content of the IP\(_3\) receptor protein in young vs. senescent HDFs and in confluent vs. proliferating young cells by Western blotting (2, 34). A highly specific anti-IP\(_3\) receptor antiserum, Ab40, raised to the purified human type I IP\(_3\) receptor protein when microsomal membranes from young (lane 1) or senescent (lane 2) HDFs were probed with Ab40 (see MATERIALS AND METHODS for details). A ~260-kDa band corresponding to the IP\(_3\) receptor protein was seen; the band obtained with senescent HDF membranes was ~48 ± 14% less intense than that obtained with young cells. Figure 6B shows that the IP\(_3\) receptor band in proliferating young HDFs was 42 ± 4% less intense than that in confluent young HDFs. Taken together, the evidence indicates a marked reduction in IP\(_3\) responsiveness and IP\(_3\) receptor content in senescent HDFs.

Moreover, we compared the production of IP\(_3\) in young and senescent cells in response to bradykinin and EGF. The two agonists were chosen because they use distinct mechanisms for IP\(_3\) production. Bradykinin interacts with a G protein-coupled receptor, resulting in the activation of membrane phospholipase C that then catalyses IP\(_3\) formation. In contrast, EGF uses a tyrosine kinase receptor that phosphorylates a phospholipase C\(_{\gamma}\)-isoform. Figure 7 shows the results obtained after a 12-h incubation protocol with two mitogens. Statistical comparisons were made between young and senescent cells by using Student’s unpaired \(t\)-test with Bonferroni’s correction for inequality for significance values <0.05. Notably, senescent cells displayed a lower IP\(_3\) production with both bradykinin and EGF.

The possibility exists that membrane receptor number had decreased in senescent cells compared with young cells. We thus examined quantitatively, using the RNAse protection assay, the expression of the thrombin and EGF receptor. The band intensity for the

### Table 3. Comparison of the effects of the mitogens, platelet-derived growth factor, and epidermal growth factor on cytosolic Ca\(^{2+}\) changes by using a variety of parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Platelet-Derived Growth Factor</th>
<th>Epidermal Growth Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Senescent</td>
</tr>
<tr>
<td>Peak cytosolic Ca(^{2+}), nM</td>
<td>261 ± 12 (26)</td>
<td>192 ± 8.4 (20)*</td>
</tr>
<tr>
<td>Response latency, s</td>
<td>84 ± 7.1 (26)</td>
<td>123 ± 17.3 (20)</td>
</tr>
<tr>
<td>Cells responding, %</td>
<td>93 (26 of 28)</td>
<td>88 (20 of 28)</td>
</tr>
<tr>
<td>Oscillations</td>
<td>89 (23 of 26)</td>
<td>75 (15 of 20)</td>
</tr>
<tr>
<td>Single responses</td>
<td>11 (3 of 26)</td>
<td>25 (5 of 20)</td>
</tr>
<tr>
<td>Number of peaks</td>
<td>2.23 ± 0.13 (26)</td>
<td>2.15 ± 0.18 (20)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Nos. in parentheses, either no. of cells tested or no. of responding cells. *\(P < 0.001; \)†\(P < 0.005; \)‡\(P < 0.05: \)by ANOVA with Bonferroni’s correction for inequality.
thrombin receptor and EGF receptor was similar in both young and senescent HDFs, indicating the lack of a significant difference between receptor expression for the respective mitogens (Fig. 8).

**DISCUSSION**

We demonstrate that, compared with young cells, senescent HDFs display larger cell spread areas; lower basal cytosolic Ca\(^{2+}\) levels; reduced IP\(_3\) formation in response to mitogens; attenuated cytosolic Ca\(^{2+}\) responses to ionomycin, mitogens and IP\(_3\); and lowered IP\(_3\) receptor protein. Reduced IP\(_3\) receptor levels have also been reported in the aging rat cerebral cortex (17, 21). Nevertheless, these receptors have a ubiquitous distribution and are of fundamental importance in Ca\(^{2+}\) homeostasis. A senescence-associated reduction in their expression may therefore explain, at least in part, the attenuated Ca\(^{2+}\) signals observed in aging cells.

We first compared basal cytosolic Ca\(^{2+}\) levels in young and senescent HDFs. The cells were either proliferating asynchronously or were arrested in a specific cell cycle phase (4). The mean basal cytosolic Ca\(^{2+}\) level of senescent HDFs was lower than that of young cells in all phases, except during G\(_2\), when the difference was reversed. We believe that these changes, albeit small, represent genuine differences because of a strict calibration protocol used for each cell to calculate its absolute cytosolic Ca\(^{2+}\) level, and high power of analysis (because of a high n value). Indeed, senescent-associated decrements in basal cytosolic Ca\(^{2+}\) may be due, in part, to the enhanced Ca\(^{2+}\) buffering, resulting possibly from increased cellular calmodulin (4). By implication, therefore, senescent HDFs synchronized in G\(_2\) may express less calmodulin, and hence a higher cytosolic Ca\(^{2+}\). Interestingly, the latter finding is consistent with markedly increased basal cellular Ca\(^{2+}\) levels documented recently in aging human erythrocytes (1).

Next, we compared the fullness of intracellular Ca\(^{2+}\) stores in young and senescent HDFs. In the absence of extracellular Ca\(^{2+}\), an ionophore, such as ionomycin, would deplete all intracellular Ca\(^{2+}\) stores and elevate cytosolic Ca\(^{2+}\) transiently. In this instance, the rate of Ca\(^{2+}\) release would depend on the store-cytosol Ca\(^{2+}\) gradient, and hence, should reflect storefullness (in the presence of near-equal cytosolic Ca\(^{2+}\) levels). The rate of Ca\(^{2+}\) release was reduced by about sevenfold in senescent HDFs. Note that a low store Ca\(^{2+}\) content would not normally result from reduced IP\(_3\) responsive Ca\(^{2+}\) release channels. It should arise from poor store refilling due to either a reduced level of, or a defect in, microsomal membrane Ca\(^{2+}\)-ATPases. Store content was further examined by using thapsigargin, a microsomal membrane Ca\(^{2+}\)-ATPase inhibitor. Thapsigargin, by blocking Ca\(^{2+}\)-ATPase and preventing store refilling, releases Ca\(^{2+}\) from the store into the cytosol. We found that thapsigargin-induced Ca\(^{2+}\) release was greater in young than senescent HDFs, indicating an expected difference in store Ca\(^{2+}\) content.

We further attempted to 1) trigger the release of Ca\(^{2+}\) specifically from IP\(_3\)-sensitive stores and 2) determine whether such agonist-stimulated Ca\(^{2+}\) release was impaired in senescent HDFs. Several mitogens, namely,
bradykinin, thrombin, PDGF, and EGF, were applied to both young and senescent HDFs. Receptors for bradykinin and thrombin generate IP₃ by coupling to phospholipase C through the classic GTP-binding protein Gₛ. In contrast, tyrosine kinase receptors for PDGF and EGF phosphorylate and activate a specific phospholipase Cγ isoenzyme (5, 14, 29, 32). Thus activation of either class of receptor by its agonist, should, through a distinct mechanism, generate IP₃, and trigger Ca²⁺ release.

Senescent and young HDFs displayed critical differences in their sensitivity to PDGF and EGF. First, the magnitude of the cytosolic Ca²⁺ signal triggered in senescent cells was attenuated markedly compared with young HDFs. Second, oscillatory cytosolic Ca²⁺ changes were much less common in senescent HDFs. Note that oscillatory Ca²⁺ transients result generally from the rapid and recurrent activation of the IP₃ receptor (3). Thus attenuated Ca²⁺ oscillations can, by themselves, suggest an attenuated IP₃ production and/or reduced IP₃ receptor numbers. Finally, cells treated with either agonist, PDGF, or EGF showed evidence of a response latency. Again, latency is reminiscent of IP₃ receptor activation and is a function of both the IP₃ formation rate and IP₃ receptor number (3). Senescent cells showed much longer latency intervals, often up to 10 min, compared with young HDFs.

We do not believe that the variation in cytosolic Ca²⁺ responses to EGF and PDGF in young cells represents cellular heterogeneity. That all cells respond to bradykinin makes cellular heterogeneity a less likely explanation for the observed variability in mitogenic responsiveness. The latter is a well-characterized phenomenon for cells, wherein tyrosine kinase receptors trigger Ca²⁺ release through IP₃ generated from phospholipase Cγ. One possible reason is that the IP₃ generation is much slower than that for receptors using the G-protein-phospholipase C pathway (3). The generated IP₃ may therefore not build up to a high enough concentration to trigger IP₃ receptor-gated Ca²⁺ release. In the case of senescent cells, this phenomenon may become exacerbated, presumably because of reduced IP₃ receptor numbers per se.

Next, we tested, directly, whether senescent HDFs displayed 1) lowered mitogen-induced IP₃ formation and 2) reduced IP₃ receptor content. Senescent cells showed attenuated IP₃ responses to both bradykinin and EGF (and marginally reduced basal IP₃ levels). We next examined, in complementary experiments, the effect of IP₃ on Ca²⁺ release, as well as on IP₃ receptor protein expression. We found that the peak cytosolic Ca²⁺ change triggered by intracellularly applied IP₃ was fourfold lower in senescent HDFs compared with young cells. In parallel, we also found that the intensity of the 260-kDa protein band seen on Western blotting was ~50% lower in senescent HDFs. Note that the antibody, Ab⁵⁰, used to probe HDF microsomal membranes has been shown, specifically, to recognize the widely distributed type I IP₃ receptor (18). These results, when taken together, are remarkably consistent with, and could possibly explain, the attenuation of mitogen-induced Ca²⁺ release in senescent HDFs.

Finally, we assessed the expression of receptors for two mitogens, EGF and thrombin using the RNase protection assay. Such a quantitative method, using GAPDH as a standard, failed to show a significant difference in receptor expression, indicating clearly that the defect in signaling was postreceptor.

Thus we provide compelling functional and biochemical evidence for the attenuation of the IP₃-Ca²⁺ activation pathway, including importantly IP₃ receptor expression in aging HDFs. We are by no means claiming that deficiency of this pathway is the sole cause of the poor responsiveness of these cells to mitogenic and hormonal stimulation. Indeed, other molecules, such as surface hormone receptors or, indeed, the cyclic AMP generation machinery (13, 31), might undergo alterations with aging. Nevertheless, the IP₃-Ca²⁺ pathway remains a fundamental component of Ca²⁺ signaling in eukaryotic cells. Its possible attenuation in other aging cells, such as myocytes, neurones, macrophages, neutrophils, and lymphocytes (10, 12, 19, 27, 28), might underlie, at least in part, their reduced sensitivity to hormonal and mitogenic activation.

Numerous questions still remain unanswered. First, we are uncertain as to whether this loss of responsiveness results from, or else underlies, cellular senescence. Second, it remains unclear whether the more global consequences of aging, such as diminished proliferation, are a consequence of an attenuated IP₃-Ca²⁺ pathway. Third, it remains to be determined whether the expression of other Ca²⁺ release channels, such as the ryanodine receptor, or indeed plasma membrane cation channels, are also reduced in senescent cells. Finally, and perhaps, more important, our results lay down a firm scientific basis for future studies on the regulation of IP₃ receptor gene expression, an area that has been explored poorly to date.

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