0014-4754/95/100976-04$1.50 + 0.20

Chronic overcrowding decreases cytoplasmic free calcium levels in T lymphocytes of aged CBA/CA mice

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Received 22 November 1994; accepted 8 February 1995

Abstract. Intracellular calcium concentration is a sensitive marker of the homeostasis of living cells, and its increase is an essential step of T lymphocyte activation. Changes in the environment provoke an adaptive stress-response of the organism. In our present work we have investigated the effect of chronic overcrowding on resting and lectin-stimulated cytoplasmic free calcium concentration of splenic T lymphocytes from young and aged CBA/CA mice (50 animals total). The animals were kept under 'normal' (68 cm^2/animal) or 'overcrowded' (22 cm^2/animal) conditions for 3 months. Young animals showed no change in resting and stimulated calcium after overcrowding. T cells from aged mice, however, displayed significantly smaller levels of both resting and lectin-stimulated intracellular calcium concentration ($p < 0.01$ each), as compared to those of the non-stressed, aged animals. This inadequate adaptation in the calcium metabolism of T lymphocytes may significantly contribute to the diminished immune response of the aged in stress.
Key words. Calcium; T lymphocytes; aging; psychosocial stress; overcrowding; animal housing; lymphocyte activation.

Intracellular calcium concentration is a sensitive marker of the homeostasis of living cells. Calcium is a second messenger, triggering a vast variety of changes in the cell. Persistent elevation of intracellular – and intranuclear – calcium concentration accompanies, and to some extent induces, the apoptosis of many cells, e.g. thymic lymphocytes[1–4]. On the other hand, a transient rise in intracellular calcium concentration is one of the first signals of T lymphocyte activation[5,6].

Overcrowding and other forms of psychosocial stress were shown to modulate the immune response in mice and rats[7,8]. Though Huie et al.[9] demonstrated a decrease in serum ionized calcium in stressed rats, our knowledge about the changes of intracellular calcium concentration after psychosocial stress is very limited.

Our earlier studies indicated that aging induces a decrease in resting levels of intracellular free calcium of human T lymphocytes[10,11]. Transient (10 days) overcrowding induced an elevated Ca-response of mouse T lymphocytes[12]. Extending these studies we have investigated the effect of persistent (3 months) overcrowding on resting and stimulated cytoplasmic free calcium concentration in splenic T lymphocytes of young and aged CBA/CA mice.

Materials and methods

Reagents and cells. Concanavalin-A (type IV), digitonin, dimethyl-sulfoxide (DMSO), EGTA, foetal calf serum (FCS), Hepes, phytohaemagglutinin, RPMI 1640 medium and succinyl-Concanavalin-A were from Sigma. TPEN (N,N,N′,N′,-tetrakis (2-pyridyl-methyl)-ethylene-diamine) and fura-2 acetoxymethyl ester (fura-2 AM) were from Calbiochem. Young (20 weeks) and old (24 months) CBA/CA mice (Lati, Gödöllö, Hungary) were kept under 'control' (68 cm^2/animal) or 'overcrowded' (22 cm^2/animal) conditions for 3 months. Splenic T lymphocytes were separated from erythrocytes by hypotonic lysis in ice-cold distilled water and from granulocytes, monocytes and B lymphocytes by adherence to plastic in RPMI 1640 medium with 10% FCS.

Measurement of intracellular calcium concentration. Intracellular calcium concentration was measured as described earlier[10,11]. Splenic T lymphocytes (5×10^6 cells/ml) were incubated with fura-2 AM at a final concentration of 2 μM in RPMI 1640 medium for 30 min at 37 °C. After a 5-fold dilution the incubation was continued for an additional 15 min. Cells were washed twice in a modified Hank's medium (143 mM NaCl, 1 mM Na_2SO_4, 5 mM KCl, 1 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 10 mM Hepes, pH 7.45) and the experiment was completed within 45 min. Fluorescence measurements were performed at a cell density of 5×10^6 cells/ml with gentle stirring using a PTI Deltascan V-1048 D101 type interfaced fluorimeter at 37 °C. Excitation and emission wavelengths were 340 (380) and 520 nm respectively, with 5 nm slits. After the initial fluorescence had been recorded, Concanavalin-A (Con-A), succinyl-Con-A, or phytohemag-

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Table. Resting and Concanavalin A-stimulated intracellular calcium concentration of splenic T lymphocytes in young (5 months) and aged (24 months) CBA/CA mice with or without chronic (3 months) overcrowding.

Group no.	Age (months)	Treatment	Resting [Ca] (nM)	Stimulated [Ca] (nM)	Difference (nM)	Relative change (% of control)
1	5	control	100.2 ± 7.9 (9)	130.0 ± 10.9 (5)	29.8	30
2	5	stressed	100.7 ± 8.0 (15)	137.9 ± 8.9 (10)	37.2	37
3	24	control	93.8 ± 6.2[a] (10)	128.4 ± 6.9[b] (9)	34.6	37
4	24	stressed	69.0 ± 5.3[a] (16)	98.7 ± 7.6[b] (12)	29.7	43

Isolated splenic T lymphocytes were treated with Concanavalin A ('stimulated' cells) and intracellular calcium concentration (Ca_i) was measured as described in 'Materials and methods'. Ca_i values from those animals which showed significant pathological changes (e.g. visible tumors of liver and spleen; 1, 3, 1 and 6 mice from groups 1 through 4, respectively) were omitted from the final calculations. The results were similar if these omissions were not made, only the level of significance decreased to a value of $p < 0.05$. Data are means ± SEM. The numbers in parentheses denote the number of animals of each experimental group. [Ca] = intracellular calcium concentration; [a,b]level of significance $p < 0.01$ (calculated by Student's t-test).

glutinin were added at final concentrations of 6, 6, and 4 µg/ml, respectively. The 1 mg/ml stock solution of Con-A contained 0.5 mM $MnCl_2$ to obtain maximal efficiency. Samples were calibrated with the digitonin-(10 µM)-EGTA (5 mM) method and the intracellular calcium concentration was calculated as described earlier[10]. Data were corrected for the aspecific lysis of fura-2 by the addition of extracellular Mn^{2+} or EGTA (see ref. 9).

Results

Resting intracellular calcium concentration (Ca_i) was not significantly different in control and in stressed young animals (cf. groups 1 and 2 in the table). Splenic T lymphocytes of old CBA/CA mice, however, showed a significant ($p < 0.01$, using Student's t-test) decrease in Ca_i after overcrowding (fig. 1, and groups 3 and 4 in the table).

If T lymphocytes were stimulated with Concanavalin-A (Con-A) at a final concentration of 6 µg/ml, cells from young animals showed a similar increase in cytoplasmic calcium, irrespective of the prior stress treatment. Lymphocytes from aged animals stressed by overcrowding reached a significantly ($p < 0.01$) smaller Ca_i level after Con-A stimulation than those from non-stressed mice (fig. 1 and the table). The Con-A-stimulated intracellular calcium concentration of T cells from aged mice was commensurate with the resting calcium levels of splenocytes from young animals. The time-course of Concanavalin-A-induced changes in Ca_i was similar in control and stressed aged animals (fig. 1). Despite the marked differences in the level of stimulated Ca_i, Con-A-induced differences in intracellular calcium concentration were similar in control and stressed old animals (34.6 and 29.7 nM, respectively, see groups 3 and 4 in the table and in figure 2. Similar effects were observed if succinyl-Concanavalin-A (6 µg/ml), or phytohemagglutinin (PHA-L, 4 µg/ml) were used as stimulants. Changes in the calcium level of T lymphocytes from adult (12 months old) CBA/CA mice were similar to those in young ones (data not shown). Chelation of extracellular calcium (by the addition of extracellular EGTA at a

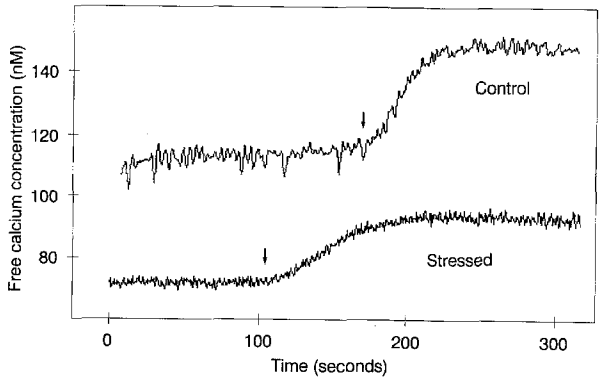

Figure 1. Time course of changes in intracellular calcium concentration after Concanavalin-A stimulation of splenic T lymphocytes from control and stressed old CBA/CA mice. Fura-2 fluorescence was measured and intracellular free calcium concentration was calculated as described in 'Materials and methods'. At the arrows Concanavalin-A was added at a final concentration of 6 g/ml. Top and bottom lines represent measurements of T lymphocytes from aged mice without ('Control'), or with overcrowding ('Stressed'), respectively. Curves are representative of a minimum of nine experiments.

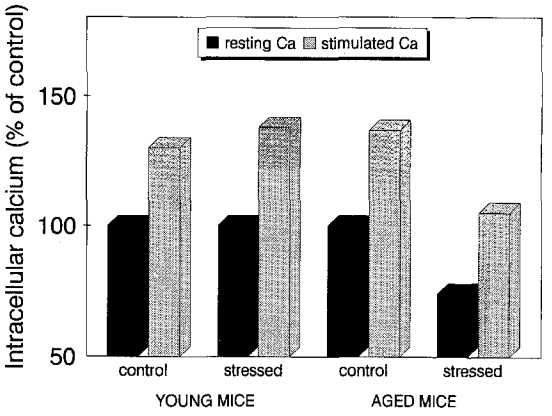

Figure 2. Percent changes of resting and Concanavalin A-stimulated intracellular calcium concentration of T lymphocytes from young and aged CBA/CA mice with or without overcrowding. Data are calculated from the Ca_i values of the table and expressed as percentages of the resting intracellular calcium levels of control young and old mice (100.2 and 93.8 nM, respectively).

final concentration of 5 mM promptly before the stimulation of the cells with Concanavalin-A) largely abolished the lectin-induced increase in intracellular calcium levels in T lymphocytes from both control and stressed young and old animals (data not shown).

Discussion

T lymphocytes of aged CBA/CA mice display a similar resting intracellular calcium concentration (Ca_i), to that in cells of young animals. On the contrary, lymphocytes from aged animals show a statistically significant decrease of Ca_i (32 nM, $p < 0.01$) after prolonged overcrowding lasting for 3 months. Lower Ca_i might be due to increased concentrations of intracellular heavy metal ions[13,14]. However, in our case this does not seem to be the case, since administration of TPEN, a heavy metal chelator which penetrates into the cells, did not change the effects observed (data not shown). There is a great variety of data on changes of resting intracellular calcium levels in lymphocytes of aged nice and humans. Decrease[10,11,15], increase[16] and no significant change[17,18] in resting Ca_i have all been reported. Variations in the level of psychosocial stress may have been the cause of these differences.

Our previous results indicated a 3-fold increase in the Ca-response of T lymphocytes of young CBA/CA mice to lectin stimulation after transient (10 days) overcrowding, compared to that of non-stressed, young animals[12]. On the contrary, persistent (3 months) overcrowding does not cause any significant changes in the difference between resting and stimulated Ca_i levels in young mice. Similarly to these observations, Monjan and Collector[7] also demonstrated that stressor-induced mitogenic stimulation of mouse splenic T lymphocytes levelled off after 1.5 months. Chronic stress may evoke different levels of adaptation.

The immune system of old animals is much more sensitive to the suppressive effect of stress than that of young subjects[19,20]. This age-dependent sensitization is generally thought to be mediated by various changes in the endocrine status of old subjects[19]. Our results may provide an alternative view or an extension of this explanation. The intracellular calcium concentration must reach a certain threshold level, around 120–150 nM, to be able to induce the activation of T lymphocytes[5,6,10]. Our results indicate that T lymphocytes of old (but not young) animals barely reach this Ca_i level after persistent overcrowding. Since the subjective stress level ('felt stress') of an aging subject is generally higher than that of a young one, this inadequate adaptation in the calcium metabolism of T lymphocytes may significantly contribute to the diminished immune response of the aged.

The mechanism of the observed changes in the calcium responsiveness of T lymphocytes from aged animals after overcrowding is not clear. Huie et al.[9] demonstrated a decrease in serum ionized calcium after persistent (3 months) overcrowding in rats. Since the intracellular calcium concentration of T lymphocytes shows parallel changes with the extracellular ionized calcium[9,13], and the 'required' (calculated to cause the 25 nM drop in intracellular calcium concentration after overcrowding in aged animals) versus observed changed in extracellular ionized calcium are in the same range (0.3 and 0.1 mM, respectively), stress-induced decrease in serum ionized calcium may significantly contribute to the decrease of Ca_i in old, stressed animals. Ghoneum et al.[20] and Hoffman-Goetz et al.[21] described various changes in the subpopulations of splenic T lymphocytes after psychosocial stress. Changes in the composition of the splenic T lymphocyte pool may also influence both the resting and stimulated intracellular calcium levels after stress.

Acknowledgments. This work was supported by research grants from the Hungarian Academy of Sciences (OTKA-T5534, T17720), the Hungarian Ministry of Social Welfare (ETT-681/93), from COST Human Exchange and Mobility programs ERB-CIPDCT 930188/940604 and from the Zsigmond Foundation. P.C. is a recipient of a Howard Hughes International Research Award. The authors are thankful to Drs Attila Fonyó and András Spät (Semmelweis University, Institute of Physiology) for the use of the fluorimetric facility, to Prof. Edit Beregi (Semmelweis University, Gerontology Center) for her valuable advice and for the help in the isolation of mouse splenic T lymphocytes, to Dr. Mária Boross (Semmelweis University, Gerontology Center) for the supervision of the animal care during overcrowding and to Mrs. Terézia Tóth for her excellent technical assistance.

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