Cytosolic calcium changes in cultured rat aortic smooth-muscle cells induced by oxyhemoglobin

KATSUNOBU TAKENAKA, M.D., HIROMU YAMADA, M.D., NOBORU SAKAI, M.D., TAKASHI ANDO, M.D., TOSHIHIKO NAKASHIMA, M.D., YASUAKI NISHIMURA, M.D., YUKIO OKANO, M.D., AND YOSHIHORI NOZAWA, M.D.

Departments of Neurosurgery and Biochemistry, Gifu University School of Medicine, Gifu, Japan

To clarify the mechanism of contractive effects in arteries caused by oxyhemoglobin, changes in the concentration of cytosolic calcium ([Ca++]i) before and after exposure to oxyhemoglobin were measured in vitro in cultured vascular smooth-muscle cells obtained from rat aorta. This was accomplished by preloading these cells with a fluorescent intracellular Ca++ probe fura-2/AM.

Oxyhemoglobin induced a significant elevation of [Ca++]i in vascular smooth-muscle cells which was sustained for 10 minutes. This response was completely abolished by chelating extracellular calcium with ethyleneglycol-bis (β-aminoethylether)-N,N′-tetra-acetic acid (EGTA). Oxyhemoglobin induced no accumulation of mass content of inositol 1,4,5-trisphosphate (IP3(1,4,5)). The oxyhemoglobin-induced elevation of [Ca++]i was not blocked by verapamil, a calcium antagonist. Serotonin induced a rapid, transient increase of [Ca++]i, followed by a sustained elevation above baseline for 5 minutes. Additions of EGTA or verapamil had a small effect on the peak height of serotonin-induced [Ca++]i elevation, but the [Ca++]i level declined more quickly to the basal level in treated compared with control cells.

These results indicate that oxyhemoglobin-induced [Ca++]i elevation caused by the influx of extracellular calcium, which is independent of the verapamil-blocked voltage-gated calcium channel. The long-lasting high elevation of [Ca++]i caused by oxyhemoglobin suggests that oxyhemoglobin may contribute to the production of abnormal contractions and/or irreversible damage in vascular smooth-muscle cells.

KEY WORDS - cytosolic calcium • oxyhemoglobin • smooth-muscle cells • serotonin • vasospasm

Cerebral vasospasm is a major complication in patients with subarachnoid hemorrhage due to ruptured aneurysms. Despite many investigations, the pathogenesis of this vasospasm has not yet been elucidated.11,20,27 It has been generally accepted that constituents of erythrocytes and substances produced during hemolysis of subarachnoid blood clots may be closely associated with cerebral vasospasm.16,17

Among hemolysate substances, oxyhemoglobin is considered to play a key role in the genesis of cerebral vasospasm because of its vasconstrictive activity in cerebral arteries and in cultured vascular smooth-muscle cells.3,4,7,22,26 However, the mechanism of contraction caused by oxyhemoglobin is still controversial.12,13,15,18,23

In general, the contraction of vascular smooth-muscle cells seems to be regulated by changes in the concentration of cytosolic free Ca++ which activates protein kinase C and the Ca++-calmodulin-dependent protein kinases.5,10,14,19 Therefore, in an attempt to clarify the mechanism of contraction in the vascular smooth-muscle cells caused by oxyhemoglobin, we measured changes in the concentration of cytosolic Ca++ in cultured vascular smooth-muscle cells exposed to oxyhemoglobin using the calcium ion chelators fura-2/AM.18

Materials and Methods

Cell Culture

Vascular smooth-muscle cells from the aorta of rats* were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum in a 10% CO2 atmosphere at 37°C as described.9 The cells were passaged weekly and cells at nine to 12 passages were used for the current study. The cells were grown in the serum-containing growth medium for 4 days to confluence.

* Rat vascular smooth-muscle cells obtained from Dr. H. Arita, Shionogi Research Laboratories, Osaka, Japan.
Oxyhemoglobin-induced changes in aortic smooth-muscle cells

![Graph showing absorption spectra of oxyhemoglobin (oxyHb) and methemoglobin (metHb).](image)

Fig. 1. Absorption spectra of oxyhemoglobin (oxyHb) preparation used in the present study. The spectra for met-hemoglobin (metHb) are also shown.

and were then serum-starved for 2 days prior to stimulation.

Preparation of Hemoglobin

Oxyhemoglobin was prepared as described previously. In brief, bovine hemoglobin Type I containing a mixture of oxyhemoglobin and methemoglobin was reduced by sodium dithionite (Na2S2O4). After removal of the reducing agent by extensive dialysis against distilled water, the purity of oxyhemoglobin was determined spectrophotometrically (Fig. 1).

Intracellular Free Calcium Signal

For measurement of the cytosolic Ca++ concentration ([Ca++]i), cells were plated at a density of 2 × 10^4 cells/chamber on the glass coverslip which adhered to the smooth lower side of a Flexiperm disc. After culture for 48 hours at 37°C, vascular smooth-muscle cells were serum-deprived as described above.

Before stimulation, the cells were washed twice and loaded with the fluorescence indicator fura-2/AM (2 μM) for 40 minutes at 37°C in 0.3 ml of serum-free DMEM, then rinsed free of the extracellular dye and incubated for 15 minutes to allow de-esterification of the dye. Fluorescence images were obtained at alternating excitation wavelengths of 340 and 360 nm through an SIT Vidicon camera, and processed by an Argus-100 image analyzer. The linear 360-nm interpolation was used and the corrected fluorescent emission intensity ratio, using 340 and 360 nm excitation with background subtraction, was monitored continuously in single cells.

Determination of Mass Content of IP3(1,4,5)

Inositol 1,4,5-trisphosphate (IP3(1,4,5)) was quantitatively measured in oxyhemoglobin- or serotonin-stimulated vascular smooth-muscle cells using a highly specific IP3(1,4,5) assay system as described previously.

Briefly, before stimulation with oxyhemoglobin or serotonin, vascular smooth-muscle cells were preincubated with DMEM containing 10 mM LiCl for 5 minutes at 37°C. The reaction was terminated at designated times by adding 10% perchloric acid; the cells were kept on ice for 20 minutes, then neutralized with ice-cold 1.5 M KOH-HEPES for 20 minutes. The samples were centrifuged at 2000 G for 10 minutes to remove KClO4 precipitate. The supernatant samples (100 μl each) were subjected to IP3(1,4,5) assay using the IP3 assay kit. The standard curve showed a linear slope from 0.19 to 25 pmol of IP3(1,4,5).

Results

Oxyhemoglobin induced a significant elevation of [Ca++]i content in all vascular smooth-muscle cells examined. There was a slow increase that reached a plateau at 1 minute, and the high [Ca++]i level did not return to the basal level even as late as 10 minutes poststimulation (Fig. 2). After exposure to oxyhemoglobin, no cells stained with 0.3% trypan blue and lactic dehydrogenase activity was not detected in the medium outside the cells, indicating that the cells were intact. Serotonin (5-hydroxytryptamine) induced a transient rise in [Ca++]i content; a peak was attained within 10 seconds, followed by a gradual decline (Fig. 3). Figure 4 illustrates the dose-dependence of the peak [Ca++]i level (ratio 340/360 nm) caused by oxyhemoglobin or serotonin. The maximum level of [Ca++]i caused by oxyhemoglobin was higher than that indicated by serotonin. The addition of 2 mM ethyleneglycol-bis(β-amineoethylether)-N,N,N'-tetra-acetic acid (EGTA) prevented any elevation of [Ca++]i caused by oxyhemoglobin (Fig. 5A and B). In contrast, the addition of 2 mM EGTA caused only slight reduction of the peak in the serotonin-induced [Ca++]i transient increase, and the [Ca++]i content declined more quickly (within 2 minutes) to the baseline level compared with control cells (Fig. 5D and E).

Verapamil (10^-7 to 10^-5 M) also failed to inhibit an oxyhemoglobin-induced ([Ca++]i) elevation (Fig. 5C). However, it reduced the duration of the serotonin-induced [Ca++]i elevation (Fig. 5F).

Incubation of vascular smooth-muscle cells with serotonin (10 μM) resulted in the rapid accumulation of IP3(1,4,5). The cellular IP3(1,4,5) content increased about fourfold above control from a resting level of 3.8 ± 1.1 pmol/10^6 cells to 15.2 ± 4.3 pmol/10^6 cells (mean ± standard error of the mean from three separate experiments) within 10 seconds after stimulation with 10 μM serotonin. In contrast, incubation of vascular...
smooth-muscle cells with oxyhemoglobin (500 μM) resulted in no accumulation of mass content of IP3(1,4,5) (Fig. 6).

Discussion

Calcium and Smooth Muscle Contraction

Cytosolic free Ca++ plays an important role in regulating the contraction of vascular smooth-muscle cells. Contraction is initiated by a variety of external stimuli which cause the cytosolic Ca++ concentration to rise to a critical level as a result of the influx of extracellular Ca++ and/or the mobilization of Ca++ from the cytosolic Ca++ store. Activation of membrane-bound phosphoinositide-specific phospholipase C leads to IP3(1,4,5)-mediated release of calcium from endoplasmic reticulum. Fura-2/AM is particularly useful in that it is highly specific for Ca++ ions. Fura-2 produces a potent fluorescent emission which allows it to be used in relatively low concentrations, thereby diminishing interference due to an inherent Ca++-buffering capacity. Because of its lipophilicity, fura-2/AM is rapidly taken up by vascular smooth-muscle cells. Once incorporated, de-esterification of the acetoxyethyl group produces free fura-2. The observed fluorescence changes in the cells correspond solely to alterations in intracellular Ca++.

Smooth Muscle Relaxation

It has been known that hemoglobin inhibits the relaxation of blood vessels due to acetylcholine, adeno-
Oxyhemoglobin-induced changes in aortic smooth-muscle cells

Fig. 5. Graphs showing typical effects of EGTA or verapamil on oxyhemoglobin (oxyHb) or serotonin-induced elevation of [Ca++] in individual fura-2-loaded vascular smooth-muscle cells. The [Ca++] levels were measured as described in the Materials and Methods section; 500 µM oxyhemoglobin (A-C) or 10 µM serotonin (D-F) was added to the cells. Cells pretreated with 2 mM EGTA for 1 minute were stimulated with oxyhemoglobin (B) or serotonin (E). Cells pretreated with 1 µM verapamil for 3 minutes were stimulated with oxyhemoglobin (C) or serotonin (F).

sine triphosphate, and nitroglycerin. Martin, et al., have postulated that hemoglobin selectively inhibits the relaxation of aorta induced by agents that increase cellular cyclic guanosine monophosphate by acting directly on smooth muscle or indirectly via a release of endothelium-derived relaxing factor. It has also been reported that hemolysate may interfere with the bio-

Fig. 6. Graph showing time courses of oxyhemoglobin (oxyHb) or serotonin-induced inositol 1,4,5-trisphosphate (IP$_3$(1,4,5)) formation in vascular smooth-muscle cells. The cells ($2 \times 10^3$ cells/assay) were pretreated with 10 mM LiCl for 5 minutes at 37°C, then stimulated with 500 µM of oxyhemoglobin (open circles) or 10 µM serotonin (closed circles) for the times indicated. The IP$_3$(1,4,5) content was determined as described in the Materials and Methods section. Values are the means of duplicate determinations from three separate experiments.

synthesis of prostaglandin I$_2$ in the vascular wall. However, there is no clear explanation for the mechanism of "contractive" effects in arteries caused by hemoglobin.

Receptor-Mediated Changes in Calcium

A plausible explanation for the receptor-mediated increase of [Ca++] content is that the activation of phosphoinositide-specific phospholipase C produces IP$_3$(1,4,5), which in turn induces intracellular calcium mobilization. Incubation of vascular smooth-muscle cells with serotonin resulted in the rapid accumulation of IP$_3$(1,4,5). In contrast, incubation of vascular smooth-muscle cells with oxyhemoglobin resulted in no accumulation of mass content of IP$_3$(1,4,5). These findings indicate that the mechanism for increasing [Ca++] was different for oxyhemoglobin and serotonin: oxyhemoglobin-induced [Ca++] elevation depended on the influx of extracellular calcium through the verapamil-insensitive calcium channel, and serotonin induced the initial intracellular calcium mobilization followed by Ca++ influx from outside the vascular smooth-muscle cells.

Organ Differences in Vascular Smooth Muscle

It is well recognized that the cerebral vasculature is different from the vasculature elsewhere in the body. Marked differences in the reactivity of cerebral and extracerebral arteries to vasoconstricting agents, such as serotonin, norepinephrine, epinephrine, angiotensin II, and vasopressin, have been demonstrated. It has been
suggested that the distribution of alpha receptors and serotonin receptors or their sensitivity to amines are different in cerebral and extracerebral arteries. In this study, we used cultured rat aortic smooth-muscle cells, not smooth-muscle cells from cerebral arteries, based on the reported evidence that oxyhemoglobin can contract not only cerebral but also extracerebral arteries. Further investigation using cultured smooth-muscle cells from cerebral arteries exposed to oxyhemoglobin is needed in order to understand the mechanism of cerebral vasospasm.

Conclusions

The present experiments demonstrated that oxyhemoglobin produced Ca\textsuperscript{2+} influx through a pathway distinct from verapamil-blocked voltage-gated calcium channels. Since oxyhemoglobin did not induce the formation of IP\textsubscript{3}, (1,4,5), which was seen in serotonin treated cells, IP\textsubscript{3}, (1,4,5)-mediated Ca\textsuperscript{2+} mobilization was not operative. These results suggest that the elevation of [Ca\textsuperscript{2+}], caused by oxyhemoglobin may, at least in part, contribute to abnormal contraction or irreversible damage in vascular smooth-muscle cells, and that the alterations may cause long-lasting contracting or myonecrosis of cerebral arteries in delayed vasospasm after subarachnoid hemorrhage.\textsuperscript{21,28}

References

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Address reprint requests to: Katsunobu Takenaka, M.D., Department of Neurosurgery, Gifu University School of Medicine, 40 Tsukasamachi, Gifu 500, Japan.

K. Takenaka, et al.