P2 purinoceptor-mediated dilations in the rat middle cerebral artery after ischemia-reperfusion

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Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H33–H41, 1999.—Endothelium-mediated dilations to selective P2Y1 and P2Y2 purinoceptor agonists [2-methylthioadenosine triphosphate (2MeS-ATP) and uridine 5'-triphosphate (UTP), respectively] were evaluated in middle cerebral arteries (MCAs) of rats after 2 h of ischemia followed by 24 h of reperfusion (I/R). MCAs were harvested, pressurized to 85 mmHg, and luminafusively perfused. 2MeS-ATP, which dilates by the synthesis and release of nitric oxide (NO), had significantly reduced maximum dilations following I/R. Reduced smooth muscle sensitivity to NO may explain the reduced dilation to 2MeS-ATP. In contrast, the dilations elicited by UTP were potentiated in that the concentration of agonist necessary to produce one-half of the maximum dilation was reduced by 75%. The potentiated dilation to UTP was the result of an endothelial factor having all the characteristics of the endothelium-derived hyperpolarizing factor (EDHF). That is, it was neither NO nor a cyclooxygenase metabolite, and its actions involved calcium-activated potassium channels and smooth muscle hyperpolarization. We conclude that the effect of I/R on endothelium-mediated dilations depends on the receptor system and the mechanism of dilation. Dilations elicited by 2MeS-ATP were attenuated, while dilations UTP were potentiated due to the upregulation of the EDHF mechanism.

ENDOTHELIAL DYSFUNCTION has previously been demonstrated in cerebral vessels following ischemia-reperfusion (I/R). After 10 min of global ischemia and 1 h of reperfusion, Mayhan et al. (23) found reduced endothelium-dependent dilations to acetylcholine, whereas endothelium-independent dilations to adenosine were preserved. Endothelium-independent constrictions to angiotensin II and serotonin were also normal, indicating that the smooth muscle responsiveness was unaltered. When the ischemia time was increased to 30 min, both acetylcholine- and adenosine-induced dilations were depressed, demonstrating impairment of both endothelium-dependent and endothelium-independent relaxations. The smooth muscle constrictor response to angiotensin II and serotonin, however, was still unaltered. Rosenblum (35) recently demonstrated in mouse cerebral arterioles that 10 min of occlusion with 10 min of reperfusion resulted in diminished acetylcholine- but not bradykinin- or sodium nitroprusside-induced dilations. These results corroborate the acetylcholine results of Mayhan et al. (23) as well as suggest that different mechanisms of endothelium-dependent dilations may be differentially affected by I/R. In a more chronic model of I/R (2 h occlusion-24 h reperfusion), Cipolla et al. (5) recently reported reduced acetylcholine-induced dilations at 24 h of reperfusion in a rat model of middle cerebral artery (MCA) occlusion-reperfusion.

The study by Rosenblum (35) points to the fact that I/R may affect some receptor systems involved with endothelial-mediated dilations and not affect others. It is therefore important to consider individual receptor systems when considering the effects of I/R on endothelial-mediated dilations.

Purine and pyrimidine nucleotides (such as ATP, ADP, and uridine 5'-triphosphate (UTP)) have recently been recognized as having major vasoactive properties in the cerebral circulation (3, 11, 16, 25, 27, 36, 38–40). In the rat MCA, stimulation of endothelial purinoceptors relaxes vascular smooth muscle via the release of nitric oxide (NO) either alone or in combination with endothelium-derived hyperpolarizing factor (EDHF) (Ref. 40 and J. P. You, T. D. Johnson, S. P. Marrelli, J. V. Mombouli, W. F. Childres, and R. M. Bryan, J.R., unpublished observations). The P2Y1 (formerly P2Y1) purinoceptor is preferentially stimulated by the physiological agonist ADP and elicits a dilation exclusively through the production of NO. The P2Y2 (formerly P2Y2) purinoceptor is preferentially stimulated by the physiological agonist ADP and elicits a dilation exclusively through the production of NO. The P2Y2 (formerly P2Y2) purinoceptor is preferentially stimulated by the physiological agonists UTP or ATP and elicits a dilation through the production of NO and EDHF. Although the effect of I/R on purinoceptor-mediated dilations has not been studied in the cerebral circulation, in models of coronary I/R the effect appears to be that of attenuated dilations to ADP (14, 30, 32, 34). From the possible differential effects of I/R on cerebral endothelium-dependent dilations (35) and the impairment of purinoceptor-mediated dilations in the coronary vasculature, we felt that it was important to evaluate the response of endothelial purinoceptors to I/R in the cerebral circulation.

The purpose of our study was to determine whether 2 h of ischemia followed by 24 h of reperfusion alters the dilations produced by stimulating purinoceptors on cerebrovascular endothelium. We tested the following two hypotheses: 1) I/R alters the dilations elicited by stimulation of endothelial P2Y1 purinoceptors on rat MCA; and 2) I/R alters the dilations elicited by stimula-
tion of endothelial P2Y₂ purinoceptors on rat MCA. The selective agonists 2-methylthioadenosine triphosphate (2MeS-ATP) and UTP were used to test the hypotheses for P2Y₁ and P2Y₂ purinoceptors, respectively.

METHODS

Animal surgery. The experimental protocol was approved by the Animal Protocol Review committee at Baylor College of Medicine. Male Long-Evans rats (275–325 g) were anesthetized with isoflurane (3%). In preliminary experiments, rats were intubated and mechanically ventilated with a Harvard small rodent ventilator. The tail artery was cannulated for measuring blood pressure and obtaining arterial blood samples for the determination of Pco₂, Po₂, and pH. Ranges for Pco₂, Po₂, and pH were maintained between 35 and 45 mmHg, >95 mmHg, and between 7.35 and 7.45, respectively. In subsequent experiments, rats were allowed to breathe spontaneously. Core body temperature was measured rectally and maintained at 37 ± 0.5°C with a temperature controller coupled to a heat lamp.

The right MCA was occluded as previously described (20, 21). Briefly, the right carotid artery was exposed in the vicinity of the carotid bifurcation. The external carotid artery was ligated and severed, and the resulting stump was gently retracted. A nylon monofilament (~242 µm diameter) was introduced into the stump and advanced, first retrogradely to the common carotid artery and then anterogradely into the internal carotid artery. Care was taken to avoid entering the pterygopalatine branch of the artery. The occluder was slowly advanced ~23–25 mm into the circle of Willis and anteriorly until slight resistance was felt. Preliminary studies in our lab demonstrated that this placement resulted in occlusion of the ostium of the MCA. After 2 h of placement, the occluder was gently removed and blood flow through the right carotid artery was restored.

The occluder was prepared by rounding the tip (~1 mm) with sandpaper and covering it with a thin coat of epoxy to create a smooth surface. Heparin (50 U) was administered before occluder insertion and after 1 h of occlusion to reduce blood clot formation. Rats were allowed to recover for 24 h. Sham-operated animals underwent the same procedure, except the occluder was removed immediately after placement.

In vitro experiments. After 24 h of reperfusion, rats were anesthetized with isoflurane and then decapitated. The brain was carefully removed from the cranial and placed in ice-cold Krebs solution. Two sections of the right MCA ~3 mm from the circle of Willis was dissected from the brain, and each was independently mounted on two glass micropipettes within a vessel chamber (Living Systems, Burlington, VT). Each vessel was assigned to either a 2MeS-ATP or UTP protocol. Each vessel was pressurized to a mean of 85 mmHg (4), and a flow of ~200 µl/min was established through the lumen. Warmed (37°C) and gassed (21% O2:5% CO2, balance N₂) physiological saline solution was circulated abluminally and perfused luminally. Buffer pH was maintained at ~7.4. The physiological saline solution had the following composition (mM): 119 NaCl, 4.7 KCl, 24 NaHCO₃, 1.18 KH₂PO₄, 1.17 MgSO₄, 0.026 EDTA, 1.6 CaCl₂, and 5.5 glucose. An agonist could be delivered preferentially to the endothelium by adding it to the luminal perfusate or to the smooth muscle by adding it to the abluminal bath. After the MCA was mounted, the chamber was placed on the stage of an inverted microscope (Nikon) equipped with a video camera and screen. Outer diameters were measured directly from the video screen (final magnification of ×600). During times of vasomotion, the average minimum diameter was recorded. For most experiments, vessel diameter was measured continuously using image-analysis software (Optimas, Bothell, WA) on a Hewlett-Packard Pentium computer. A custom macro was written to allow acquisition at a frequency of 1.1 Hz. Concentration-response curves (CRCs) were run to luminal application of 2MeS-ATP and UTP and abluminal application of 2-nitroso-N-acetylpenicillamine (SNAP), an exogenous NO donor. CRCs to 2MeS-ATP and UTP were also conducted in the presence of N^̅-nitro-L-arginine methyl ester (L-NAME) (10 µM, 50 µM, or 1 mm) or N^̅-nitro-L-arginine (L-NNA) (50 µM) or after the removal of the endothelium by luminal perfusion with air (8–10 min). (19, 40). Additionally, UTP CRCs were performed in the presence of indomethacin (10 µM) + L-NAME (10 µM), charybdotoxin (100 nM) + L-NAME (50 µM), or 5,1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 µM) + L-NAME (50 µM). SNAP CRCs were performed after endogenous NO production had been eliminated with L-NAME (10 µM). Unless stated otherwise, all inhibitors were given both luminally and abluminally in the concentration reported.

Electrophysiology. In another subset of sham and I/R MCAs, smooth muscle membrane potential (V̅), and diameter were simultaneously measured (You et al., unpublished observations) following L-NAME incubation (50 µM) and UTP (1 and 10 µM) administration. Briefly, glass microelectrodes (diameter 1 mm) were pulled using a Flaming/Brown model P-87 micropipette puller (Sutter, Novato, CA) and filled with 3 M KCl (electrode resistances ranged from 50 to 80 MΩ). The potential difference between the KCl electrode and an Ag/AgCl reference electrode (submersed in the vessel bath) was measured with a Dagan 8700 Cell Explorer (Dagan, Minneapolis, MN). Output was displayed on a Tektronix 5223 Digitizing Oscilloscope. Criteria for successful impalements included a sharp drop in the voltage upon entering the smooth muscle cell (SMC) and an unchanged tip resistance upon exiting the SMC. The site of impalement was moved frequently to avoid excessive tissue damage. For each MCA, Eₜₚ was measured in the presence of L-NAME and after the luminal administration of UTP. A single Eₜₚ value for each condition in a given MCA was obtained by averaging two to six different SMC impalements. Then value (see Fig. 8) refers to the number of animals studied and not the number of impalements.

Histology. After the right MCA (ipsilateral to the injury) was removed, the brains were placed in a rat brain matrix (Braintree Scientific, Braintree, MA) and sectioned coronally in 2-mm sections for evaluation of tissue viability. The sections were incubated in a 2% triphenyltetrazolium chloride (TTC) solution for 30 min and placed in a Formalin solution for at least 24 h (1). Viable tissue stained deep red, whereas the lesion area due to the occlusion remained white. Lesion volumes were evaluated using image analysis (MCID, Imaging Research, St. Catharines, Ontario, Canada). Confirmation of lesion by TTC was a prerequisite for all "I/R" vessels.

Drugs. UTP, L-NAME, L-NNA, TTC, charybdotoxin, and indomethacin were purchased from Sigma (St. Louis, MO). SNAP and 2MeS-ATP were obtained from Research Biochemicals International (Natick, MA). ODQ was obtained from Tocris (Ballwin, MO). Stock solutions of UTP, 2MeS-ATP, L-NAME, charybdotoxin, and SNAP were prepared in distilled water, aliquotted, and then stored at −20°C. ODQ was dissolved in pure EtOH and stored at −20°C. L-NNA stock solutions were prepared daily in hot distilled water. Indomethacin stock solution was prepared daily by dissolving in a 15 mM Na₂CO₃ solution. Stock solutions were diluted to at least 1,000-fold in solution.
Analysis of data. All data are presented as means ± SE. Tone for the MCAs is defined as

\[
\text{tone (\%) } = \frac{(D_{\text{max}} - D_t)/D_{\text{max}}}{100}
\]

where \(D_t\) is the vessel diameter and \(D_{\text{max}}\) is the maximum diameter. Both \(D_t\) and \(D_{\text{max}}\) were obtained at 85 mmHg luminal pressure. For dilations, data are presented as percent diameter change of the MCAs and were calculated by the following formula

\[
\% \text{diameter change } = \frac{(D - D_{\text{rest}})/(D_{\text{max}} - D_{\text{rest}})}{100}
\]

where \(D\) is the diameter after the addition of the vasodilator, \(D_{\text{rest}}\) is the diameter before drug addition, and \(D_{\text{max}}\) is the maximum diameter. The maximum diameter was the diameter immediately on pressurization to 85 mmHg. Preliminary studies demonstrated that "maximum diameter" corresponded to the diameter obtained in Ca-free buffer at 85 mmHg. For comparison of the concentration-response curves, the two-way repeated-measures analysis of variance (2-way RM-ANOVA) was used followed by a post hoc Student-Newman-Keuls test when appropriate. Individual dose-response curves were fitted to a hyperbolic curve (Marquardt-Levenberg algorithm) in Sigmaplot Software (Jandel Scientific, San Rafael, CA) with the formula

\[
f(x) = (\text{Max}) \cdot x/EC_{50} + x
\]

where \(f(x)\) is the dilation for the MCA for a given concentration of UTP, \(\text{Max}\) is the maximal dilation, and \(EC_{50}\) is the concentration of agonist necessary to produce one-half of a maximum dilation. For comparisons of absolute diameters, maximum diameter changes, and \(EC_{50}\) values (following log transformation), a Student's \(t\)-test was used. Differences were considered significant at \(P < 0.05\).

RESULTS

Brain lesions were verified for all animals by TTC staining and quantified in a subset of animals. The mean lesion volume (108 ± 26 mm\(^3\), \(n = 11\)) represented ~13% of the right cerebral hemisphere and generally involved both the striatum and cortex. Sham brains showed no evidence of lesion by TTC staining.

Contribution of basal release of NO to resting tone. Maximum diameters, the diameters immediately on pressurization to 85 mmHg, for sham and I/R vessels were 298 ± 6 µm (\(n = 17\)) and 303 ± 3 µm (\(n = 33\)), respectively (not significant, \(P = 0.41\)). During the 60-min equilibration period, the vessels spontaneously developed a tone of 27% (sham) and 28% (I/R, Fig. 1). As stated in METHODS, tone is defined as the percent constriction from the maximum diameter at 85 mmHg. After inhibition of NO synthase with 10 µM \(L\)-NAME (Ref. 40 and You et al., unpublished observations), tone in sham vessels was significantly greater than that in I/R vessels (41 ± 2% (\(n = 13\)) vs. 35 ± 2% (\(n = 20\)), respectively, \(P = 0.01\), Fig. 1). Increasing the \(L\)-NAME concentration 100-fold to 1 mM in I/R vessels did not further increase the tone (35 ± 2%, \(n = 3\), data not shown). Removal of the endothelium, in the presence of 10 µM \(L\)-NAME, similarly produced tones of 43 ± 2% (\(n = 7\)) and 36 ± 2% (\(n = 6\)) for sham and I/R vessels, respectively (\(P = 0.02\), Fig. 1). Thus removal of the endothelium did not result in a greater constriction than the addition of \(L\)-NAME alone for either group.

CRCs to the abluminal administration of the NO donor SNAP showed that there was a significant interaction between the group (sham vs. I/R) and SNAP concentration (\(P = 0.03\), 2-way RM-ANOVA) (Fig. 2). Note that \(L\)-NAME was given before the SNAP CRC to eliminate possible differences due to different rates of endogenously produced NO in the two groups. A significant effect between groups approached but did not reach statistical criteria (\(P = 0.09\)). However, the power of the statistical analysis for a group effect was 0.24, a value less than the desired power of 0.8, making a definitive conclusion regarding group differences difficult. Nevertheless, it appears that a decreased sensitiv-
vessels did not demonstrate a statistically significant

8) vessels (Fig. 5). Whereas the CRCs for sham and I/R
did not alter the L-NAME-insensitive component. In con-

The involvement of NO in the dilation to UTP was
evaluated by inhibiting NO synthase with L-NAME and

The effects of NO synthase inhibition and removal of
endothelium on CRCs to 2MeS-ATP are shown in Fig.
4, A (sham) and B (I/R). In sham vessels, L-NAME (10
µM), L-NNA (50 µM), or removal of the endothelium
almost completely abolished the dilations to 2MeS-ATP
(Fig. 3A). In I/R vessels, 10 µM L-NAME did not inhibit
the dilation to the same magnitude as in sham vessels
(P = 0.002, 2-way RM-ANOVA compared with sham,
Fig. 3, A and B). However, 50 µM of either L-NAME or
L-NNA further suppressed the dilation to nearly the
same level as the dilation in sham vessels. For all
practical purposes, removal of the endothelium in I/R
vessels abolished the dilation to 2MeS-ATP.

P2Y2-mediated dilations. CRCs to luminally adminis-
tered UTP were performed on sham (n = 5) and I/R (n =
8) vessels (Fig. 5). Whereas the CRCs for sham and I/R
vessels did not demonstrate a statistically significant
group difference (P = 0.15, 2-way RM-ANOVA), there
was a significant interaction between the group (sham
vs. I/R) and the concentration (P < 0.01). Thus the
effect of I/R on the UTP CRC was dependent on the
concentration of UTP. Comparison of the dilation be-
tween groups at individual UTP concentrations re-
vealed that the interaction was due to a significantly
greater dilation in I/R vessels at 10^{-7} M UTP compared
with that of sham vessels (P < 0.05, Student-Newman-
Keuls method). Further analysis, where individual CRCs were fitted to a
hyperbolic function (see METHODS), indicated that the
significant interaction was a result of a 20% suppres-
sion of the maximum response to 2MeS-ATP in I/R
vessels (Table 1).

Table 1. EC50 values and maximum diameters to 2MeS-ATP and UTP for sham and I/R middle cerebral arteries

<table>
<thead>
<tr>
<th></th>
<th>2MeS-ATP</th>
<th>UTP</th>
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<tr>
<td></td>
<td>EC50, nM</td>
<td>Max</td>
</tr>
<tr>
<td>Sham</td>
<td>23 ± 9</td>
<td>102 ± 1</td>
</tr>
<tr>
<td>I/R</td>
<td>11 ± 3</td>
<td>81 ± 7*</td>
</tr>
<tr>
<td></td>
<td>344 ± 187</td>
<td>101 ± 2</td>
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<tr>
<td></td>
<td>86 ± 24*</td>
<td>99 ± 2</td>
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Data are presented as means ± SE; n in parenthesis refers to
number of animals. The concentration required to produce a half-
maximal dilation (EC50) to 2-methylthioadenosine triphosphate
(2MeS-ATP) or uridine 5’-triphosphate (UTP) was calculated by
fitting the data to a hyperbolic curve equation. Percentage of maxi-
mum diameter is represented as Max. *P ≤ 0.05 by t-test sham vs.
ischemia-reperfusion (I/R). EC50 values are compared following log
transformation.

The involvement of NO in the dilation to UTP was
evaluated by inhibiting NO synthase with L-NAME and
L-NNA. In sham vessels (Fig. 6A), 10 µM L-NAME or 50
µM L-NNA completely blocked the dilation at 10^{-7} M
UTP and attenuated the dilation at 10^{-5} M by ~90% with-
out suppressing the maximal dilation occurring at
10^{-5} M. This response in the presence of L-NAME (10
µM) was consistent with data from naive animals in our
laboratory (Ref. 40 and You et al., unpublished observa-
tions), demonstrating that the sham surgery alone did
not alter the L-NAME-insensitive component. In con-
trast to the sham vessels, dilations in I/R vessels were
near maximum at 10^{-6} M UTP in the presence of
L-NAME (10 µM and 1 mM), L-NNA (50 µM), or 50 µM
L-NAME in combination with ODQ, a guanylate cyclase
inhibitor (Fig. 6B). There were no significant differ-
ces in the response to UTP among the L-NAME
groups (with or without ODQ) and L-NNA group in I/R
vessels. Thus the potentiated dilations in I/R vessels at
10^{-6} M UTP persisted when NO synthase alone or in
combination with guanylate cyclase was completely
inhibited.

For the purpose of comparison, the sham and I/R
responses to UTP in the presence of 10 µM L-NAME are
plotted together in Fig. 6C. In the presence of L-NAME,
dilations to UTP were significantly potentiated in I/R
vessels compared with those of the sham vessels (P <
EC₅₀ values for the sham and I/R responses were 4.0 ± 0.6 and 0.6 ± 0.3 µM UTP, respectively (P = 0.001, t-test). Similarly, in the presence of 50 µM L-NAME, sham and I/R CRCs were also significantly different (P = 0.002, 2-way RM-ANOVA; see L-NAME curves Fig. 6, A and B). Thus the I/R curve was shifted approximately sevenfold to the left.

The dilations to UTP were endothelial dependent because removal of the endothelium completely abolished the dilation in both sham and I/R groups (Fig. 6, A and B).

To evaluate a possible role of cyclooxygenase metabolites in the L-NAME-insensitive dilation in I/R vessels, CRCs to UTP were conducted in the presence of 10 µM L-NAME plus 10 µM indomethacin, an inhibitor of cyclooxygenase. This concentration of indomethacin has been shown to be effective in blocking cyclooxygenase-dependent dilations in the rat MCA (9). Indomethacin had no effect on the dilations to UTP in the presence of L-NAME (n = 4, data not shown). Thus cyclooxygenase metabolites, such as prostacyclin, were not responsible for the potentiated dilation to UTP in I/R vessels.

CRCs to UTP were conducted in the presence of L-NAME (50 µM) or L-NAME (50 µM) plus charybdotoxin (100 nM). Charybdotoxin is an inhibitor of calcium-sensitive potassium (K₉Ca) channels in cerebral arterial smooth muscle at the above concentration (Ref. 2 and You et al., unpublished observations). Charybdotoxin completely inhibited the L-NAME-insensitive component of the dilation (Fig. 7; n = 3 each, P = 0.03, 2-way RM-ANOVA).

To determine whether hyperpolarization accompanied the L-NAME-insensitive dilation in I/R vessels, membrane potentials of individual SMCs and vessel diameters were simultaneously measured in MCAs (see METHODS). Figure 8 shows the diameters (top) and corresponding membrane potentials (bottom) for sham and I/R MCAs. The E₉m for each condition of each MCA was derived by averaging from two to six SMC impalements of the individual vessel. In the presence of L-NAME (50 µM), the diameter of sham vessels was significantly less than the diameter for I/R vessels.
These data are in complete agreement with the differences in tone for sham and I/R vessels after L-NAME administration (Fig. 1). The significantly smaller diameter in sham vessels corresponded to and was likely partially produced by the more depolarized vascular smooth muscle (32 ± 2 vs. 36 ± 1 mV, respectively; P = 0.02, t-test). In sham MCAs, UTP 10⁻⁶ M had no significant effect on the diameter or Eₘ (32 ± 1 mV). However, the same concentration of UTP dilated and produced a 10-mV hyperpolarization (to 46 ± 4 mV) in I/R MCAs (P = 0.02, t-test). This dilation at 10⁻⁶ M UTP corresponded to a near-maximal dilation of the vessel. In agreement with results presented in Fig. 6, sham MCAs maximally dilated to 10⁻⁵ M UTP, and this dilation was accompanied by a 12-mV hyperpolarization (to 44 ± 2 mV) (P = 0.002, t-test, Fig. 8).

DISCUSSION

The purpose of the present study was to determine whether 2 h of ischemia followed by 24 h of reperfusion produced alterations in the dilations elicited by stimulating 1) P₂Y₁ purinoceptors or 2) P₂Y₂ purinoceptors on the endothelium of rat middle cerebral arteries.
Larger cerebral vessels, such as the MCA in the rat, contribute upwards of 45–50% of the overall resistance within the cerebral circulation (12, 13). Unlike in many peripheral tissues, these larger cerebral arteries play a major role in control of blood flow (7, 8, 22, 24, 37).

We report three major findings from this study. First, I/R produced selective smooth muscle alterations. Second, I/R attenuated dilations to the P2Y1-selective agonist UTP via an upregulation of the EDHF component of the dilation. Thus the effect of 2 h of ischemia and 24 h of reperfusion on endothelial-mediated dilations was dependent on the receptor system.

I/R produced smooth muscle alterations. After pressurization to 85 mmHg, sham and I/R vessels developed the same amount of tone (Fig. 1) giving the “appearance” of normal function in I/R vessels during the resting state. However, more in-depth studies revealed that vascular smooth muscle was actually altered following I/R and that two separate alterations offset one another to give the “appearance” of normal tone in the resting state.

After the inhibition of NO synthase, I/R MCAs contracted significantly less than sham MCAs (Fig. 1). Removal of the endothelium, another means of eliminating NO, confirmed the NO synthase inhibition findings (Fig. 1) and indicated that the smooth muscle itself differed between sham and I/R MCAs. Furthermore, I/R reduced smooth muscle sensitivity to NO (SNAP) (Fig. 2), and the smooth muscle was significantly more hyperpolarized (4 mV) compared with shams (Fig. 8). It has been shown that small hyperpolarizations can result in a significant decrease in Ca flux (29) and therefore significantly influence vessel diameter. Thus the decreased smooth muscle sensitivity to NO was presumably offset by a smooth muscle hyperpolarization resulting in similar resting tones between sham and I/R MCAs when basal NO is present (Fig. 1, baseline). These alterations were very similar to those occurring after severe traumatic brain injury in the rat (10).

I/R attenuated dilations to P2Y1-selective agonist 2MeS-ATP. After I/R, maximum dilations elicited by 2MeS-ATP were attenuated ~20% without a significant alteration of the EC50 (Fig. 3 and Table 1). Attenuation of the maximum dilation was apparently responsible for the statistically significant interaction between the groups and concentration of 2MeS-ATP (Fig. 3 and Table 1). The reduced sensitivity to NO in I/R vessels (Fig. 2) may, at least partially, explain the attenuated dilations following I/R. However, neither attenuated NO release from the endothelium nor removal of NO by reactive oxygen species can be ruled out as also contributing to the attenuated dilation following I/R. Interestingly, a fivefold increase in L-NAME was required to effectively abolish the dilation to 2MeS-ATP in I/R vessels compared with sham vessels (Fig. 4). Although we cannot fully explain this greater requirement for L-NAME in I/R vessels, it could reflect 1) an altered uptake of L-NAME, 2) an increased expression of NO synthase (15), or 3) a partial shift of the dilation to one involving an L-NAME-sensitive, ATP-sensitive potassium (KATP) channel mechanism (17, 18).

The attenuation of P2Y1-mediated dilations are consistent with responses to I/R in the coronary artery (14, 31). In these studies, occlusion/reperfusion of the coronary artery resulted in attenuated dilations to ADP (a P2Y1 agonist) for at least 48 h (14) in one study and 12 wk in another study (31). However, in contrast to our findings, these studies found no alterations in smooth muscle responses to NO donors.

I/R potentiated dilations to the P2Y2-selective agonist UTP by an increase in the EDHF component of the dilation. Contrary to P2Y1-mediated dilations, P2Y2-mediated dilations were potentiated following I/R; that is, I/R MCAs were significantly more sensitive to UTP than sham MCAs. The EC50 in I/R vessels was 25% of that in sham vessels, whereas the maximum dilation was not significantly altered (Fig. 5 and Table 1). Our results strongly support the idea that the potentiation was due to the upregulation of the EDHF component of the UTP-mediated dilation. EDHF is defined as a relaxing factor other than NO or a cyclooxygenase metabolite, which is released from the endothelium and...
relaxes the vessel by hyperpolarizing the vascular smooth muscle. This hyperpolarization is due to activation of a potassium channel (26). Our laboratory has previously shown that the L-NAME-insensitive component of the UTP-mediated dilation in naive MCAs is EDHF (You et al., unpublished observations). We now show that this EDHF component is upregulated following I/R to produce the increased sensitivity (or potentiation) to UTP.

The component producing the potentiation following I/R exactly fits the definition of EDHF. First, the dilation of I/R MCAs, like that of sham MCAs, is completely endothelium dependent (Fig. 6, A and B). Second, neither NO nor a cyclooxygenase metabolite such as prostacyclin was involved (see Fig. 6 and RESULTS). The dilation in I/R MCAs was shifted approximately 10-fold to the left following NO synthase inhibition or NO synthase plus cyclooxygenase inhibition compared with that of sham MCAs after NO synthase inhibition. Because the ability to completely inhibit NO synthase (6) and the selectivity of arginine analogs for NO synthase (17, 18) has been recently questioned, we used a combination of methods to inhibit NO or the effect of NO. In I/R MCAs, the dilations to UTP in the presence of L-NAME (10 μM to 1 mM) either alone or in combination with ODQ, an inhibitor of guanylate cyclase, or L-NNA alone were still significantly potentiated compared with those of the NO synthase inhibition in sham MCAs. Third, the dilation was accompanied by a significant hyperpolarization of the vascular smooth muscle (Fig. B). Finally, the dilation could be abolished by charybdotoxin, a blocker of the K_{Ca} channel.

To our knowledge, this study is the first to show an upregulation of the EDHF mechanism in a pathological condition. At this time, we cannot be certain if this upregulation is due to increased EDHF production and/or release or due to a greater smooth muscle sensitivity to EDHF. Others have shown increased K_{Ca} channel dependence for agonist stimulation during hypercholesterolemia (28) and endotoxemia (33). Although EDHF is generally believed to act via a K_{Ca}-like channel, the involvement of EDHF was not confirmed in the latter studies.

In summary, 2 h of ischemia followed by 24 h of reperfusion resulted in a potentiated response to the P2Y_{2} purinoceptor system (UTP) and an attenuated response of the P2Y_{1} purinoceptor system (2MeS-ATP). Whereas the former was potentiated by a mechanism involving EDHF, the latter was characterized by an attenuated NO-mediated dilation. EDHF upregulation or potentiation is not a generalized response for all receptor systems following I/R because it did not occur with the P2Y_{1} purinoceptor. It follows that the effect of I/R is not a ubiquitous response but rather is dependent on the individual receptor system and/or mechanism of dilation studied. Furthermore, because individual receptors may have different mechanisms in different species, generalizations regarding the effect of I/R should be based on mechanisms rather than solely on receptors.


