Altered Endothelial Ca\textsuperscript{2+} Regulation After Ischemia/Reperfusion Produces Potentiated Endothelium-Derived Hyperpolarizing Factor–Mediated Dilations

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Background and Purpose—Endothelium-derived hyperpolarizing factor (EDHF)–mediated dilations are potentiated after several pathologies, including ischemia/reperfusion (I/R). However, no study to date has addressed the mechanism by which this potentiation occurs. This study tested the hypothesis that potentiated EDHF-mediated dilations are due to altered endothelial Ca\textsuperscript{2+} handling after I/R.

Methods—Rat middle cerebral arteries (MCAs) were isolated after 2 hours of MCA occlusion and 24 hours of reperfusion (or sham surgery). This model has been previously demonstrated to produce potentiated EDHF-mediated dilations. MCAs were studied in a pressurized/perfused vessel chamber equipped for the simultaneous measurement of endothelial Ca\textsuperscript{2+} (with fura 2) and artery diameter. Measures were made after luminal administration of UTP (P2Y\textsubscript{2} purinoceptor agonist), 2 MeS-ATP (P2Y\textsubscript{1} purinoceptor agonist), and Br-A23187 (receptor-independent Ca\textsuperscript{2+} ionophore) for sham and I/R MCAs.

Results—I/R resulted in significantly potentiated UTP-mediated dilations (through a P2Y\textsubscript{2} purinoceptor) and endothelial Ca\textsuperscript{2+} responses in the presence of \textit{N}\textit{G}-nitro-\textit{L}-arginine methyl ester (L-NAME) and indomethacin. Endothelial Ca\textsuperscript{2+} and diameter responses were also significantly potentiated with 2 MeS-ATP (through a P2Y\textsubscript{1} purinoceptor) when L-NAME and indomethacin were absent. Br-A23187, a receptor-independent Ca\textsuperscript{2+} ionophore, produced significantly potentiated endothelial Ca\textsuperscript{2+} responses after I/R in the presence of L-NAME/indomethacin. Evaluation of artery diameter as a function of endothelial Ca\textsuperscript{2+} demonstrated no differences between sham and I/R groups.

Conclusions—These findings demonstrate that I/R results in augmented endothelial Ca\textsuperscript{2+} responses that appear to be downstream of the receptor level. Moreover, these data suggest that this augmented Ca\textsuperscript{2+} response contributes to the potentiated EDHF-mediated dilations after I/R. (\textit{Stroke}. 2002;33:2285-2291.)

Key Words: brain ■ calcium ■ cerebral arteries ■ cerebral ischemia, transient ■ endothelium ■ endothelium-derived hyperpolarizing factor ■ middle cerebral artery ■ middle cerebral artery occlusion ■ rats...
clear that an elevation of endothelial Ca\textsuperscript{2+} is necessary.\textsuperscript{13–16} Furthermore, recent data have demonstrated that a specific endothelial Ca\textsuperscript{2+} threshold exists that, when met, initiates an EDHF-mediated dilation.\textsuperscript{17} Thus, agents that increase Ca\textsuperscript{2+} above the EDHF threshold result in the production of EDHF-mediated dilations, whereas those that fail to increase Ca\textsuperscript{2+} to the threshold do not. Along these lines, one might also expect the potency of EDHF-producing agonists to vary depending on conditions that affect endothelial Ca\textsuperscript{2+} regulation. For example, one might expect potentiated EDHF-mediated dilations under conditions that favor greater increases in endothelial Ca\textsuperscript{2+}.

Given the critical role of Ca\textsuperscript{2+} in normal EDHF-mediated dilations, it is reasonable to suspect that alterations in endothelial Ca\textsuperscript{2+} regulation might account for the potentiated EDHF-mediated dilations after I/R. Therefore, the following studies were designed to evaluate the hypothesis that endothelial Ca\textsuperscript{2+} regulation is altered after I/R, thus resulting in amplified Ca\textsuperscript{2+} responses and potentiated dilations for EDHF-dependent agonists. It was predicted that endothelial Ca\textsuperscript{2+} would reach the EDHF-producing threshold at lower agonist concentrations in I/R arteries compared with shams. To test this hypothesis, endothelial Ca\textsuperscript{2+} was increased via 2 separate receptor systems (P2Y\textsubscript{1} and P2Y\textsubscript{2}, purinoceptors) and through a receptor-independent mechanism (Br-A23187) in pressurized rat middle cerebral arteries (MCAs). Selective measurement of endothelial Ca\textsuperscript{2+} was performed in pressurized MCAs from sham and I/R rats with the use of recently developed fluorescence techniques.\textsuperscript{17,18} Simultaneous measurement of artery diameter permitted the evaluation of endothelial Ca\textsuperscript{2+} in the vasodilatory responses.

Materials and Methods

All experiments were approved by the Animal Protocol Review Committee at Baylor College of Medicine. A total of 29 male Long-Evans rats were used for these studies (weight, 250 to 350 g). Rats were anesthetized with 3% isoflurane before any procedures.

MCA Occlusion/Reperfusion Surgery

Rats were maintained with 1.5% to 2.5% isoflurane through a nose cone and allowed to breathe freely during surgery. Rats were also treated with 2% lidocaine at the site of surgery before the surgical procedure. Core body temperature was measured rectally and maintained at 37°C with a temperature controller coupled to a heating pad.

The right MCA was occluded for 2 hours, as previously described.\textsuperscript{7,19} In brief, the right carotid artery was exposed in the area of the carotid bifurcation. The external carotid artery was tied off, and a photomultiplier detector. The photomultiplier detector was equipped with a 540/40-nm bandpass filter to selectively measure artery diameter. The infrared light also passed through the dichroic mirror to the beam splitter. Approximately 20% of the fluorescence/infrared signal was diverted to a charge-coupled device (CCD) camera with the remaining diverted to a photomultiplier detector. The photomultiplier detector was converted to intracellular free Ca\textsuperscript{2+} (\([Ca^{2+}]_i\)) on the basis of the following equation:

\[
[Ca^{2+}]_i = \beta \times K_d (R - R_{\text{max}})/(R_{\text{max}} - R),
\]

where \(\beta\) is the ratio of the 380 fluorescence unbound to bound Ca\textsuperscript{2+}, \(R\) is the \(R_{340/380}\), \(R_{\text{max}}\) is the \(R_{340/380}\) in saturating Ca\textsuperscript{2+} conditions, and \(R_{\text{min}}\) is the \(R_{340/380}\) in Ca\textsuperscript{2+}-free conditions. Values for these constants were obtained from a series of in situ calibration curves yielding values as follows: \(\beta = 5.218\), \(R_{\text{max}} = 2.017\), and \(R_{\text{min}} = 0.1365\). The dissociation constant for fura 2 to Ca\textsuperscript{2+} (\(K_d\)) in intact cerebral arteries was 282 nmol/L.\textsuperscript{20}

Concentration-Response Curves to Luminal Agents

Concentration-response curves were performed with the use of luminaly administered UTP, 2-methylthioadenosine 5’-triphosphate...
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(2 MeS-ATP), and Br-A23187. A manifold system was used to substantially reduce the dead volume of the luminal tubing and thus greatly speed the rate of getting the drug from the reservoir to the endothelium. Concentration-response curves to UTP and Br-A23187 were performed in the presence of \(N^\text{a}-\text{nitro-L-arginine (L-NAME; 50 \mu\text{mol/L}}\) and indomethacin (10 \(\mu\text{mol/L}\)) to rule out the effects of NO and PGI\(_2\) formation, respectively. Concentration-response curves to 2 MeS-ATP did not include L-NAME/indomethacin.

**Histology**

After 24 hours of reperfusion, the brain was evaluated for the presence of ischemic injury with 2,3,5-triphenyltetrazolium chloride (TTC). After removal of the MCA segment, the brain was placed in a rat brain matrix (Braintree Scientific) and sliced into 2-mm coronal sections. The sections were incubated in 2.5% TTC for at least 30 minutes. Areas with profound ischemic injury remained white, whereas viable tissue stained red.\(^7,21\) Brains were scored on a scale from 0 to 4 as follows: 0, no lesion; 1, small lesion confined to the striatum; 2, lesion largely consuming the striatum; 3, lesion consuming the striatum and involving some cortex; and 4, lesion consuming the striatum and cortex. I/R arteries were used only from rats with an injury score of 3 or 4. Note that this reflects stricter inclusion criteria for I/R arteries compared with previous studies, which included animals with scores of 1 through 4.\(^7,22\)

**Chemicals and Buffer Compositions**

All drugs and chemicals were obtained from Sigma with the exception of fura 2-AM and pluronic F-127 (Teflabs) and Br-A23187 (Molecular Probes). The Krebs’ buffer (PSS) consisted of the following (in mmol/L): 119 NaCl, 4.7 KCl, 21 NaHCO\(_3\), 1.17 MgSO\(_4\), 1.17 MgSO\(_4\), 0.026 EDTA, 1.6 CaCl\(_2\), and 5.5 glucose.\(^18\) The buffer was bubbled continuously with 20% O\(_2\)/5% CO\(_2\)/balance N\(_2\) to yield a pH of 7.4 at 37°C.

**Statistical Methods**

Values are reported as mean±SE. Single measurements between groups were compared with Student’s \(t\) test. When multiple measurements were performed (such as for a concentration-response curve), a 2-way repeated-measures ANOVA was used to determine whether a statistical difference existed between groups. When group differences did exist, further evaluation of individual differences was performed with a Tukey test. Significance was defined as \(P<0.05\).

Changes in artery diameter are reflected by the following equation:

\[
\text{% Diameter Change} = \frac{(D_{\text{drug}} - D_{\text{base}})}{(D_{\text{max}} - D_{\text{base}})} \times 100,
\]

where \(D_{\text{drug}}\) is the diameter of the artery after administration of an agonist, \(D_{\text{base}}\) is the resting diameter, and \(D_{\text{max}}\) is the maximal diameter of the artery (equivalent to Ca\(^{2+}\)-free diameter).

**Results**

On pressurization (85 mm Hg) and establishment of luminal flow (100 \(\mu\text{L/min}\)), sham and I/R MCAs developed spontaneous tone over the course of 40 to 60 minutes. The percent tone at the end of the equilibration period was similar between the 2 groups (Table). The addition of L-NAME/indomethacin resulted in a significant increase in tone in the sham group but not in the I/R group. Tone in the presence of L-NAME/indomethacin was significantly greater in the sham group (38±3% versus 25±2% tone; \(P<0.01\)). I/R MCAs were not further constricted with a constricting agent because of the likely alterations that would result in endothelial Ca\(^{2+}\).\(^23\)

To determine whether altered endothelial Ca\(^{2+}\) responses might account for the potentiated EDHF-mediated dilations after I/R, concentration-response curves to luminal UTP (10\(^{-7}\) to 10\(^{-5}\) mol/L) were performed in the presence of L-NAME and indomethacin. Dilations to UTP in the presence of L-NAME/indomethacin have been demonstrated to be EDHF dependent and potentiated after I/R in this preparation.\(^7,24\) MCA diameter changes and endothelial [Ca\(^{2+}\)]\(_i\) were recorded simultaneously in fura 2–loaded arteries for both sham and I/R groups. Figure 1 shows representative experiments for both sham and I/R MCAs in response to UTP. Note that endothelial Ca\(^{2+}\) increased more for a given concentration of UTP.
UTP in I/R MCAs compared with shams (Figure 1, bottom). In addition, note the potentiated dilation in the I/R MCA at 10 μmol/L UTP (Figure 1, top). Interestingly, the dilations at 1/6 mol/L UTP appeared more transient than the corresponding Ca²⁺ response in the I/R group. These data are summarized in Figure 2. From Figure 2, it is evident that while resting Ca²⁺ was similar between sham and I/R groups (n=5 each), UTP produced a significantly greater increase in endothelial Ca²⁺ in the I/R MCAs for a given concentration of UTP. The horizontal dashed line indicates the Ca²⁺ threshold (340 nmol/L) for EDHF-mediated dilations17 (and see Figure 4). Significant group differences between sham and I/R exist for both percent diameter change and endothelial [Ca²⁺] (2-way repeated-measures ANOVA). *P<0.05 for individual comparisons between sham and I/R groups. Data are mean±SE of steady state values.

UTP in I/R MCAs compared with shams (Figure 1, bottom). In addition, note the potentiated dilation in the I/R MCA at 10⁻⁶ mol/L UTP (Figure 1, top). Interestingly, the dilations at 1 μmol/L UTP appeared more transient than the corresponding Ca²⁺ response in the I/R group. These data are summarized in Figure 2. From Figure 2, it is evident that while resting Ca²⁺ was similar between sham and I/R groups (n=5 each), UTP produced a significantly greater increase in endothelial Ca²⁺ in the I/R MCAs for a given concentration of UTP. The horizontal dashed line indicates the Ca²⁺ threshold (340 nmol/L) for EDHF-mediated dilations from a previous study17 and the present study (Figure 4, bottom). Endothelial Ca²⁺ in response to 1 μmol/L UTP in the I/R group exceeds the Ca²⁺ threshold for EDHF-mediated dilations, whereas Ca²⁺ from the sham group does not. The dilatory response to UTP was also significantly potentiated in the I/R group. In particular, dilations at 1 μmol/L UTP were significantly greater in the I/R group. Although endothelial Ca²⁺ was considerably higher in the I/R group at 10 μmol/L UTP, differences in percent diameter change did not exist between the groups because of maximal dilations that occurred in each group.

To determine whether the potentiated Ca²⁺ and diameter responses were specific to the receptor system used above, an additional agonist, 2 MeS-ATP, was evaluated. UTP and 2 MeS-ATP operate through 2 separate receptor systems and dilatory mechanisms. UTP stimulates a P₂Y₁ purinoceptor that results in an increase in endothelial Ca²⁺ through an inositol 1,4,5-triphosphate (IP₃) – dependent mechanism with the subsequent production of NO and EDHF.24-25 2 MeS-ATP stimulates a P₂Y₁ purinoceptor that produces an increase in endothelial Ca²⁺ in an IP₃ – independent manner with subsequent release of NO alone under normal conditions.25-27 Figure 3 represents summary data for concentration-response
reach significance. The second part that contributes to this apparent discrepancy is due to the fact that the endothelial [Ca^{2+}] at 10^{-3} mol/L 2 MeS-ATP falls right around the Ca^{2+} threshold for NO-mediated responses in these arteries (220 nmol/L Ca^{2+}). Therefore, increases in endothelial Ca^{2+} that fail to reach the threshold of 220 nmol/L Ca^{2+} do not produce an increase in diameter, whereas increases above the threshold do. Importantly, only relatively small increases from baseline in endothelial Ca^{2+} are necessary to reach that threshold. Furthermore, because a threshold exists, a linear relationship between Ca^{2+} and diameter would not be expected. Rather, significant increases in diameter would be expected once Ca^{2+} entered the threshold range. In this way, relatively small changes in endothelial Ca^{2+} (that do not reach statistical difference) could result in a more amplified difference in diameter responses (that does reach statistical difference).

To determine whether the potentiated Ca^{2+} and diameter responses were dependent on receptor-mediated events, concentration-response curves were conducted with Br-A23187 in the presence of L-NAME and indomethacin. Br-A23187 is a nonfluorescent Ca^{2+} ionophore used to increase endothelial [Ca^{2+}], through a non-receptor-dependent process. Concentration-response curves to luminally delivered Br-A23187 (1 to 6 μmol/L) resulted in a significantly greater increase in endothelial Ca^{2+} for a given concentration of Br-A23187 (Figure 4, top; n=4 each).

Data from individual experiments with Br-A23187 and UTP were combined and plotted as percent diameter versus endothelial Ca^{2+} (Figure 4, bottom). The data derived from UTP and Br-A23187 did not differ from each other and were therefore combined into 1 plot. Two things are apparent from these data. First, there is close overlap of the sham and I/R data. Fitting of the data to a Gompertz equation shows that a similar relationship between endothelial [Ca^{2+}], and diameter exists between sham and I/R groups. Note that the relationship is flat at the beginning (where the Ca^{2+} threshold has not yet been reached) and at the end (where the artery reaches maximal dilation). This similar relationship between endothelial [Ca^{2+}], and diameter between groups suggests that I/R does not affect the endothelial Ca^{2+} threshold for initiating the EDHF-mediated dilations. Second, there is a sharp transition between no dilation and maximal dilation for a given steady state endothelial [Ca^{2+}], (Figure 4, bottom). This steep response reflects the rather narrow endothelial [Ca^{2+}], range for no activation to maximal activation of the EDHF-dependent mechanism. A previous study has demonstrated that this Ca^{2+} range starts at approximately 340 nmol/L,

In summary, the present study has shown that I/R increases endothelial Ca^{2+} concentrations in a non-receptor-dependent manner. Furthermore, increases in Ca^{2+} do not affect the endothelial Ca^{2+} threshold for NO-mediated responses. However, increases in Ca^{2+} above the threshold do result in significant increases in diameter. The lack of data regarding the Ca^{2+} threshold for NO-mediated responses after I/R makes it difficult to determine whether Ca^{2+} is independent of NO-mediated responses.

**Discussion**

A growing number of recent studies have reported upregulated EDHF-mediated dilations after a variety of pathological conditions, including I/R, hypercholesterolemia, and congestive heart failure. However, none of these studies has addressed the mechanism by which EDHF-mediated responses are potentiated. Presumably, this lack of data regarding the potentiated EDHF mechanism has been due in part to
the incomplete understanding of the mechanism by which EDHF-mediated responses occur.

Although there is still considerable debate regarding the identity of EDHF (or whether EDHF is a factor per se), it is generally accepted that EDHF-mediated responses are initiated by an increase in endothelial Ca\(^{2+}\). Furthermore, recent data from this laboratory have demonstrated that an endothelial Ca\(^{2+}\) threshold exists that, when met, initiates an EDHF-mediated dilatation. The threshold for EDHF-mediated dilations is higher than that for NO-mediated dilations (340 versus 220 nmol/L), explaining why NO-mediated responses are typically found to precede EDHF-mediated responses. Additionally, it was shown that a normally non-EDHF-producing agonist (2 MeS-ATP) could elicit an EDHF-mediated response in conditions in which 2 MeS-ATP–mediated endothelial Ca\(^{2+}\) responses were augmented in order to reach the critical Ca\(^{2+}\) threshold. Thus, it appears that endothelial [Ca\(^{2+}\)]critically regulates the EDHF-mediated response. In the present study it was hypothesized that an augmented endothelial Ca\(^{2+}\) response accounts for the potentiated EDHF-mediated response after I/R. This hypothesis was addressed by simultaneously measuring endothelial Ca\(^{2+}\) and artery diameter in pressurized arteries.

Two separate and distinct endothelial purinoceptors (P2Y\(_{1}\) and P2Y\(_{2}\)) were used in addition to a Ca\(^{2+}\) ionophore (Br-A23187) in order to increase endothelial Ca\(^{2+}\). UTP, which is selective for P2Y\(_{1}\) purinoceptors, has been demonstrated to produce both NO- and EDHF-mediated responses in the rat MCA. Br-A23187, which is selective for P2Y\(_{2}\) purinoceptors, produces dilations through an exclusively NO-dependent mechanism. Release of Ca\(^{2+}\) through the P2Y\(_{2}\) purinoceptor is believed to depend on the production of IP\(_{3}\), whereas that of the P2Y\(_{1}\) purinoceptor does not. Br-A23187 elevates endothelial Ca\(^{2+}\) in a receptor-independent fashion, thus bypassing any effect of receptor number or coupling.

Potentiated endothelial Ca\(^{2+}\) responses were found with the use of both receptor-dependent and -independent mechanisms after I/R. The augmented Ca\(^{2+}\) release found in the present study with both UTP and 2 MeS-ATP might suggest either (1) upregulation of both P2Y\(_{1}\) and P2Y\(_{2}\) purinoceptors, (2) potentiation of a common mechanism between the 2 receptor systems, or (3) potentiation of the Ca\(^{2+}\) response downstream of receptor stimulation. However, the additional finding of potentiated Ca\(^{2+}\) responses with the Ca\(^{2+}\) ionophore (Br-A23187) suggests that the potentiation of the Ca\(^{2+}\) response occurs downstream of the receptors. Since increased Ca\(^{2+}\) responses were found with receptor-dependent mechanisms as well as with receptor-independent mechanisms, it would appear that the potentiated Ca\(^{2+}\) is due to more fundamental changes in Ca\(^{2+}\) handling. For instance, there could be a greater increase of [Ca\(^{2+}\)] (from internal stores or from external sources), or there could be a reduced removal of [Ca\(^{2+}\)] (to internal stores or extrusion across the plasma membrane) after I/R. Either one or both of the above possibilities would result in augmented Ca\(^{2+}\) responses.

On the basis of the aforementioned findings, it would be tempting to conclude that the effects of I/R appear to exclude the possibility of greater Ca\(^{2+}\) release from internal stores.

The reasoning behind such a conclusion would be based on the assumptions that Br-A23187 acts purely as a direct mediator of Ca\(^{2+}\) influx and that the 2 MeS-ATP response does not involve an IP\(_{3}\)-mediated Ca\(^{2+}\) increase. However, such a conclusion might be incorrect for the following 2 reasons. First, in addition to its ionophoretic properties, Br-A23187 has been suggested to stimulate Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) channels secondary to depletion of internal store Ca\(^{2+}\). This release of Ca\(^{2+}\) from internal stores may be due to the ability of Br-A23187 to promote the liberation of inositol phosphates such as IP\(_{3}\). Second, while 2 MeS-ATP does not appear to involve a significant IP\(_{3}\)-dependent mechanism under normal circumstances, one cannot assume that IP\(_{3}\) is not involved in pathological circumstances. Thus, while the present data demonstrate significant alterations in Ca\(^{2+}\) handling after I/R, further studies will be needed to identify where that alteration occurs.

One additional possibility that could contribute to the potentiated dilations after I/R is that of Ca\(^{2+}\) sensitivity. Although the aforementioned data clearly demonstrate that potentiated Ca\(^{2+}\) responses occur, it is possible that an increased Ca\(^{2+}\) sensitivity could also contribute to the potentiated dilations. To evaluate the possibility of altered Ca\(^{2+}\) sensitivity, percent diameter change was plotted against the Ca\(^{2+}\) sensitivity threshold. Thus, the data are suggestive that increased Ca\(^{2+}\) sensitivity does not play a measurable role in the potentiated EDHF-mediated dilations. However, given the inherent limitations of the bioassay system, one cannot completely rule out the possibility of multiple offsetting factors (such as in the vascular reactivity to EDHF) downstream of Ca\(^{2+}\).

In summary, potentiated EDHF-mediated dilations after I/R appear to result from augmented endothelial Ca\(^{2+}\) responses. These augmented Ca\(^{2+}\) responses are not receptor dependent but rather appear to result from a more fundamental alteration in endothelial Ca\(^{2+}\) regulation. Additional studies will be required to determine the specific mechanism by which endothelial Ca\(^{2+}\) regulation is altered.

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References


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