Effects of Aging on Serum Ionized and Cytosolic Free Calcium
Relation to Hypertension and Diabetes

Mario Barbagallo, Ligia J. Dominguez, Giuseppe Licata, Lawrence M. Resnick

Abstract—Elevated cytosolic free calcium (Ca\textsuperscript{i}) and reciprocally reduced, extracellular ionized calcium (Ca-ion) levels are observed in both hypertension and non–insulin-dependent diabetes mellitus (NIDDM). Because the changes of vascular function and insulin sensitivity in these conditions resemble the changes associated with “normal” aging, we wondered to what extent similar alterations in calcium metabolism occur with aging per se in the absence of overt hypertension or diabetes. We therefore measured platelet Ca\textsubscript{i} levels by spectrofluorometry and serum Ca-ion levels in normotensive, nondiabetic, healthy, normal, elderly (>65 years old) subjects, mean age ±SEM, 72.2±1.5 years old (n=11); in healthy, normal, young (<65 years old) adults, 46.1±2.3 years old (n=12); in 10 young adult hypertensives, 48.6±1.9 years old; and in 10 normotensive NIDDM subjects, 49.2±1.6 years old. Platelet Ca\textsubscript{i} levels were higher (104.5±4.9 versus 80.2±1.8 mmol/L, \(P<0.01\)) and Ca-ion levels lower (1.212±0.010 versus 1.236±0.011 mmol/L, \(P<0.05\)) in normal elderly compared with young control subjects, but normal elderly Ca\textsubscript{i} and Ca-ion levels were indistinguishable from those in hypertensive (Ca\textsubscript{i} 107.5±3.6 mmol/L, Ca-ion 1.210±0.009 mmol/L) and NIDDM (Ca, 110.7±4.7 mmol/L, Ca-ion 1.204±0.014 mmol/L) subjects. In normal subjects, significant correlations were found between platelet Ca\textsubscript{i} levels and age (\(r=0.655, P<0.01\)) and between Ca\textsubscript{i} levels and systolic blood pressure (\(r=0.733, P<0.001\)). We conclude that aging is associated with alterations of Ca\textsubscript{i} and Ca-ion levels resembling those changes present at any age in hypertension and type 2 diabetes. We hypothesize that these alterations of calcium metabolism underlie the predisposition to the alterations of blood pressure and insulin sensitivity characteristic of “normal” aging. The data also suggest that studies of the aging process should be limited to subjects with normal blood pressure and glucose tolerance.

Key Words: calcium ■ platelets ■ aging ■ hypertension ■ diabetes mellitus

Calcium ions, both intracellular and extracellular, are critically important for a wide spectrum of cellular processes, including skeletal, cardiac, and vascular smooth muscle contraction; neural excitability; and stimulus-secretion coupling in all endocrine and exocrine tissues.\textsuperscript{1,2} In the steady state, altered calcium and magnesium levels are present in essential hypertension and in non–insulin-dependent diabetes mellitus (NIDDM).\textsuperscript{2} Elevated basal cytosolic free calcium (Ca\textsubscript{i}) levels, as well as defective membrane binding and transport kinetics of calcium, have been identified in platelets,\textsuperscript{3} erythrocytes,\textsuperscript{4} lymphocytes,\textsuperscript{5} and adipocytes\textsuperscript{6} of hypertensive subjects, in whom blood pressure levels were closely and directly related to Ca\textsubscript{i} content.\textsuperscript{3,4} In diabetes, even in the absence of overt hypertension, Ca\textsubscript{i} levels were similarly elevated,\textsuperscript{4} and defective calcium handling has been found in all diabetic tissues tested thus far, including cardiac and skeletal muscle, arteries, kidney, liver, erythrocytes, osteoblasts, adipocytes, and platelets.\textsuperscript{7} Published data on the effects of normal aging on serum calcium levels are scanty and contradictory,\textsuperscript{8–11} although aging is also associated with an increasing arterial stiffness and/or blood pressure,\textsuperscript{12} reduced glucose tolerance,\textsuperscript{13} and general alterations of calcium metabolism.\textsuperscript{14} We thus wondered to what extent these age-related changes could be a reflection of alterations in the extracellular and intracellular distributions of free calcium similar to those we had previously demonstrated in hypertensive and NIDDM subjects.

The present investigation was undertaken to examine the effect of age on serum ionized calcium (Ca-ion) and cytosolic Ca\textsubscript{i} concentrations in elderly normotensive, nondiabetic, healthy subjects compared with younger control normotensive, nondiabetic subjects and young adult hypertensive and NIDDM subjects. Our preliminary results indicate that “normal” aging per se is indeed associated with altered calcium distributions, increased cytosolic Ca\textsubscript{i}, and reciprocally decreased serum Ca-ion, that are indistinguishable from those,
independent of age, that are associated with hypertension and/or NIDDM.

**Methods**

Forty-three subjects were studied consecutively at the Institute of Internal Medicine and Geriatrics of the University of Palermo, Palermo, Italy. Four groups of patients were studied: 23 were healthy, normal subjects, arbitrarily divided into 2 groups by age as being <65 years old (n = 12, 46.1 ± 2.3 years, mean ± SEM) and >65 years old (n = 11, 72.2 ± 1.5 years old); 10 were young, age-matched, adult essential hypertensive subjects (48.6 ± 1.9 years old); and 10 were young, age-matched, adult normotensive NIDDM subjects (49.2 ± 1.6 years old).

Normal elderly subjects compared with young subjects, excluding age matching, exhibited no differences in gender, race, blood pressures, or body mass index (Table 1). Essential hypertension was diagnosed on the basis of blood pressures >150/90 mm Hg measured on 3 different occasions, in the absence of history, physical examination, or laboratory evidence of secondary forms of hypertensive disease. Hypertensive subjects refrained from taking medication for at least 3 weeks before the study and from diuretic therapy for at least 6 months before the study, which in our previous experience is a sufficient time to achieve stable ion levels. None had significant renal dysfunction, as assessed by serum creatinine levels. Excluding blood pressure levels, no differences in age, gender, race, or body mass index were present among hypertensive compared with normotensive subjects (Table 1). All NIDDM subjects were treated with diet therapy only and had never been treated before with insulin or hypoglycemic agents.

After an overnight fast, 20 mL of heparinized blood was drawn to measure cytosolic Ca2+ levels in platelets with a Perkin-Elmer LS-50 spectrofluorometer, as described by Grinkiewicz et al. Ten milliliters of blood were drawn into a heparinized tube. Platelet-rich plasma was separated by centrifugation at 180g for 15 minutes. The platelets were washed once, recentrifuged, and resuspended in physiological salt solution containing (in mmol/L) 145 NaCl, 5 KCl, 10 HEPES, 1 MgSO4, 0.5 NaH2PO4, and 6 glucose, pH 7.4. Fura 2-acetoxymethyl ester (3 μmol/L; Calbiochem) was added to the above-mentioned calcium-poor medium to prevent platelet aggregation and incubated for 30 minutes at 37°C. After removing the fura 2 dyes by centrifugation, platelets were resuspended in the same solution without fura 2 and incubated for an additional 30 minutes. At this time, 1.5 mmol/L CaCl2 was added to the platelet suspension for the calcium measurement.

The excitation wavelengths were set at 340/380 nm, and the emission wavelength was set at 505 nm. Cytosolic Ca2+ was calculated as described by Grinkiewicz et al. by using a Ca2+ fura 2 K0.25 of 224 mmol/L. Maximum intensities were determined by lysing the cells with Triton X-100. Minimum intensities were determined by adding 20 mmol/L EGTA.

**Statistical Analysis**

All values are reported as the mean ± SEM. One-way ANOVA and subsequent post hoc tests (Super-Anova, Abacus Concepts Inc) were used to assess the significance of the differences in values that were measured in normal, young, control subjects compared with elderly, hypertensive, and NIDDM subjects. Pearson’s correlation coefficients were used to analyze linear correlations between variables. Differences were considered statistically significant at a probability value <0.05.

**Results**

Clinical and laboratory characteristics of the study subjects are reported in Tables 1 and 2, respectively. Cytosolic Ca2+ levels in normal, elderly subjects were significantly higher (104.5 ± 4.9 nmol/L) compared with those in younger control subjects (80.2 ± 1.8 nmol/L, P < 0.01) but were indistinguishable from those in younger hypertensive (107.5 ± 3.6 nmol/L) and young NIDDM (110.7 ± 4.7 mmol/L) subjects (Table 2), who also exhibited significantly elevated Ca2+ values (P < 0.01) compared with young, normal control subjects (Figures 1 and 2). Conversely, extracellular serum Ca-ion levels were significantly lower in elderly versus young normal subjects (1.212 ± 0.010 versus 1.236 ± 0.011 mmol/L, P < 0.05). This finding paralleled the same behavior for hypertensive and NIDDM subjects, who displayed lower Ca-ion levels compared with age-matched, normal control subjects (hypertensives 1.210 ± 0.009 mmol/L and NIDDM 1.204 ± 0.014 mmol/L; P < 0.01 versus young controls). As for Ca2+ levels, serum Ca-ion levels in normal, elderly subjects with respect to hypertensive and/or diabetics subjects were indistinguishable (Figure 1 and Table 2).

For all normal subjects, platelet Ca2+ levels were significantly and directly related to age (r = 0.655, P < 0.01) and systolic blood pressure (r = 0.733, P < 0.001), whereas extracellular serum Ca-ion values were reciprocally and inversely related to age (r = –0.455, P < 0.01; Figures 2A and 2B).

**Discussion**

The present data reveal altered distributions of extracellular and intracellular free calcium attributable to age per se.
Specifically, elevated platelet Ca and lower serum Ca-ion levels were observed in normotensive, nondiabetic, elderly, healthy subjects compared with healthy, younger control subjects. Interestingly, these “normal” elderly values were indistinguishable from values observed among subjects who, albeit younger, had either essential hypertension or NIDDM. These latter subjects also exhibited elevated Ca and lower Ca-ion values compared with age-matched, normotensive, nondiabetic controls.

“Normal” aging in the absence of hypertension, as well as arterial hypertension itself, are both associated with many similar alterations of cardiovascular and metabolic function. Thus, increased arterial stiffness and/or blood pressure, increased peripheral vascular resistance, and increased left ventricular mass are common in hypertensive patients and in healthy, normotensive, elderly subjects. Similarly, abnormalities of carbohydrate metabolism, such as hyperinsulinemia, insulin resistance, altered glucose tolerance, and/or frank NIDDM also occur more frequently with increasing age. Conversely, hypertension and NIDDM, even at a younger age, display the “premature” ionic changes of aging and may thus be considered diseases of accelerated vascular aging.

However, the biological basis for these alterations and how both hyperinsulinemia/insulin resistance and hyperglycemia are linked to the hypertension and vascular disease associated with NIDDM and aging remains undefined. We have

<table>
<thead>
<tr>
<th>Laboratory Parameters</th>
<th>Young Normals</th>
<th>Elderly Normals</th>
<th>HTN</th>
<th>NIDDM</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPG, mg/dL</td>
<td>89.5±3.1</td>
<td>96.1±4.0</td>
<td>97.2±2.3</td>
<td>143.6±5.9*</td>
</tr>
<tr>
<td>FPI, μU/mL</td>
<td>12.6±3.1</td>
<td>16.8±2.1</td>
<td>17.2±2.8</td>
<td>22.4±6.8</td>
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<td>AUC-glucose, mg/dL</td>
<td>534.5±28.0</td>
<td>582.6±38.6</td>
<td>580.9±26.8</td>
<td>1151.7±116.2*</td>
</tr>
<tr>
<td>Ca, nmol/L</td>
<td>80.2±1.8</td>
<td>104.5±4.9*</td>
<td>107.5±3.6*</td>
<td>110.7±4.7*</td>
</tr>
<tr>
<td>Ca-ion meq/L</td>
<td>1.236±0.011</td>
<td>1.212±0.010†</td>
<td>1.210±0.009†</td>
<td>1.204±0.014†</td>
</tr>
<tr>
<td>Serum Ca²⁺, meq/L</td>
<td>9.6±0.11</td>
<td>9.5±0.13</td>
<td>9.5±0.12</td>
<td>9.3±0.14</td>
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<tr>
<td>Serum Mg²⁺, meq/L</td>
<td>1.95±0.10</td>
<td>1.85±0.10</td>
<td>1.83±0.08</td>
<td>1.82±0.09</td>
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<tr>
<td>Serum Na⁺, meq/L</td>
<td>140.8±0.4</td>
<td>143.6±0.7†</td>
<td>141.0±0.4</td>
<td>142.3±1.0</td>
</tr>
<tr>
<td>Serum K⁺, meq/L</td>
<td>4.5±0.07</td>
<td>4.4±0.13</td>
<td>4.1±0.06†</td>
<td>4.1±0.10†</td>
</tr>
<tr>
<td>BUN, mg/dL</td>
<td>14.9±1.4</td>
<td>16.2±1.1</td>
<td>16.9±1.3</td>
<td>17.6±1.5</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.89±0.06</td>
<td>0.96±0.06</td>
<td>0.96±0.14</td>
<td>0.98±0.113</td>
</tr>
</tbody>
</table>

HTN indicates hypertension; FPG, fasting plasma glucose; FPI, fasting plasma insulin; AUC, area under the curve; and BUN, blood urea nitrogen.

*p < 0.01 vs young normals.
†p < 0.05 vs young normals.

Figure 1. Effect of age on platelet cytosolic free calcium (A) and ionized calcium (B) levels in healthy, young adults (NLY), elderly subjects (NLE), young hypertensive (HTN) subjects, and young type 2 diabetic (NIDDM) subjects. *P < 0.05 vs NLY.

Figure 2. Relationship between age and platelet cytosolic free calcium (A) and ionized calcium (B) levels in normal young and elderly subjects (n=23).
previously suggested that these linked abnormalities could be explained, at least in part, on a cellular ionic basis and have proposed an “ionic hypothesis” in which lower intracellular free magnesium and/or elevated free calcium levels are a cellular “lesion” shared in common by all tissues. This ionic lesion, as part of a final, common pathway mediating blood vessel tone, pancreatic insulin secretion, peripheral tissue insulin sensitivity, sympathetic nerve activity, etc in turn represents a necessary though not sufficient condition for the emergence of elevated blood pressure, insulin resistance, and the other clinical manifestations subsumed by the term “syndrome X.”2,4,18–22 In support of this hypothesis, we demonstrated that the height of blood pressure, the degree of peripheral insulin resistance, and the ambient blood glucose values in hypertensive and NIDDM subjects4,18,19 are predicted by the quantitative deviations of Ca, and cytosolic free magnesium from average normal values.4

Our present results are consistent with and extend our previous work, showing that the elevated Ca, and the lower Ca-ion levels present in healthy, elderly subjects are not distinguishable from those occurring in young, essential hypertensives or NIDDM subjects. These results are also consistent with the literature, wherein elevated Ca,6,7 and either reduced or normal levels of extracellular Ca-ion have been reported previously in elderly subjects.8–11 Thus, it appears that aging per se, in common with hypertension and NIDDM, exhibits a similar maldistribution of calcium between extracellular and intracellular sites, with more calcium accumulating in the cytosol as a reflection of less calcium in the circulation. As is true for hypertension and diabetes, this defect in elderly subjects may help to explain and be a necessary condition underlying the increasing incidence of hypertension and predisposition to insulin resistance and diabetes characteristic of what has always been considered “normal” aging (Figures 1 and 2). Indeed, increased Ca levels as well as suppressed intracellular free magnesium levels induce insulin resistance22,23 and inhibit insulin-dependent glucose transport24 without changing insulin binding or tyrosine kinase activity.

What mechanism(s) can account for this cytosolic Ca rise with age? One consideration, although such data were not obtained in our subjects, is that the progressive decrease of dietary calcium intake with age prevalent in Western societies may be an initiating factor. This may lead to lower serum Ca-ion levels, which remain within the normal range only at the expense of chronically higher circulating levels of calcium-regulating hormones such as parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D (1,25D). These hormones facilitate cellular calcium uptake, not only in gastrointestinal and renal tissues, but in cardiovascular tissues as well. Indeed, 1,25D stimulates the L-channel calcium current in vascular smooth muscle cells25 and epidemiologically is a strong correlate of blood pressure in a general population.26 Conversely, the ability of oral calcium supplementation to lower blood pressure is strongly correlated with the concomitant suppression of circulating 1,25D levels.27 Thus, lower average calcium intakes and compensatorily higher circulating levels of various calcium-regulating hormones that facilitate calcium uptake from the extracellular space would not only help to explain the higher average Ca levels observed here but also the lower average plasma renin activity levels characteristic of older populations.28 Indeed, low-renin essential hypertensive subjects display significantly lower Ca-ion levels coupled with higher PTH and 1,25D levels.29,30 An intriguing question resulting from this is whether increased dietary calcium intake would reverse these calcium ionic shifts observed with aging.

Second, the small differences that we observed in extracellular Ca-ion levels among normal, elderly subjects may themselves be significant. Indeed, extracellular calcium appears to influence ongoing intracellular Ca, reactivity to various stimuli. In particular, we have recently shown that this phenomenon, referred to in the literature as the “membrane-stabilizing effect of calcium”31,32 or calcium “gating its own channels,”73 may also operate at physiological calcium concentrations, i.e., within the normal range of extracellular Ca-ion concentrations.34 Thus, the rise in Ca levels induced by intravenous calcium infusions, which elevated Ca-ion levels only slightly within the normal range, were closely and inversely related to the basal, preinfusion Ca-ion level. The lower the basal extracellular Ca-ion, the greater was the rise in Ca, and the higher the extracellular Ca-ion, the less was the rise in Ca.34 This phenomenon, which we have termed “calcium-inhibitable calcium entry,” has recently been supported by in vitro studies in lymphocytes.35

Certain caveats to the interpretation of our data should also be considered. First, a comparison with other cell types to substantiate our present ion measurements should be made, especially in those tissues participating more directly in the vascular and metabolic diseases more prevalent with aging. Second, although serum creatinine levels were not significantly different among the groups, creatinine clearance values would have been a preferable way of ensuring comparability among the groups. Last, exclusion of the assessment of dietary mineral content, which might have provided additional mechanistic clues underlying our present data, makes it unclear to what extent our results reflect something necessarily intrinsic to the aging process or rather merely a potentially reversible common accompaniment of it.

In summary, the present study demonstrates a maldistribution of intracellular and extracellular calcium in healthy, aged subjects that is also found in young hypertensive and diabetic subjects and that may represent a pathophysiological lesion connecting these conditions with aging. To what extent these findings result from (1) dietary-influenced alterations in hormonal systems such as calcium-regulating hormones; (2) sodium volume and vascular hormone systems such as the renin-angiotensin-aldosterone system or the sympathetic nervous system; or (3) altered cell membrane calcium binding or availability or other mechanisms is unknown. Further studies are clearly needed to provide insight into those processes regulating the cellular ionic environment, their derangements with age, and their relation to senescence.

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