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Hypochlorite Raises Intracellular Free Ca^{2+} in Primary Cultured Smooth Muscle Cells of Rat Aorta: Participation of Cellular Signaling Pathways

Jian-Ping Liu[1], Weimin Liu[2], Wenyan Li[1], Tao Zheng[1], Bella T Altura[1,3-5] and Burton M Altura[1,3-5*]

[1]Department of Physiology and Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, NY, USA

[2]Department of Anatomy and Cell Biology, State University of New York, Downstate Medical Center, Brooklyn, NY, USA

[3]The Center for Cardiovascular and Muscle Research, State University of New York, Downstate Medical Center, Brooklyn, NY, USA

[4]School of Graduate Studies in Molecular and Cellular Science, State University of New York, Downstate Medical Center, Brooklyn, USA

[5]Department of Medicine, State University of New York, Downstate Medical Center, Brooklyn, NY, USA

***Corresponding author:** Professor BM Altura, Suny Downstate Medical Center, Brooklyn, New York, USA, Tel: +1 -718 -270 -2194; Fax: +1 -718-270 3103; E-mail: baltura@downstate.edu

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Abstract

It has been reported that the local concentration of hypochlorite potentially exceeds 100 µM/l under pathological conditions *in vivo* and could cause vasoconstriction and damage of blood vessels in the microcirculation. Hypochlorite is found in atherosclerotic lesions and has been implicated in a number of cardiovascular disease processes. During moderate inflammatory conditions, the concentration of hypochlorite can reach as high as 340 µM. Thus, hypochlorite might be implicated in some pathological conditions such as inflammatory diseases, hypertension, heart diseases and stroke. The present study was designed to investigate the effects of three different protein kinase C inhibitors (bisindolylmaleimide I, staurosporine and Gö6979), PD-098059 (an inhibitor of extracellular signal-regulated kinases), genistein (an inhibitor of protein tyrosine kinase) and wortmannin {an antagonist of phosphatidylinositol 3-kinases (PI_3Ks)} on intracellular free Ca^{2+} ($\{Ca^{2+}\}_i$) in primary, cultured rat aortic smooth muscle cells exposed to hypochlorite. The present investigation represents the first demonstration, to our knowledge, that hypochlorite increases intracellular Ca^{2+} concentration (from approximately 78 ± 11.4 nM to 206.3 ± 23.8 nM) in rat aortic smooth muscle cells. All the protein kinase C inhibitors (PD-098059, genistein and wortmann) significantly inhibited the elevation in $\{Ca^{2+}\}_i$ induced by hypochlorite at different concentrations of 200, 400 and 800 µM from control levels. Our data suggest that protein kinase C as well as extracellular signal-regulated kinases, protein tyrosine kinases and PI3Ks, may play essential roles in hypochlorite-triggered signal transduction pathways in vascular smooth muscle cells in both normal and pathological conditions.

Keywords: Hypochlorite; Rat aorta; PKC inhibitor; ERK inhibitor; Protein tyrosine kinases inhibitor; PI_3Ks antagonist; Ca^{2+}, Intracellular free; Smooth muscle cells; Signal transduction

Introduction

Hypochlorite is one of the most aggressive oxidants of reactive oxygen species which can be produced by the myeloperoxidase (MPO)-hydrogen peroxide (H_2O_2)-chloride system [1-4]. MPO, a member of the hem-peroxidase-cyclooxygenase superfamily, is abundantly expressed in neutrophils, and to a lesser extent in monocytes, and certain types of macrophages. MPO participates in innate immune defense mechanisms through formation of microbicidal reactive oxidants and diffusible radical species. A unique activity of MPO is its ability to use chloride as a co-substrate with hydrogen peroxide to generate chlorinating oxidants such as hypochlorous acid, a potent antimicrobial agent [4]. Hypochlorite can be released to the outside of cells, where it may attack normal tissues and cells, and thus contribute to the pathogenesis of disease processes [3-5]. It is known that hypochlorite plays important roles in both normal and pathological conditions. Reactive oxygen species are continuously generated *in vivo*, and an excessive level of these species are considered to be responsible for several pathophysiological processes, partly by affecting vascular tone [6]. Hydrogen peroxide serves as a substrate for myeloperoxidase, an enzyme that is released by activated neutrophils during inflammatory processes, as seen, for instance, in reperfusion injury and atherosclerosis [7]. Myeloperoxidase catalyzes the oxidation of chloride by hydrogen peroxide, yielding hypochlorite, an extremely potent oxidant [4]. Under pathological conditions, hypochlorite may be formed in relatively high concentrations. It has been

documented that myeloperoxidase is a component of human atherosclerotic lesions [2] and certain inflammatory conditions [5]. Up to 40% of the H2O2 generated by activated leukocytes is used to form hypochlorite, and the local concentrations of hypochlorite can potentially exceed 100 µM/l under pathological conditions *in vivo* [1,8-10]. During moderate inflammatory conditions, the concentration of hypochlorite can reach as high as 340 µM [5]. The hypochlorite concentrations in our studies could be considered as biologically/pathologically-relevant to some diseases such as atherosclerosis and inflammation *in vivo*.

Previous studies from our labs have shown that hypochlorite can modulate vascular tone directly or, indirectly, leading to vasodilation or vasoconstriction [11,12]. Protein kinase C, mitogen-activated protein kinase (MAPK), and nonreceptor tyrosine kinase have been demonstrated to play essential roles in H_2O_2-tiggered signal transduction pathways in vascular smooth muscle cells [13,14]. It is, however, unclear whether hypochlorite acts on vascular smooth muscle cells via similar or different mechanisms/pathways. The current study was undertaken to investigate the effects of hypochlorite on vascular smooth muscle cells of rat aorta and to gain insight into the underlying mechanisms of its action, with special attention given to potential signal transduction mediators generated by hypochlorite.

Materials and Methods

Primary cell culture and hypochlorite treatment

The experiments were performed on single vascular smooth muscle cells of aorta, which were obtained from male Wistar rats (250–350 g) after pentobarbital sodium anesthesia and sacrifice. The procedures employed to isolate and culture rat aortic SMCs have been reported [15]. Briefly, the SMCs were cultured in Dullbecco's modified Eagle's medium, mixed 1:1 with Ham's nutrient mixture F-12 at 37˚C in a humidified atmosphere composed of 95% air and 5% CO_2 [15]. The culture medium contained 10% fetal calf serum, 100 U/ml penicillin, and 100µg streptomycin. More than 97% of the primary cultured cells were SMCs as confirmed by immunostaining with a monoclonal α-smooth muscle actin antibody [15]. Cells were regularly subcultured with 0.05% trypsin and seeded onto 12 mm circular coverslips. Cells at the second to sixth passages, at 80% confluence, were used for our experiments. Twenty-four hours before hypochlorite treatment, the medium was replaced with 1% serum medium. Hypochlorite was diluted in phosphate-buffered saline (PBS; pH 7.3) and was directly added to the culture medium at final concentrations ranging from 100 to 800 µM. Control samples were treated with PBS (pH 8.3) only. All of the experiments described above were performed under normal laboratory light, and approved by the Animal Care and Use Committee of our institution.

Intracellular Ca^{2+} measurements

Intracellular Ca^{2+} ($\{Ca^{2+}\}_i$) was measured in isolated primary SMCs in the presence or absence of hypochlorite, using the Ca^{2+}-sensitive membrane-permeant fluorescent dye fura 2-AM (acetoxymethylester of 1-2-{5-carboxyoxazol-2-yl}- 6-aminobenzofuran-5-oxy-2-{2'-amino-5'methylphenoxy} ethane-N,N,N',N'-tetra-acetic acid [15], according to previously established methods [16,17]. Monolayers of the aortic SMCs, grown on the coverslips, were loaded with 2.0 µM fura 2-AM and 0.12% pluronic acid F-127 (60 mins, 37˚C). The monolayers were washed two to three times with PBS and 20 mM HEPES (pH 7.4) and incubated with this buffer at room temperature until ready to use. The monolayers were inserted in a leakproof coverslip holder. Buffer was added to the monolayer on the coverslip. The coverslip holder was mounted onto the stage of a temperature-controlled Nikon TMS inverted microscope with a long working distance Nikon Fluor objective (n.a. 0.5), attached to a 300-W xenon light source and the CCD camera for image acquisition. Buffer (control) and hypochlorite were added to the monolayers. The primary cultured aortic SMC monolayers, preloaded with fura 2-AM, were excited alternatively, at 340 and 380 nm, and the emission intensity was recorded at 510 nm using a silicon-intensified target camera [17]. Background autofluorescence for both excitation wavelengths was acquired from blanks for each experiment and subtracted from each pair of images separately before ratioing. Fluorescence ratios (R) were obtained by dividing the 340 nm image by the 380 nm image. No image misalignments occurred when those two ratiometric images were superimposed. The resulting images were then used to calculate $[Ca^{2+}]_i$ in smooth muscle cells using external standards containing 2.54 mM Ca^{2+} and 0 mM Ca^{2+} plus 10 mM EGTA for maximum (Rmax) and minimum (Rmin) fluorescence ratios of the 340-nm and 380-nm images. $[Ca^{2+}]_i$ was calculated according to the following equation [18]:

$$[Ca^{2+}]_i = \frac{Kd \times B \times (R - R\min)}{(R\max - R)}$$

A Kd of 224 nM was used for the fura-2/Ca^{2+} complex [16]. B is the ratio of fluorescence intensity of fura-2 to Ca^{2+} : fura-2 complex excited at 380 nm.

Drugs and chemicals

The following pharmacological agents were purchased from Sigma Chemical (St. Louis, Mo., USA): sodium hypochlorite, bisindolylmaleimide I HCl, staurosporine and genistein. The fura 2-AM was purchased from Molecular Probes (Eugene, Ore., USA). Dimethyl sulfoxide (DMSO), Gö6976, PD-98059 and wortmannin were purchased from CALBIOCHEM (La Jolla, Calif., USA). All other organic and inorganic chemicals were obtained from Fisher Scientific (Fair Lawn, N.J., USA) and were of the highest purity.

Calculations and statistical analyses

Where appropriate, results are expressed as mean ± S.E.M of at least 35-40 cells each. Statistical evaluation of the results

was carried out by analysis of Student t tests and ANOVA. The results were considered significant at a P value of <0.05.

Results

Effects of hypochlorite on [Ca2+]i in isolated smooth muscle cells

The quantitative effects of hypochlorite on $[Ca^{2+}]_i$ in SMCs isolated from rat aorta were determined by using the direct technique of Ca^{2+} visualization in single cells as revealed by the digital imaging microscope using fura 2-AM [16]. The control basal level of $[Ca^{2+}]_i$ was 78.57 ± 11.43 nM. As shown in **Figure 1**, hypochlorite elicited a concentration-dependent increase in $[Ca^{2+}]_i$ within 30 seconds, the threshold being ~100 µM; 800 µM hypochlorite resulted in an almost a 2.6-fold rise in $[Ca^{2+}]_i$.

Effects of PKC antagonists on hypochlorite induced elevations in $[Ca^{2+}]_i$

The response of three protein kinase C antagonists on hypochlorite- induced $[Ca^{2+}]_i$ changes in SMCs isolated from rat aorta were determined by using the direct technique of Ca^{2+} visualization in single cells as revealed by the digital imaging microscope using fura 2-AM [16]. **Figures 2-4** illustrate that preincubation of primary cultured smooth muscle cells from rat aorta with staurosporine (1 µM), bisindolylmaleimide I (1 µM) and Gö6979 (1 µM) effectively prevents hypochlorite - induced elevation in $[Ca^{2+}]_i$. The control basal level of $[Ca^{2+}]_i$ in **Figures 1-3** were 82.75 ± 16.26 nM, 66.22 ± 25.03 nM, 67.24 ± 2.89 nM, respectively. Somewhat to our surprise, the inhibitory effects of bisindolymaleimide I on hypochlorite induced elevations in $[Ca^{2+}]_i$ displayed concentration-dependent effects **(Figure 2).**

Effects of PD-098059, genistein and wortmannin on hypochlorite induced elevations in $[Ca^{2+}]_i$

Figures 5-7 summarize the results of our experiments with hypochlorite and PD-098059, genistein and wortmannin on $[Ca^{2+}]_i$ in primary, cultured rat aortic smooth muscle cells. Hypochlorite, in the concentration range of 100–800 µM, significantly increased $[Ca^{2+}]_i$ in a concentration-dependent manner. The control basal level of $[Ca^{2+}]_i$ in **Figures 4-6** were 97.4 ± 12.6 nM, 79.42 ± 3.39 nM, and 78.41 ± 22.92 nM, respectively. Preincubation of primary cultured smooth muscle cells from rat aorta with PD-098059 (2 µM), genistein (10 µM) and wortmannin (1 µM) significantly prevents hypochlorite-induced elevation in $[Ca^{2+}]_i$. These three inhibitors inhibited almost completely the rises of $[Ca^{2+}]_i$ induced by hypochlorite at the high concentrations (400 µM and 800 µM). Using 100 µM hypochlorite, on the preincubated cells, no changes in $[Ca^{2+}]_i$ were observed from control levels.

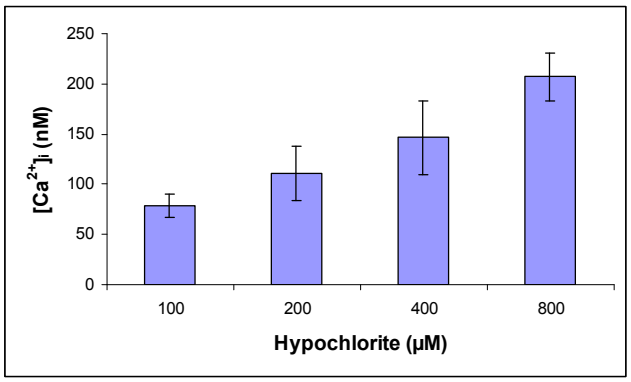

Figure 1 Hypochlorite results in elevation in intracellular free Ca2+ ($[Ca^{2+}]_i$) concentration in rat aortic SMCs. The data were obtained 30 seconds after treatment with hypochlorite. Each result indicates the mean ± SD (n=210–240 cells).

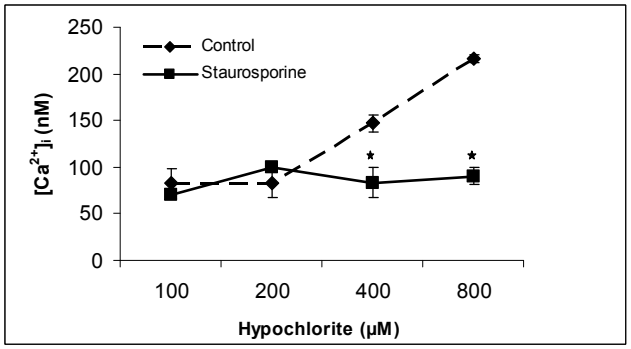

Figure 2 Responses of intracellular free Ca2+ ($[Ca^{2+}]_i$) concentration in rat aortic SMCs to hypochlorite at 20-30 min after exposure of these cells to staurosporine (1 µM). The data were obtained 30 seconds after treatment with hypochlorite. Each result indicates the mean ± S.E.M. Staurosporine significantly inhibited the elevation in [Ca2+]i induced by hypochlorite at concentrations of 400µM and 800µM from control, *P < 0.05 (Student's t test). n ≥ 35-40 cells each.

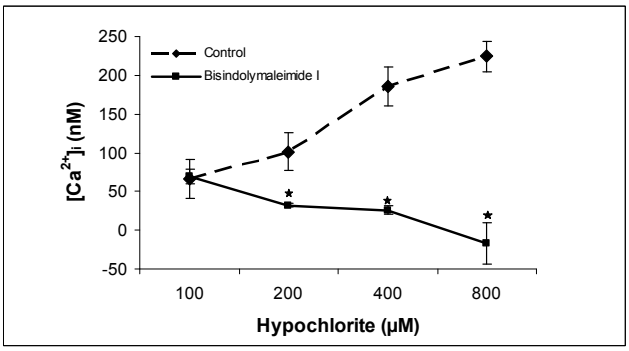

Figure 3 Responses of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentration in rat aortic SMCs to hypochlorite at 20-30 min after exposure of these cells to bisindolymaleimide I (1 μM). The data were obtained 30 seconds after treatment with hypochlorite. Each result indicates the mean ± S.E.M. Bisindolymaleimide I significantly inhibited the elevation in $[Ca^{2+}]_i$ induced by hypochlorite at concentrations of 200 μM, 400 μM and 800 μM (*P < 0.05, Student's t- test). n ≥ 35-40 cells each.

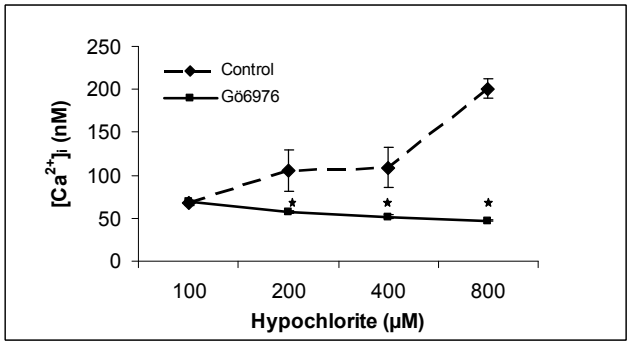

Figure 4 Responses of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentration in rat aortic SMCs to hypochlorite at 20-30 min after exposure of these cells to Gö-6976 (1 μM). The data were obtained 30 seconds after treatment with hypochlorite. Each result indicates the mean ± S.E.M., Gö-6976 significantly inhibited the elevation in $[Ca^{2+}]_i$ induced by hypochlorite at concentrations of 200 μM, 400 μM and 800 μM (*P<0.05, Student's t -test). n ≥ 35-40 cells each.

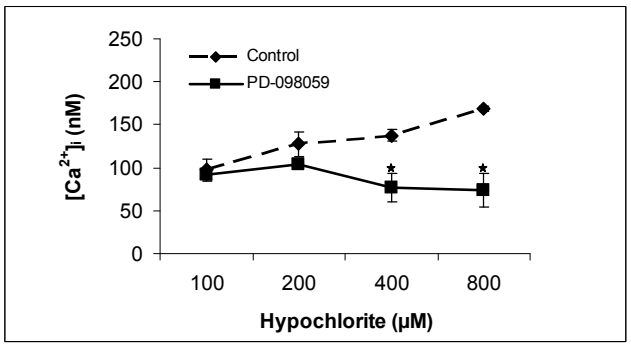

Figure 5 Responses of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentration in rat aortic SMCs to hypochlorite at 20-30 min after exposure of these cells to PD-098059 (2 μM). The data were obtained 30 seconds after treatment with hypochlorite. Each result indicates the mean ± S.E.M. PD-098059 significantly inhibited the elevation in $[Ca^{2+}]_i$ induced by hypochlorite at concentrations of 400 μM and 800 μM(*P<0.05, Student's t- test). n ≥ 35-40 cells each.

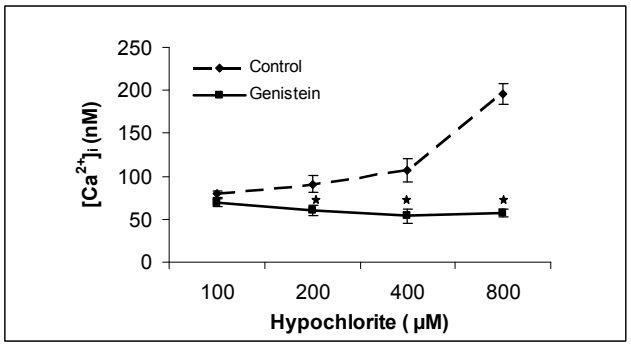

Figure 6 Responses of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentration in rat aortic SMCs to hypochlorite at 20-30 min after exposure of these cells to genistein (10 μM). The data were obtained 30 seconds after treatment with hypochlorite. Each result indicates the mean ± S.E.M. Genistein significantly inhibited the elevation in $[Ca^{2+}]_i$ induced by hypochlorite at concentrations of 200- 800 μM (*P<0.05, Student's t -test). n ≥ 35-40 cells each.

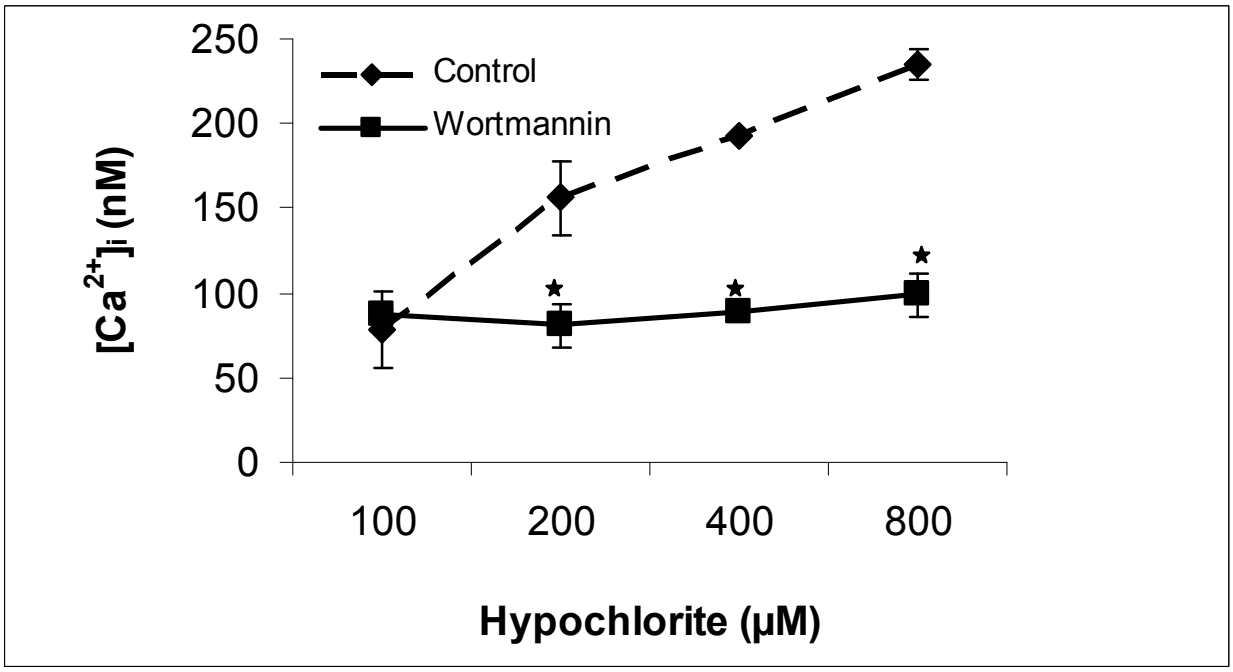

Figure 7 Responses of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) concentration in rat aortic SMCs to hypochlorite at 20-30 min after exposure of these cells to wortmannin (1 μM). The data were obtained 30 seconds after treatment with hypochlorite. Each result indicates the mean ± S.E.M. Wortmannin significantly inhibited the elevation in $[Ca^{2+}]_i$ induced by hypochlorite at concentrations of 200- 800 μM (*P<0.05, Student's t- test). n ≥ 35-40 cells each.

Discussion

The present investigation represents the first demonstration, to our knowledge, that:

- Hypochlorite increases free intracellular Ca^{2+} concentration (i.e., from approximately 80 nM to 210 nM in rat aortic smooth muscle cells).
- Antagonists of three different protein kinase C inhibitors (bisindolylmaleimide I, staurosporine and Gö6979), PD-098059, genistein and wortmannin produced striking inhibitory effects on the elevation in $[Ca^{2+}]_i$ induced by hypochlorite when the SMCs were exposed to different concentrations(200, 400 and 800 μM), thereby giving us some insight into the potential contribution of four diverse cellular-signaling pathways to hypochlorite-induced Ca^{2+} mobilization in rat aortic smooth muscle cells.

Previous studies from our lab have shown that hypochlorite-induced contractions are mainly mediated via the elevation of intracellular free Ca^{2+} levels [11,12]. Multiple cellular signal pathways are known to participate in mechanisms of vasoconstriction, such as activation of protein kinase C (PKC), protein tyrosine kinase, MAPK, and PI_3Ks [11-13,19-21].

PKC, a family of Ca^{2+}-sensitive and Ca^{2+}-insensitive phospholipid-dependent protein kinases, that are present in high concentrations in vascular smooth muscle, have been shown to play important roles in cellular signal transduction [11,13,14,19,20,22]. However, up until the present study, no experimental evidence has appeared on the possible role of PKC in hypochlorite-induced Ca^{2+} mobilization in rat aortic smooth muscle cells. In the present work, the elevation in $[Ca^{2+}]_i$ induced by hypochlorite were found to be completely inhibited in the presence of two potent PKC antagonists (bisindolylmaleimide I and staurosporine) and one selective PKC_α and $PKC_{â1}$ antagonist (Gö6976). This suggests that the inhibitory effect of bisindolylmaleimide I, staurosporine and Gö6976 is mediated through the activation of PKC. PKC isoforms, probably PKC_α (possibly $PKC_{\beta1}$ as well) may be a pivotal signaling mechanism by which hypochlorite acts on the aortic smooth muscle cells to raise $[Ca^{2+}]_i$. This is, thus, similar to that found for H_2O_2 in rat aortic rings and cerebral arteries [13,14] and hydroxyl radicals in rat aortic rings [20].

It has been reported that PI_3Ks are a group of enzymes that act directly to provide biochemical links between a novel phosphatidylinositol pathway and nonreceptor tyrosine kinases that appear to modulate Ca^{2+} channels through activity of these PI3Ks [23]. Up to now, there has been no information regarding PI_3Ks on hypochlorite-induced Ca^{2+} mobilization in rat aortic smooth muscle cells. In this paper, we report that one potent antagonist of PI3Ks (wortmannin) completely attenuated the elevation in $[Ca^{2+}]_i$ induced by hypochlorite, suggesting the possible involvement of products of PI3Ks in such responses. Approximately 20 years ago, we demonstrated that H_2O_2-induced contractions [14,22,24] and hydroxyl radical-induced contractions [20] were found to be somewhat dependent on PI3Ks as well.

Previous studies have shown that protein tyrosine phosphorylation is an essential component in signal

transduction pathways in smooth muscle cells [7,25]. Our present data also demonstrate that genistein, an inhibitor of protein tyrosine kinase [25], which is an enzyme for catalyzing protein tyrosine phosphorylation, significantly inhibited the elevation in $[Ca^{2+}]_i$ induced by hypochlorite in rat aortic SMCs, which indicates the likely involvement of protein tyrosine phosphorylation in hypochlorite-triggered signal transduction pathways in vascular smooth muscle cells. The conclusion is supported by other investigators who have demonstrated that oxidative stress can promote protein tyrosine phosphorylation in smooth muscle cells [13,14,20,22,24,26].

Mitogen-activated protein kinases [MAPKs], also called extracellular signal-regulated kinases (ERKs)] are constituents of numerous signal transduction pathways, and are activated by protein kinase cascades [27]. Several studies have been reported which indicate MAPK responds to diverse stimuli, including reactive oxygen species, and can transduce signals from the cell membrane to the nucleus [11,13,14,21,22,26,28,29]. Activation of MAPK plays an important role in modulating vascular tone [14,19, 21,22]. In this investigation, the specific MAPK antagonist, PD-098059 [12], significantly inhibited the elevation in $[Ca^{2+}]_i$ induced by hypochlorite in rat aortic SMCs. This suggests that activation of MAPK is probably an important pathway in hypochlorite-mediated action on rat aortic smooth muscle cells [30-37].

Conclusion

In conclusion, three different protein kinase C inhibitors (bisindolylmaleimide I, staurosporine and Gö6979), PD-098059, genistein and wortmannin significantly inhibited the elevation in [Ca2+]i induced by hypochlorite at different concentrations, i.e., from 200 to 800 µM , which would suggest that protein kinase C, extracellular signal-regulated kinases, protein tyrosine kinases and PI_3Ks probably play essential roles in hypochlorite-triggered signal transduction pathways in vascular smooth muscle cells in both normal and pathological conditions.

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