L-Serine decreases basal Ca\textsuperscript{2+} in rat aortic vascular smooth muscle cells

Saswati Tripathy and Ramesh C Mishra

Department of Pharmacology, College of Medicine, University of Saskatchewan, Saskatoon, Canada.

ABSTRACT

L-serine decreases mean arterial pressure (MAP) in both normotensive and hypertensive rats. However, the molecular mechanism is not clear. Therefore, we examined the effect of L-serine on intracellular calcium (Ca\textsuperscript{2+}) in cultured aortic vascular smooth muscle cells (VSMC) isolated from normotensive and hypertensive rat models. Ca\textsuperscript{2+} fluorescence intensity was studied by using confocal imaging. L-serine evoked a concentration dependent (5-30 µM) decrease in basal Ca\textsuperscript{2+} fluorescence intensity in normotensive rat models. This decrease in basal Ca\textsuperscript{2+} fluorescence intensity level was also profound in hypertensive rat models despite the fact that basal Ca\textsuperscript{2+} fluorescence intensity was higher in these models compared to the normotensive rats. We also observed L-serine (20-30 µM) decreases nuclear Ca\textsuperscript{2+} fluorescence intensity in both the rat models. In presence of a neutral amino acid transporter inhibitor, 2-amino-2-norbornane-carboxylic acid (BCH), L-serine did not have any effect on basal Ca\textsuperscript{2+} fluorescence intensity in VSMC of either rat models, suggesting the inhibition of amino acid transporter blocked the entry of L-serine in to the cell to have an effect. Hence this data indicates the possible role of L-serine in regulation of intracellular Ca\textsuperscript{2+} in these rat models.

Key words: L-serine; Basal Ca\textsuperscript{2+}; VSMC; Cytoplasm; Nucleus; Fluo 3-AM

INTRODUCTION

L-serine is a non essential amino acid, it can be synthesized in the body from dietary sources and from other amino acids as well.\textsuperscript{1} The possible therapeutic potential of L-serine in the management of neurological disorder such as chronic fatigue syndrome, depression and schizophrenia has already been examined.\textsuperscript{2} We have already shown that acute intravenous administration of L-serine evokes a dose dependent fall in MAP which is more pronounced and profound in hypertensive rat models compared to normotensive rats.\textsuperscript{3, 4} L-serine induces VSMC relaxation probably by opening potassium channels present in the endothelium.\textsuperscript{4} However, the complete mechanism of the vascular relaxant effect of L-serine is not known. In disease state such as hypertension and diabetes, there is a evidence of increase in vascular tone due to progressive endothelial dysfunction where endothelial nitric oxide (NO) bioavailability is compromised.\textsuperscript{5, 6} In hypertensive conditions, it has been reported earlier that basal intracellular...
Ca$^{2+}$ in aortic VSMC is elevated.\textsuperscript{7, 8} Regulation of intracellular Ca$^{2+}$ plays an important role in contraction and relaxation of VSMC.\textsuperscript{8, 9} Ca$^{2+}$-calmodulin binding and subsequent stimulation of myosin light chain phosphorylation contributes to the VSMC contraction.\textsuperscript{10} On the other hand, Ca$^{2+}$ removal from the cytosol and activation of myosin light chain phosphatase followed by subsequent inactivation of myosin light-chain kinase, initiates the process of VSMC relaxation.\textsuperscript{11} Both contraction and relaxation process of VSMC involves a complex and dynamic regulation of intracellular Ca$^{2+}$.\textsuperscript{10, 11} Recent development of confocal imaging using calcium sensitive fluorescent dyes is a useful tool to study and analyze the changes in intracellular calcium level in VSMC.\textsuperscript{12, 13} In the present study, we used confocal microscopy to investigate whether L-serine has an effect on regulation of intracellular Ca$^{2+}$ in aortic VSMC isolated from normotensive and hypertensive rat models.

**METHODOLOGY**

**Animals:** The present protocols were approved by the University of Saskatchewan Animal Care committee, and conformed to the guidelines for the care and use of Laboratory animals stipulated by the Canadian Council on Animal Care. Normotensive male Sprague-Dawley, Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) of 11 weeks old were obtained from Charles River (St. Constant, Quebec, Canada). WKY rats were used as control group for SHR since they are from outbreed WKY rats. A group of seven Sprague-Dawley and WKY rats received the nitric oxide synthase (NOS) inhibitor, N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME, 0.7 mg/ml in drinking water ad libitum for 5 days) to evoke drug induced hypertension.\textsuperscript{14, 15} The rest of the rats received plain water.

**VSMC Culture:** After 5 days of treatment, aortic VSMC were isolated from L-NAME treated, normotensive and age matched SHR rats. Aortic VSMC are cultured under sterile conditions as described previously.\textsuperscript{3, 4, 14, 15, 16, 17} Briefly rats were exposed to anhydrous diethyl ether for induction of the anesthesia and euthanized. All subsequent procedures were conducted under sterile conditions inside a laminar flow hood. The thoracic aorta was dissected out and the adventitia was carefully removed. After the intimal layer was gently scraped off, the vessels were cut longitudinally and incubated for 30 min at 37°C in Hank’s balanced salt solution (HBSS) containing 0.2 \% type-II collagenase, 0.025 \% type-IV elastase and 0.02 \% soyabean trypsin inhibitor. This process ensured the successful removal of endothelial cells. The vessels were further cleaned to remove the remaining adventitia, fat, etc. using a teflon scraper. The cleaned vessels were cut into small pieces (1-2 mm length) and digested in the fresh HBSS for 45-60 min at 37°C. The suspension was centrifuged at 200 \* g for 5 min. The pellets were resuspended in 10 ml of Dulbecco’s Modified Eagle’s Medium (DMEM) with 10 \% (v/v) calf serum, 10 \% (v/v) horse serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and neomycin (1µg/ml). The dispersed cells were carefully plated into a 100 \* 20 mm culture dish and incubated at 37°C in a humidified (5 \% CO$_2$ and 95 \% O$_2$) incubator. After the cells had adhered sufficiently to the bottom of the cell culture dish (3-4 days), the non-attached cells were washed away and replaced with fresh culture medium. When the cells reached the stage of confluence, (within 6-8 days), the culture medium was removed and the cells were treated with 0.06 \% trypsin for 2 to 3 min at 37°C until the cells started to show signs of detachment. This stage was identified, morphologically, under a microscope. Trypsin was quickly neutralized by adding Ca$^{2+}$ containing 10 ml DMEM to the dish, and the cells were washed once with DMEM solution.
Fresh culture medium was added to the culture dish and the cells were detached by scraping manually with a teflon scraper, and finally resuspended by triturating with a transfer pipette. Then cells were replated in the culture medium at a split ratio of 1:3 in 20 * 1 mm culture dishes. The culture medium was changed in every 2 to 3 days. The cells became confluent in 3 to 4 days for each passage. In the entire study, cells up to 5th passage were used. Smooth muscle cell morphology was routinely assessed by examining the cells under microscope. Most importantly, more than 95 % cells showed positive immunofluorescent staining with α-actin antibody, confirming the typical smooth muscle cell characteristics of adherent cells. 

**Measurement of Ca^{2+} fluorescence intensity**

**Fluo 3-AM loading:** Ca^{2+} fluorescence intensity was determined by using the fluorescent probe fluo 3-AM as described previously. The cells were loaded with fluo 3-AM by adding the fluo 3-AM (4 μM) from a 1 mM stock in dry dimethyl sulfoxide (DMSO) to Krebs HEPES buffer containing (mM): (NaCl 145, KCl 5, CaCl$_2$.2H$_2$O 1.2, glucose 10, HEPES 10 concentration and 0.2 % bovine serum albumin (BSA, pH 7.4). An equal amount of pluronic F-127 was also added along with fluo 3-AM into the Krebs HEPES buffer to facilitate the solubility of fluo 3-AM and to ensure effective loading. To measure the Ca^{2+} fluorescence intensity by a confocal microscope, VSMC were grown on 18 * 18 mm glass cover slips, maintained in DMEM for 3 to 4 days and transferred to serum free medium for 24 hours prior to the experiment. The cells were washed once in Krebs HEPES buffer and subjected to fluo 3-AM loading for 30 min. After a period of 30 min incubation, the cells were washed twice with Krebs HEPES buffer, and then were incubated with L-serine for 10 min. Fluorescence images of the intracellular Ca^{2+} were taken with the help of a laser-scanning confocal microscope (Olympus FV300 Confocal Laser Scanning Biological Microscope) at an excitation/emission wavelength of 488-550 nm, equipped with an argon ion laser. For excitation of the fluorescent probe, the 488 nm argon ion laser line was directed to the sample. All the parameters like photomultiplier tube (PMT) setting, off set, gain, pinhole size, image size, zoom factor, contrast and brightness were carefully optimized and maintained constant for the entire series of experiment. Fluorescent signals emitted by the aortic VSMC were scanned with a scan size of 512 * 512 pixels. At least 40 cells were analyzed for each concentration of L-serine. Fluorescence intensity was measured and calculated by using the image ‘J’ software. Fluorescence intensity responses were expressed as relative unit.

BCH (2 mM), a neutral amino acid transporter inhibitor, was prepared in Kreb’s HEPES buffer where we replaced NaCl with LiCl since extracellular sodium opens the sodium channels located in the cell membrane and facilitate L-serine entry in to the cell. After being loaded with fluo-3 for 30 min, the cells were washed twice with Kreb’s HEPES buffer and allowed to incubate with BCH for 10 min. Then the cells were incubated with L-serine (30 μM) for 10 min. After 10 min, cells were taken for measurement of Ca^{2+} fluorescence.

**Materials:** The fluorescent probes fluo 3-AM and F-127 were purchased from Molecular Probes Co (Eugene, OR, U.S.A). All other cell culture materials were purchased from Sigma Aldrich, Canada Ltd.

**Statistics:** Results are expressed as mean ± SEM of 7 rats per group. Comparisons between groups were performed by using one-way analysis of variance (ANOVA) followed by Dunnett’s test. The difference was considered statistically significant when $P < 0.05$. 

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RESULTS

Effect of L-serine on basal Ca$^{2+}$ fluorescence: L-serine significantly decreased basal Ca$^{2+}$ fluorescence intensity in primarily cultured rat aortic VSMC from both normotensive and hypertensive rats (Table 1). We observed there was no significant difference in basal Ca$^{2+}$ fluorescence and L-serine mediated decrease in Ca$^{2+}$ fluorescence in Sprague-Dawley and WKY rats (Table 1). However, there is a significant difference in basal Ca$^{2+}$ fluorescence between normotensive WKY vs. L-NAME treated WKY and SHR strains (76 ± 4.3 vs. 154 ± 3.1 and 112 ± 4.4, n = 7, P < 0.001, Figure 1 A, B, C, and Table 1). Incubation of aortic VSMC with L-serine, dose dependently decreased Ca$^{2+}$ fluorescence at a concentration range of (5-30 µM), whereas in higher concentration (50-250 µM), Ca$^{2+}$ fluorescence intensity goes back to the control level. L-serine mediated decrease in Ca$^{2+}$ fluorescence is concentration dependent and shows a biphasic effect. A maximal decrease in Ca$^{2+}$ fluorescence was observed at a concentration of L-serine (30 µM) in normotensive as well as in hypertensive rat models. Control rats (76 ± 4.3 vs. 52 ± 5.2, n = 7, P < 0.05), L-NAME treated hypertensive rats (154 ± 3.1 vs. 117 ± 3.4, n = 7, P < 0.001), and SHR (112 ± 4.4 vs. 73 ± 2.9, n = 7, P < 0.001, Figure 1 A, B, C, and Table 1). Note: There was no significant difference to L-serine evoked response between chronic vs. acute L-NAME treatment in VSMC (data not shown).

Effect of L-serine in nuclear Ca$^{2+}$ fluorescence: It is interesting to note that in normotensive and both the hypertensive rat models, fluorescence imaging shows a profound decrease in nuclear Ca$^{2+}$ intensity when the cells were incubated with L-serine (30 µM) for 10 min (Figure 2 A, B, C). L-serine mediated decrease in nuclear Ca$^{2+}$ fluorescence was significant in all the three rat models. Normotensive WKY control rats (49 ± 6.0 vs. 26 ± 5.1, n = 7, P < 0.01), L-NAME treated hypertensive WKY rats (45 ± 4.8 vs. 23 ± 2.9, n = 7, P < 0.001) and SHR (102 ± 6.1 vs. 75 ± 3.0, n = 7, P < 0.001, Figure 2 A, B, C). This decrease in nuclear Ca$^{2+}$ fluorescence intensity was 53 % in WKY, 51 % in L-NAME treated WKY, and 73 % in SHR. Thus, data collected from our study indicates that there was a significant difference in basal nuclear Ca$^{2+}$ fluorescence in SHR compared to control WKY as well as L-NAME treated WKY rats (102 ± 6.1 vs. 49 ± 6.0, and 45 ± 4.8, n = 7, P < 0.001). D-serine an isomer of L-serine did not have any significant effect in basal or nuclear Ca$^{2+}$ fluorescence intensity compared to L-serine.

Effect of L-serine in presence of BCH: Aortic VSMC isolated from normotensive and hypertensive rats were incubated for 10 min with BCH (2 mM), a known neutral amino acid transporter inhibitor. BCH blocked L-serine mediated decrease in basal Ca$^{2+}$ fluorescence intensity (Figure 3 A, B, C) in the VSMC of all the three rat models.
Figure 1. Compares Ca\(^{2+}\) fluorescence intensity before and 10 min after incubation with L-serine (5-30 μM) in the aortic VSMC. (A) WKY rats, (B) L-NAME treated WKY rats, and (C) SHR. Each bar represents mean ± SEM, expressed as relative unit. *\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\) vs. control value (\(n = 7\)).
Figure 2. Compares nuclear Ca\(^{2+}\) fluorescence intensity before and 10 min after incubation with L-serine (20 µM and 30 µM) in the aortic VSMC. (A) WKY rats, (B) L-NAME treated WKY rats, and (C) SHR. Each bar represents mean ± SEM, expressed as relative unit. *\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\) vs. control value (\(n = 7\)).
Figure 3. Compares basal Ca$_{2+}$ fluorescence intensity with L-serine (30 µM) in the presence and absence of BCH (2 mM) in the aortic VSMC. (A) WKY rats, (B) L-NAME treated WKY rats, and (C) SHR. Each bar represents mean ± SEM value, expressed as relative unit. *P < 0.05, and ***P < 0.001 vs. control value (n = 7).
Figure 4. (A) Representative confocal images compare the basal $\text{Ca}^{2+}$ fluorescence intensity in the presence and absence of L-serine (30 μM) in the aortic VSMC. (A) Normotensive WKY rats, (B) L-NAME treated WKY rats, and (C) SHR ($n = 7$).
Table 1: Compares basal $\text{Ca}^{2+}$ fluorescence before and 10 min after incubation with L-serine (5-250 $\mu$M) in 12 week old male SD, L-NAME treated SD, WKY, L-NAME treated WKY and SHR strain. Each data point is in mean ± SEM ($n=7$) each.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>L-serine</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>5 $\mu$M</td>
</tr>
<tr>
<td>SD rat</td>
<td>81 ± 5.3</td>
<td>74 ± 4.0</td>
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<tr>
<td>SD rat (L-NAME)</td>
<td>158±3.3††</td>
<td>150 ±4.7</td>
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<tr>
<td>WKY rat</td>
<td>76 ± 4.3</td>
<td>71 ± 4.7</td>
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<tr>
<td>WKY rat (L-NAME)</td>
<td>154±3.1††</td>
<td>147 ±2.8</td>
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<tr>
<td>SHR</td>
<td>112±4.4††</td>
<td>88 ± 4.5</td>
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</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to control group.
†† $P < 0.001$ compared to respective control group.

SD, Sprague-Daley; WKY, Wistar-Kyoto; SHR, Spontaneously Hypertensive Rat

DISCUSSION

Calcium is an intracellular and extracellular ion which is known to serve as a second messenger, and thus plays a crucial role in smooth muscle cell function.23 Membrane bound voltage gated $\text{Ca}^{2+}$ channels in smooth muscle cells facilitate the extracellular $\text{Ca}^{2+}$ influx, whereas the intracellular $\text{Ca}^{2+}$ acts on inositol triphosphate (IP$_3$) and ryanodine (Ry) receptors to open the channel and release the $\text{Ca}^{2+}$ from sarcoplasmic reticulum (SR).24 After the development of $\text{Ca}^{2+}$ fluorescent dyes, $\text{Ca}^{2+}$ fluorescence studies have become an exciting field of study.25 Fluo 3-AM is a $\text{Ca}^{2+}$ sensitive fluorescence indicator widely used to study the rapid fluctuation in intracellular $\text{Ca}^{2+}$ fluorescence in aortic VSMC.24, 27 Our present observation revealed that L-serine but not its D-isomer, significantly decreased the basal $\text{Ca}^{2+}$ fluorescence intensity in aortic VSMC isolated from age matched normotensive and hypertensive rat models. L-Serine mediated decrease in basal $\text{Ca}^{2+}$ fluorescence intensity was dose dependent at a concentration range of (5-30 $\mu$M) and attained maximal decrease at 30 $\mu$M. Interestingly, L-serine at a concentration range of (50-250 $\mu$M) did not decrease the basal $\text{Ca}^{2+}$ fluorescence intensity; instead, dose dependently increased basal $\text{Ca}^{2+}$ fluorescence which is close to the original basal level. Thus, this shows a biphasic response, which is consistent with our in vitro studies.3 In our in vitro study, L-serine evoked a vasodilator response at a concentration range of (10-200 $\mu$M) in third order mesenteric arterioles isolated from L-NAME treated hypertensive Spraugh-Dawley rats. However, further increase in the dose of L-serine evoked a dose dependent contraction in the same tissue preparation. Plasma concentration of L-serine is (118 ± 14) $\mu$M and intracellular concentration is (10 ± 4) $\mu$M.2, 28 The dose at which L-serine evoked a vasodilator response seems to be
pharmacological. In the current study, L-serine decreases basal $\text{Ca}^{2+}$ fluorescence intensity and attains maximum at 30 µM which is pharmacological and consistent with our previous report. Our present study demonstrated that there is an elevation of basal $\text{Ca}^{2+}$ fluorescence intensity in L-NAME pretreated hypertensive and SHR strains, compared to their respective control group. Despite elevation in basal $\text{Ca}^{2+}$ fluorescence intensity in hypertensive rats, L-serine significantly decreased the basal $\text{Ca}^{2+}$ fluorescence intensity in both the hypertensive rat models. Most importantly, L-serine also decreases nuclear $\text{Ca}^{2+}$ fluorescence intensity in a lower but pharmacological dose range. Our study also shows that in higher dose range, L-serine reversed the decreased $\text{Ca}^{2+}$ fluorescence intensity to base line. Changes in $\text{Ca}^{2+}$ concentration in the nucleus regulates cell viability, gene expression and is responsible for a wide range of activities. Although it is not clear from the present study how L-serine is decreasing nuclear $\text{Ca}^{2+}$ selectively, it is possible that it might be directly binding to selective nuclear enzyme, protein, or receptor of its own, thereby decreasing nuclear $\text{Ca}^{2+}$ fluorescence intensity. Since we do not have clear evidence, to address this issue, more studies are required. However, in the presence of BCH, an inhibitor of neutral amino acid transporter, L-serine mediated decrease in basal and nuclear $\text{Ca}^{2+}$ fluorescence intensity was completely blocked in VSMC isolated from both normotensive and hypertensive rats. From this data, it is evident that this decrease in basal and nuclear $\text{Ca}^{2+}$ fluorescence intensity is solely mediated by L-serine, L-serine being an amino acid, can be transported into the cell through sodium channels by a sodium dependent mechanism. To rule out this possibility, we replaced NaCl with LiCl in Krebs HEPES buffer to ensure that L-serine was not transported through sodium ion channels into the cell in presence BCH.

In conclusion, the present data is new and novel. L-serine, but not D-serine decreases basal and nuclear $\text{Ca}^{2+}$ fluorescence intensity selectively in aortic VSMC isolated from normotensive WKY and hypertensive rats. This data supports our previous reports that L-serine has vasodilator and antihypertensive effects in hypertensive state.

CONFLICT OF INTEREST: The authors declare no conflict of interest.

REFERENCES


