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THE NITRIC OXIDE DONOR, SODIUM NITROPRUSSIDE, INCREASED INTRANUCLEAR AND CYTOSOLIC FREE CALCIUM CONCENTRATION IN SINGLE PU5-1.8 CELLS

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Summary: In the present study, the effect of sodium nitroprusside (SNP), a nitric oxide (NO) releasing agent, on the intracelluar free calcium concentration ($[Ca^{2+}]i$) was studied. Our results demonstrate that SNP could increase $[Ca^{2+}]i$ in single PU5-1.8 cells, a murine macrophage cell line, as determined by confocal laser scanning microscope with calcium green-1 fluorescent probe. In the presence of hemoglobin, a NO scavenger, the effect of SNP on the Ca^{2+} level was suppressed. Our data also show that the intranuclear Ca^{2+} ($[Ca^{2+}]n$) was always higher than those in cytosol. Stimulation of cells with 1mM SNP induced an immediate increase of $[Ca^{2+}]n$ with a delayed rise of cytosolic Ca^{2+} ($[Ca^{2+}]c$). Interestingly, when cells were subsequently treated with 2mM SNP, an increase of $[Ca^{2+}]c$ with a similar pattern to the rise $[Ca^{2+}]n$ was observed. In Ca^{2+}-free buffer, SNP also raised the $[Ca^{2+}]n$ and $[Ca^{2+}]c$. When Ca^{2+} was added to the buffer, a second peak of $[Ca^{2+}]n$ and several spikes of $[Ca^{2+}]c$ were observed. These results thus indicate that 1) NO may increase both $[Ca^{2+}]n$ and $[Ca^{2+}]c$ in PU5-1.8 cells; 2) the increase of $[Ca^{2+}]n$ requires both internal and external Ca^{2+}. Moreover, the diverse responses in these cells suggest that the regulation of Ca^{2+} in these areas may be different.

Receptor-mediated generation of inositol (1, 4, 5) trisphosphate (IP_3) evokes changes of intracellular free Ca^{2+} concentration in a variety of cells (1). The increase of Ca^{2+} was found to be important in a number of cellular functions including hormone secretion, fertilization, neurotransmitter release, muscle contraction and immune response. In the past few years, it was found that the regulation of intracellular Ca^{2+} is very complicated. With an aid of fluorescent microscopic imaging technique (2), spatial and temporal changes of cytosolic Ca^{2+}, ranging from

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Abbreviations: Nitric oxide (NO), Sodium nitroprusside (SNP), Intracelluar free Ca^{2+} level ($[Ca^{2+}]i$), Intranuclear free Ca^{2+} level ($[Ca^{2+}]n$), Cytosolic free Ca^{2+} level ($[Ca^{2+}]c$).

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Ca^{2+} oscillations to Ca^{2+} microdomains, has been observed (3, 4). More recently, data have indicated that emptying of intracellular Ca^{2+} stores releases a novel small messenger, calcium influx factor, that stimulates Ca^{2+} influx (5). On the other hand, attempts have been made to study the Ca^{2+} regulation inside the nucleus (6, 7). For example, receptors for IP_3 have been found to localize to the nuclear envelope in neurons (8). Nuclei isolated from hepatocytes release Ca^{2+} in response to IP_3 (9). Moreover, activation of nuclear protein kinase C accelerated the IP_3-mediated Ca^{2+} release in isolated rat liver nuclei (10). These suggest that the calcium events in the nucleus may be independent of and different from the one in the cytosol. In light of this, we attempt to investigate whether a nuclear Ca^{2+} signalling system exists in living cells. In the present study, we demonstrate that sodium nitroprusside (SNP), a nitric oxide (NO) releasing agent (11), was able to induce an increase of Ca^{2+} in nucleus and in cytosol. Results also indicate that the increase of intranuclear free calcium level ($[Ca^{2+}]n$) requires both Ca^{2+} release from internal pools and calcium influx from external source.

Materials and Methods

Chemicals: The following chemicals were used: Calcium green-1/AM (Molecular Probes), Sodium nitroprusside and hemoglobin (Sigma).

Cell culture: PU5-1.8 cells derived from Balb/C mice were obtained from American Type Culture Collection (Maryland). Cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco) at 37°C, 5% CO_2. The generation time of PU5-1.8 cells is about 24h at 37°C, 5% CO_2 in the presence of 10% FCS. Before experiments, cells were cultured on a steriled coverslip (Fisher) overnight at 37°C, 5% CO_2 in serum-free RPMI medium.

Determination of intracellular Ca^{2+} level by confocal laser scanning microscopy: Calcium green-1, a Ca^{2+} fluorescent indicator, was used to monitored changes of intracellular Ca^{2+} (12). PU5-1.8 cells grown on coverslips were loaded with 1μM calcium green-1/AM at room temperature for 30min in Na^+-Hepes buffer (in mM: 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Hepes, final pH 7.2). After washing, cells were incubated in 0.5ml Na^+-Hepes buffer or Ca^{2+}-free buffer (in mM: 140 NaCl, 5 KCl, 2 EGTA, 1 $MgCl_2$, 10 glucose and 10 Hepes, final pH 7.2) and the change of calcium was measured with confocal imaging system at room temperature. At various time intervals, agonists were added as indicated. Images of 256 x 256 pixel were acquired at 2.5sec intervals on a Multiprobe 2001 confocal laser scanning microscope (Molecular Dynamics) fitted with an Argon laser (8mW) and a Nikon diaphot inverted microscope. For the fluorescence determination, an excitation filter with 488nm wavelength and a long pass emission filter of 510nm were used. Cells were scanned using a 60X oil objective with 1.4 numerical aperture (Nikon). The voltage for the photomultiplier tube (PMT) was set around 650mV and the diameter of the pinhole before the PMT was 50μm. At the end of some experiments, the voltage of the PMT detector was tuned down until no

fluorescent image was obtained. Subsequently, acridine orange (1μg/ml final concentration) was added to the cells to determine the location of nucleus. Images were processed and the averaged fluorescent intensity in the nucleus and in the cytosol was calculated. For the pseudocolour images, black-blue represents a low fluorescence of calcium green-1 while orange-red illustrates a high degree of fluorescence.

Results and Discussion

Macrophage cell line, PU5-1.8 cells, loaded with a Ca^{2+}-sensitive fluorescence indicator, calcium green-1, were stimulated with SNP and responses of cells at the same optical section were observed under a confocal laser scanning microscope. Fig.1 demonstrates the effect of SNP on the intracellular free calcium level ($[Ca^{2+}]i$) of 5 individual cells. Pre-treatment of cells with 30μM hemoglobin (Hb), a NO scavenger (11), did not alter the resting $[Ca^{2+}]i$. Subsequently, SNP of 1mM was added and a small rise of $[Ca^{2+}]i$ was obtained in almost all the cells in the presence of Hb. The cells were then washed and re-stimulated with 1mM SNP again. After washing, SNP could induce a more significant increase in $[Ca^{2+}]i$ in the absence of Hb. These observations suggest that NO could elicit an increase in $[Ca^{2+}]i$ in PU5-1.8 cells.

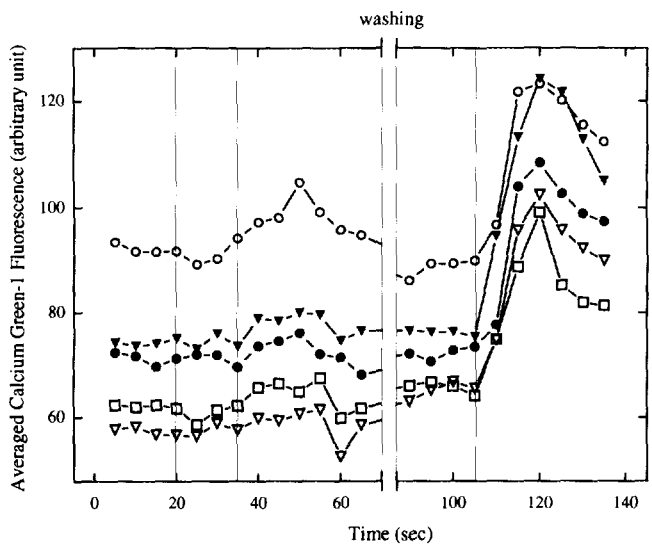

Fig.1. Effect of sodium nitroprusside (SNP) on the intracellular free calcium level in five single PU5-1.8 cells loaded with calcium green-1. Sequential images of the same optical section of cells are obtained at 2.5sec intervals and the averaged fluorescence of the whole section was calculated. Hairlines at 20, 35 and 105 sec illustrate the exposure of cells to 30μM Hb, 1mM and 1mM SNP. The break in the x-axis represents the time interval for washing.

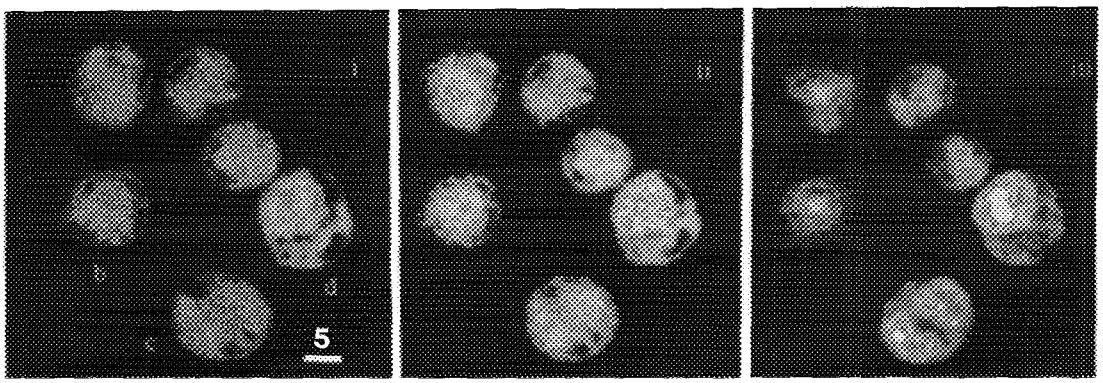

Fig.2. Confocal fluorescence pseudocolour images of calcium green-1 loaded PU5-1.8 cells before and after sodium nitroprusside (SNP) stimulation. Panel (i) & (ii) demonstrate the pseudocolour image before and 15sec after SNP (1mM) stimulation. Panel (iii) is the pseudocolour image of cells with acridine orange staining to show the location of nucleus.

As shown in Fig.2i & Fig.4i, fluorescence intensities of the cells before stimulation were a little bit higher in the central region. From the image of cells stained with acridine orange (Fig.2iii & Fig.4iii), the regions of bright fluorescence were found to be nucleus of PU5-1.8 cells. In Ca^{2+}-containing buffer, addition of SNP to cells induced a significant increase of calcium green-1 fluorescence in the nuclear region (Fig.2ii). The temporal responses of cells labelled with 'b' to 'd' shown in Fig.2 are illustrated in Fig.3a-c accordingly. These cells were chosen for further analysis because they had a relatively clear boundary between nucleus and cytosol. In general, the calcium level in the nuclear region was always higher than those in cytosol even after SNP stimulation. This observation is similar to those found in B-lymphocytes (13) but in contrast to the one in frog neuron that no difference between cytosolic and nuclear Ca^{2+} was observed (14). As illustrated in Fig.3a-c, treatment of cells with SNP (1mM) induced a steady increase of $[Ca^{2+}]c$ and an immediately biphasic increases in $[Ca^{2+}]n$ (Fig.3a-c). These observations coincide well with the findings of 3T3 cells that change of $[Ca^{2+}]n$ did not passively follow the changes in $[Ca^{2+}]c$ (15). In the same experiment, however, subsequent addition of a higher dose of SNP (2mM) produced a faster and more marked rise in the $[Ca^{2+}]n$ (Fig.3a-c). Obviously, a simultaneous increase of $[Ca^{2+}]c$ with similar pattern to the nuclear Ca^{2+} rise was found in PU5-1.8 cells (Fig.3a-c). These diverse and dose-dependent responses with SNP (1mM & 2mM) suggest that the machinery in the cytosol and in the nucleus which regulates the intracellular Ca^{2+} may be different. In our experiments, although we did not determine the NO concentration

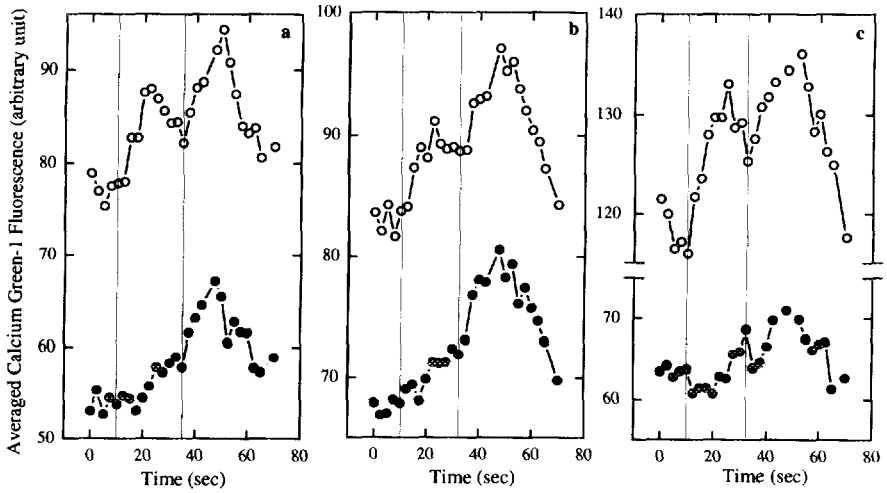

Fig.3. Time course of the fluorescence changes of calcium green-1 loaded PU5-1.8 cells with treatment as indicated in Fig.2. The averaged fluorescent intensity of the cells in the nuclear region (○) and in the cytosol (●) was calculated. Panels (a) to (c) represent the cells labelled with 'b' to 'd' in Fig.2, respectively. Hairlines at 10 and 35sec illustrate the exposure of cells to 1mM and 2mM SNP as indicated in Fig.2.

in our system, however, SNP is known to release NO in normal buffer and a NO level of about 450nM was found in 1mM SNP kept under room temperature (11). Moreover, the SNP-mediated response was suppressed by the presence of Hb. Thus, the sharp rise of $[Ca^{2+}]i$ level especially with a higher dose of SNP stimulation suggests a very fast-moving signal transducer, possibly the NO molecules, interacts with the intracellular Ca^{2+} regulating system(s) to increase the intracellular calcium in cytosol and nucleus. However, the earlier and more significant rise of the $[Ca^{2+}]n$ after SNP (1mM) activation in the presence of external Ca^{2+} as shown in Fig.3 suggest that the sensitivity of Ca^{2+} pools to the signal molecules and/or the mechanisms of regulation of Ca^{2+} in these two compartments may be different.

The Ca^{2+} activity of PU5-1.8 cells in response to SNP in Ca^{2+}-free buffer was also studied. As shown in Fig.4i, the resting intranuclear Ca^{2+} was higher. The temporal response of cells labelled with 'b' to 'd' are shown in Fig.5a-c, respectively. When the cells were challenged with SNP (2mM), a rise of $[Ca^{2+}]n$ and $[Ca^{2+}]c$ were observed (Fig.4ii) although the extent of the rise in $[Ca^{2+}]c$ was smaller (Fig.5a-c). Upon addition of 2μM Ca^{2+} to the bathing buffer, a small rise in the $[Ca^{2+}]n$ followed by a decrease was observed (Fig.5a-c). On the other hand, several spikes of $[Ca^{2+}]c$ were obtained after exogenous Ca^{2+} addition. At the end of the experiment, addition of ionomycin increased Ca^{2+} level in both compartments

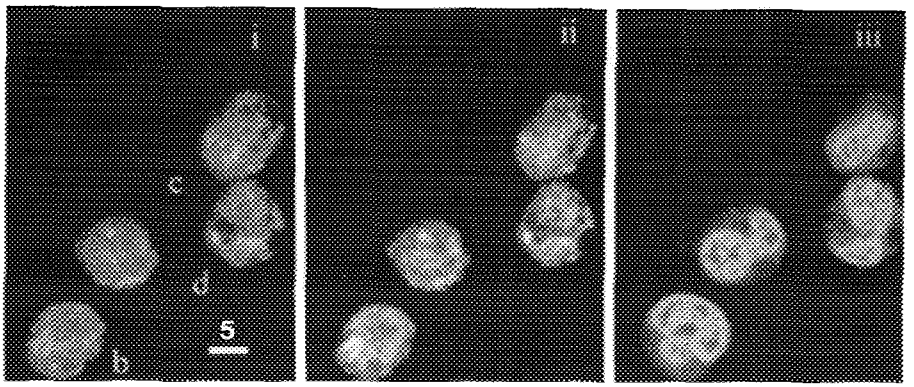

Fig.4. Confocal fluorescence pseudocolour images of calcium green 1-loaded PU5-1.8 cells treated with sodium nitroprusside (SNP) in Ca^{2+}-free buffer. Panel (i) & (ii) illustrate the cells before and 20sec after SNP (2mM) treatment. Panel (iii) is the fluorescent image of cells with acridine orange staining to show the location of nucleus.

illustrating that the cells were still functional. These results indicate that both the increase of $[Ca^{2+}]n$ and $[Ca^{2+}]c$ require a supply of Ca^{2+} from internal and external sources. Moreover, no significant increase in $[Ca^{2+}]c$ before the rise of $[Ca^{2+}]n$ after addition of external Ca^{2+} suggests that there may be some transport systems transporting the external Ca^{2+} directly to the nucleus. Alternatively, the external Ca^{2+} may be transported into intracellular Ca^{2+} pools first without passing through

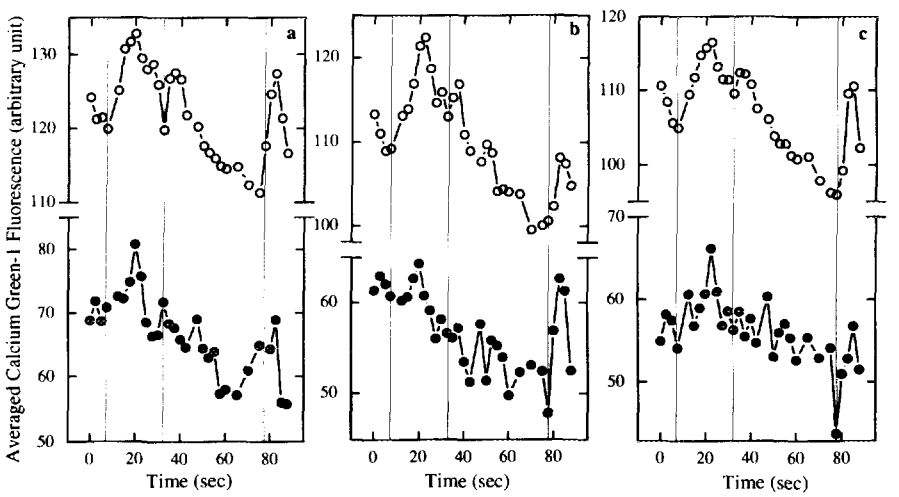

Fig.5. Time course of the fluorescence changes of calcium green 1-loaded PU5-1.8 cells treated with SNP (2mM) as indicated in Fig.4. The averaged fluorescent intensity of the cells in the nuclear region (○) and in the cytosol (●) was calculated. Panels (a) to (c) represent the cells labelled with 'b' to 'd' in Fig.4, respectively. Hairlines at 7.5, 32.5 and 77.5sec illustrate the exposure of cells to 2mM SNP, 2mM Ca^{2+} and 1μg/ml inomycin as indicated in Fig.4.

the cytoplasm and the Ca^{2+} is then forwarded to the nuclear region. In addition, when the cells were stimulated with SNP in the Ca^{2+}-containing buffer (Fig.3), the spikes of the $[Ca^{2+}]c$ were not so obvious as compared to those in Ca^{2+}-free buffer with Ca^{2+} re-addition (Fig.5). These may be explained by the capacitative model that depletion of the intracellular Ca^{2+} stores by the first SNP stimulation in the Ca^{2+}-free buffer may enhance the Ca^{2+} influx (16, 17). Once Ca^{2+} inside the cells, Ca^{2+} may be transferred among Ca^{2+} pools to generate Ca^{2+} spikes in the cytosol as indicated by our earlier data that calcium could induce calcium release and uptake in PU5-1.8 cells (18). Alternatively, the increase in $[Ca^{2+}]c$ may be partially due to the Ca^{2+} efflux from the nuclear region.

At present, we do not know the relationship between the cytosolic and nuclear Ca^{2+} regulation. The physiological significance of the NO-mediated calcium rise in the nucleus in PU5-1.8 cells is also unclear. However, a recent finding indicates that NO induces apoptosis and DNA fragmentation in murine peritoneal macrophages (19). Thus, it seems likely that the rise of intranuclear Ca^{2+} induced by NO may play a role to activate the endonuclease for the DNA fragmentation. The signalling pathway for these observations awaits further investigation.

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