Arachidonic Acid Metabolites Contribute to the Irreversible Depolarization Induced by In Vitro Ischemia

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Tanaka, E., S. Niiyama, S. Sato, A. Yamada, and H. Higashi. Arachidonic acid metabolites contribute to the irreversible depolarization induced by in vitro ischemia. J Neurophysiol 90: 3213–3223, 2003. First published August 13, 2003; 10.1152/jn.00542.2003. Intracellular recordings were made from hippocampal CA1 neurons in rat slice preparations. Superfusion with oxygen- and glucose-deprived medium (in vitro ischemia) produced a rapid depolarization ~5 min after the onset of the superfusion. Even when oxygen and glucose were reintroduced immediately after rapid depolarization, the membrane depolarized further (persistent depolarization) and reached 0 mV (irreversible depolarization) after 5 min from the reintroduction. The pretreatment of the slice preparation with a phospholipase A₂ (PLA₂) inhibitor, para-bromophenacyl bromide, or a cytochrome P-450 inhibitor, 17-octadecynoic acid, significantly restored the membrane to the preexposure potential level after the reintroduction of oxygen and glucose. The administration of 14,15-epoxyeicosatrienoic acid or 20-hydroxyeicosatetraenoic acid did not change the latency of the rapid depolarization and did not allow the membrane potential to recover after the ischemic exposure. In contrast, after pretreatment with cyclooxygenase or lipoxygenase inhibitors, such as indomethacin, resveratrol, Dup-697, nordihydroguaiaretic acid, and 3,4-dihydroxyphenyl ethanol, a minority of neurons tested showed posts ischemic recovery from the persistent depolarization. Improved recovery was also seen after treatment with the free radical scavengers, edaravone and α-tocopherol. These results suggest that the activation of the arachidonic acid cascade via PLA₂ and the free radicals produced by arachidonic acid metabolism contribute to the irreversible depolarization produced by in vitro ischemia.

INTRODUCTION

In the mammalian plasma membrane, the sn-2 position of phospholipids is enriched with arachidonic acid (Farooqui et al. 1997; Sapirstein and Bonventre 2000). Lyso phospholipids and arachidonic acid are therefore mainly produced by the hydrolysis of the ester bond at the sn-2 position of glycerophospholipids with phospholipase A₂ (PLA₂). PLA₂ isozymes are classified into four subtypes and type VI PLA₂ isozymes are cytosolic. The type VI PLA₂ isozymes are further classified into two groups, either Ca²⁺-dependent or -independent. The initiation of cerebral anoxia or ischemia immediately accumulates free fatty acids including arachidonic acid in the brain in a time-dependent manner (Bazan 1970; Siesjo and Katsura 1992). Arachidonic acid produced by the activation of PLA₂ can be metabolized by lipoxygenase (LOX) isozymes, cyclooxygenase (COX) isozymes, and cytochrome P-450 isozymes to leukotrienes, prostaglandins, and other eicosanoids, respectively (Sapirstein and Bonventre 2000). The cytochrome P-450 isozymes are divided into two groups by their functions, α-hydroxylases and epoxygenases (Roman 2002). In the rat brain, α-hydroxylases produce mainly 20-hydroxyeicosatetraenoic acid (20-HETE), whereas epoxygenases produce mainly 14,15-epoxyeicosatrienoic acid (14,15-EET) (Alkayed et al. 1996; Amruthesh et al. 1993; Roman 2002). In vivo experiments show the accumulation of leukotrienes and prostanoids in the mammalian brain during and after complete cerebral ischemia (Moskowitz et al. 1984; Stevens and Yaksh 1988). In humans, both the leukotriene C₄- and prostaglandin-E₂-like activity increase in acute cerebral ischemia patients (Akatani et al. 1991). The enzymes that metabolize arachidonic acid also produce oxygen radicals (superoxyl and hydroxyl radicals) (Paller and Jacob 1994; Siesjo and Katsura 1992). Oxygen radicals produce wide-ranging cellular effects, such as lipid peroxidation, inactivation of enzyme, damage to cytoskeleton, and DNA damage (Kontos 2001). It is therefore possible that activation of arachidonic acid metabolism induce severe neuronal damage or death during ischemia and after reperfusion.

Recently, several lines of evidence support the proposal that the activation of PLA₂ and the subsequent arachidonic acid metabolites are closely related to the neuronal cell death produced by brain ischemia. Administration of an inhibitor of COX isozymes before exposure to ischemia reduces the area of infarction produced by either occlusion of common carotid arteries or a middle cerebral artery in gerbils or mice (Sasaki et al. 1988). The administration of an inhibitor for LOX isozymes also attenuates the neuronal death produced by occlusion of carotid arteries in gerbils (Rao et al. 1999). In rat hippocampal slice cultures, LOX inhibitors also attenuate the injury caused by oxygen and glucose deprivation (Arai et al. 2001b). Moreover, PLA₂ or COX-2 knockout mice show a reduction in the infarcted area produced by occlusion of the middle cerebral artery in comparison with wild-type mice (Bonventre et al. 1997; Iadecola et al. 2001). In CA1 pyramidal neurons of the rat hippocampal slices, superfusion with oxygen- and glucose-deprived medium (in vitro ischemia) produce a rapid depolarization ~5 min after the onset of the ischemic exposure (Tanaka et al. 1997). When
oxygen and glucose are reintroduced immediately after rapid depolarization, the membrane depolarizes further (persistent depolarization) and reaches 0 mV (irreversible depolarization) after 5 min from the onset of the reintroduction: as a result, the neurons show no functional recovery. The persistent depolarization is a Ca\(^{2+}\)-dependent process that is mediated by the activation of ionotropic glutamate (Glu) receptors and the Ca\(^{2+}\)-induced Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) store sites (Yamamoto et al. 1997). Moreover, blebs appear on the cell body of the CA1 pyramidal neuron 1 min after starting the reintroduction of oxygen and glucose, and the cell body becomes swollen 3 min after (Tanaka et al. 1999). It is therefore possible that during the persistent depolarization, the Ca\(^{2+}\) dependent PLA\(_2\) may be activated and thereby induce the neuronal damage. Nevertheless, the critical metabolic pathway of arachidonic acid cascade for generating the irreversible depolarization is still unclear.

In the present study, we examined whether or not PLA\(_2\) and the subsequent arachidonic acid metabolites contribute to the generation of the irreversible depolarization induced by in vitro ischemia. Inhibitors for PLA\(_2\) have been administered to the rat hippocampal slices, and the potential changes in CA1 neurons during and after ischemic exposure have been compared between the slices with no administration (control) and the slices administered these drugs. In addition, to clarify the metabolic pathway of arachidonic acid cascade, which leads to the irreversible change, we have examined effects of an inhibitor for LOX, COX, or cytochrome P-450 isozymes on the potential changes after in vitro ischemia.

**METHODS**

All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan and had the approval of the Institutional Animal Use and Care Committee in Kurume University. Wistar rats (male, 250–350 g; 8–12 wk old) were rapidly decapitated under ether anesthesia, and the forebrains were removed and placed in chilled (4–6°C) Krebs solution that was aerated with 95% O\(_2\)-5% CO\(_2\). The composition of Krebs solution was (in mM) 117 NaCl, 3.6 KCl, 2.5 CaCl\(_2\), 1.2 MgCl\(_2\), 1.2 NaH\(_2\)PO\(_4\), 25 NaHCO\(_3\), and 11 glucose. The hippocampus was dissected and then sliced (thickness of 400 μm) from intracellular Ca\(^{2+}\) store sites (Yamamoto et al. 1997). Moreover, blebs appear on the cell body of the CA1 pyramidal neuron 1 min after starting the reintroduction of oxygen and glucose, and the cell body becomes swollen 3 min after (Tanaka et al. 1999). It is therefore possible that during the persistent depolarization, the Ca\(^{2+}\) dependent PLA\(_2\) may be activated and thereby induce the neuronal damage. Nevertheless, the critical metabolic pathway of arachidonic acid cascade for generating the irreversible depolarization is still unclear.

The drugs used were (from Sigma) para-bromophenacyl bromide (para-BPB), arachidonic acid, 17-octadecyenoic acid (17-ODA), α-to-copherol, allopurinol; (from Funakoshi) nordihydroguaiaretic acid (NDGA), 3,4-dihydroxyphenyl ethanol, indomethacin, resveratrol, Dup-697, 20-hydroxycosatetraenoic acid (20-HETE), 14,15-eicosatetraenoic acid (14,15-EET); and edaravone (gift from Mitsubishi Welpharma). All drugs were dissolved in the perfusate and applied by bath application. The slices were pretreated with media containing test compounds for 20 min before ischemic exposure.

The latency of the rapid depolarization was measured from the onset of superfusion to onset of the rapid depolarization estimated by extrapolating the slope of the rapid depolarization to the slope of the slow depolarization (Tanaka et al. 1997). Recovery after the reintroduction of oxygen and glucose is defined as follows: no recovery, 30–60 min after reintroduction the membrane potential lay between 0 and −19 mV; complete recovery, the membrane potential was more negative than −60 mV; partial recovery, membrane potential repolarized to a value between −20 and −59 mV (Yamamoto et al. 1997).

All quantitative results were expressed as the means ± SD. The number of slices examined is given in parentheses. A one-way ANOVA with the Scheffé post hoc test was used to compare the data. Statistical significance was determined at the level of P < 0.05 unless otherwise indicated.

**RESULTS**

**Effects of PLA\(_2\) inhibitor on the irreversible depolarization produced by in vitro ischemia**

Hippocampal CA1 pyramidal neurons with stable membrane potentials more negative than −60 mV were used for the following studies. The resting membrane potential and the apparent input resistance in CA1 neurons were −70 ± 5 mV (n = 297) and 38 ± 10 MΩ (n = 297), respectively. To elucidate the effects of PLA\(_2\) on the potential change after in vitro ischemia, broad-spectrum inhibitors of PLA\(_2\), para-BPB, and aristolochic acid were administered to the slice preparations. The administration of aristolochic acid at concentrations >100 μM induced a hyperpolarization of a few millivolts, whereas para-BPB (3–100 μM) or arachidonic acid (50–100 μM) did not induce any potential change before ischemic exposure. Figure 1A shows the typical potential changes in CA1 neurons during and after in vitro ischemia in the presence and absence (control) of para-BPB (10 μM) or aristolochic acid (100 μM). In the control condition, in vitro ischemia produced a sequence of potential changes consisting of an initial hyperpolarization, a slow depolarization, and a rapid depolarization (Fig. 1A top). When oxygen and glucose were reintroduced immediately after generating the rapid depolarization, the membrane depolarized further (persistent depolarization) and reached 0 mV. The membrane never showed a restoration to the potential level before exposure to in vitro ischemia (irreversible depolarization). The latency and the maximal slope of the rapid depolarization were 5.2 ± 1.0 min and 9.6 ± 3.7 mV/s (n = 96), respectively. The pretreatment of the slices with para-BPB (100 μM) or arachidonic acid (100 μM) did not significantly change the latency and the maximal slope of rapid depolarization. After the reintroduction of oxygen and glucose, however, both para-BPB and arachidonic acid restored the membrane potential either partially or completely to the preexposure level in a concentration-dependent manner (Fig. 1, A and B, and Table 1). The membrane 30 min after the reintroduction significantly repolarized in the presence of para-BPB (10–100 μM) or arachidonic acid (100 μM) in comparison with the absence of the drugs (Fig. 1C and Table...
of para-precipitate; we therefore administered 17-ODA at concentra-
significantly change the latency and the maximal slope of rapid
after the reintroduction of oxygen and glucose. Each column shows the mean
A
not change the membrane potential. Figure 2
vitro ischemia in the absence and presence of 17-ODA (10

by PLA 2 activation thus contributed to the generation of the
irreversible depolarization (cf. Fig. 6).

Effects of inhibitors for LOX, COX, and cytochrome P-450
on the irreversible depolarization produced by in vitro ischemia

We next examined effects of enzymes for arachidonic acid
metabolism on the irreversible depolarization produced by in
vitro ischemia. Solutions containing the cytochrome P-450
inhibitor, 17-ODA, at concentrations >10 μM produced a fine
precipitate; we therefore administered 17-ODA at concentra-
tions ≤10 μM. The administration of 17-ODA (1–10 μM) did
not change the membrane potential. Figure 2A shows the
typical potential changes in CA1 neurons during and after in
vitro ischemia in the absence and presence of 17-ODA (10
μM). The pretreatment of the slices with 17-ODA did not
significantly change the latency and the maximal slope of rapid
depolarization. After the reintroduction, 17-ODA restored the
membrane potential either partially (8 of 16 neurons) or com-
pletely (2 neurons) to the preexposure level in a concentration-
dependent manner (Fig. 2B). The membrane significantly re-
polarized toward the preexposure level in the presence of
17-ODA (10 μM) 30 min after reintroduction in comparison to
the control condition (Fig. 2C, Table 1). On the other hand,
LOX or COX inhibitors showed a tendency to restore the
membrane potential toward the preexposure level. As shown in
Fig. 3A, three of nine neurons showed either a partial or
complete recovery in the presence of a broad LOX inhibitor,
NDGA (100 μM). Four of nine neurons showed a partial or
complete recovery in the presence of a 5- and 12-LOX inhib-
itor, 3,4-dihydroxyphenyl ethanol (20 μM). Three of 10 neu-
rons showed either a partial or complete recovery in the pres-
ence of COX2 inhibitor, Dup-697 (5 μM). Two of 11 neurons
showed a partial recovery in the presence of nonselective COX
inhibitor, indomethacin (100 μM). One of eight neurons
showed a partial recovery in the presence of COX1 inhibitor,
resveratrol (100 μM). Two of eight neurons showed a partial
recovery in the presence of a xanthine oxidase inhibitor, allo-
purinol (300 μM). Nevertheless, the membrane potential 30
min after the reintroduction of oxygen and glucose did not
show a significant restoration to the preexposure level in the
presence of NDGA (100 μM), 3,4-dihydroxyphenyl ethanol
(20 μM), Dup-697 (5 μM), indomethacin (100 μM), resvera-

trol (100 μM), or allopurinol (300 μM; Fig. 3B, Table 1). The
pretreatment of the slices with LOX, COX, or xanthine oxidase
inhibitors did not significantly change the latency and the
maximal slope of rapid depolarization. In addition, the admin-

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TABLE 1. Effects of the inhibitors of the arachidonic acid cascade and scavengers of free radicals on the recovery potential after in vitro ischemia

<table>
<thead>
<tr>
<th>Enzyme Blocker</th>
<th>Recovery Potential, mV</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>−3.0 ± 4.4 (96)</td>
</tr>
<tr>
<td>para-BBP 3 µM</td>
<td>−23.7 ± 31.1 (8)</td>
</tr>
<tr>
<td>10 µM</td>
<td>−44.5 ± 31.4 (14)**</td>
</tr>
<tr>
<td>100 µM</td>
<td>−47.4 ± 22.0 (9)**</td>
</tr>
<tr>
<td>Aristolochic acid 50 µM</td>
<td>−7.5 ± 9.8 (7)</td>
</tr>
<tr>
<td>100 µM</td>
<td>−27.0 ± 19.3 (16)*</td>
</tr>
<tr>
<td>17-ODA 1 µM</td>
<td>−11.3 ± 6.9 (9)</td>
</tr>
<tr>
<td>3 µM</td>
<td>−18.8 ± 14.3 (10)</td>
</tr>
<tr>
<td>10 µM</td>
<td>−26.3 ± 24.2 (16)*</td>
</tr>
<tr>
<td>NDGA 100 µM</td>
<td>−22.5 ± 24.6 (9)</td>
</tr>
<tr>
<td>3,4-Dihydroxyphenyl ethanol 20 µM</td>
<td>−21.8 ± 27.4 (9)</td>
</tr>
<tr>
<td>Indomethacin 100 µM</td>
<td>−6.3 ± 13.3 (11)</td>
</tr>
<tr>
<td>Resveratrol   100 µM</td>
<td>−6.6 ± 12.8 (8)</td>
</tr>
<tr>
<td>Dup-697 5 µM</td>
<td>−19.7 ± 33.0 (10)</td>
</tr>
<tr>
<td>Allopurinol   300 µM</td>
<td>−10.1 ± 14.4 (8)</td>
</tr>
<tr>
<td>Edaravone     100 µM</td>
<td>−14.0 ± 21.0 (7)</td>
</tr>
<tr>
<td>300 µM</td>
<td>−27.6 ± 21.7 (14)*</td>
</tr>
<tr>
<td>α-Tocopherol 10 µM</td>
<td>−15.1 ± 18.5 (8)</td>
</tr>
<tr>
<td>50 µM</td>
<td>−32.5 ± 26.4 (16)**</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD with values of n shown in parentheses. * P < 0.05. ** P < 0.01, one-way ANOVA with Scheffé post hoc comparisons. para-BBP, para-bromophenacyl bromide; PLA2, phospholipase A2; 17-ODA, 17-octadecynoic acid; NDGA, nordihydroguaiaretic acid; LOX, lipooxygenase; COX, cyclooxygenase.

Effects of free radical scavengers on the irreversible depolarization produced by in vitro ischemia

Cytochrome P-450, LOX, and COX produce oxygen radicals during the arachidonic acid metabolism. To elucidate the effects of oxygen radicals on the irreversible depolarization, free radical scavengers, edaravone and α-tocopherol, were administered. Figure 5A shows the typical potential changes in CA1 neurons during and after in vitro ischemia in the presence and absence of edaravone or α-tocopherol. The pretreatment of the slices with either edaravone (300 µM) or α-tocopherol (50 µM) did not significantly change the latency and the maximal slope of rapid depolarization. After the reintroduction, edaravone and α-tocopherol restored the membrane potential either partially or completely to the preexposure level in a concentration-dependent manner (Fig. 5A and B). The membrane 30 min after the reintroduction was significantly repolarized in the presence of edaravone or α-tocopherol in comparison with the control condition (Fig. 5C, Table 1). From these results, it is concluded that the oxygen radicals generated from arachidonic acid metabolism via cytochrome P-450 isoforms produce the irreversible depolarization following in vitro ischemia (cf. Fig. 6).

DISCUSSION

In rat hippocampal CA1 neurons, PLA2 inhibitors, para-BBP and aristolochic acid, significantly restored the membrane potential after the in vitro ischemia toward the preexposure level in the majority of neurons tested. A cytochrome P-450 isoform inhibitor, 17-ODA, also significantly restored the membrane potential in the majority of neurons tested. On the other hand, arachidonic acid metabolites (14,15-EET and 20-HETE) via cytochrome P-450 isoforms did not either accelerate or inhibit the generation of the rapid depolarization and the irreversible depolarization after in vitro ischemia. These results indicate that the reaction mediated by cytochrome P-450 isoforms contributes to the generation of the irreversible depolarization (Fig. 6). The free radical scavengers, α-tocopherol and edaravone, significantly restored the membrane to the preexposure level. This result indicates that the oxygen radicals produced by metabolism of arachidonic acid via cytochrome P-450 isoforms are responsible for the generation of the irreversible depolarization induced by in vitro ischemia.

Effects of PLA2 inhibitors on the irreversible depolarization after the reintroduction

The present results show that PLA2 inhibitors, such as para-BBP and aristolochic acid, provide protection against the generation of the irreversible depolarization, and their action is comparable to previous reports that reported that in a hippocampal slice culture, para-BBP reduces the propidium iodide uptake after oxygen and glucose deprivation (Arai et al. 2001a). The aristolochic acid partially restored the membrane toward the preexposure level after the reintroduction of oxygen.
and glucose. Because IC₅₀ value of the aristolochic acid for PLA₂ activity in human neutrophil is 40 μM (Rosenthal et al. 1989), the concentration (100 μM) used for the hippocampal slices in the present study seems to be reasonable. A previous report showed the intracellular Ca²⁺ concentration to be markedly elevated at the generation of the rapid depolarization (Tanaka et al. 1997). Both the ionotropic-Glu-receptor-mediated Ca²⁺ influx and the Ca²⁺-induced Ca²⁺ release result in the generation of the persistent depolarization (Yamamoto et al. 1997). Moreover, the cell membrane of the CA1 neuron may be irreversibly damaged during the persistent depolarization because blebs appear on the cell body of the CA1 pyramidal neuron 1 min after starting the reintroduction of oxygen and glucose, and the cell body becomes swollen 3 min after that (Tanaka et al. 1999). Arai et al. (2001a) has also shown that arachidonyl trifluoromethyl keton, a Ca²⁺-dependent PLA₂ inhibitor, reduces neuronal cell death in a hippocampal slice culture after oxygen and glucose deprivation. It is therefore possible that the redistributed arachidonic acid induces the accumulation of Glu in the interstitial space, which may produce a further increase in [Ca²⁺], and augment the neuronal damage. In fact, we previously reported that in rat hippocampal CA1 neurons, the ionotropic Glu receptor antagonists, 2-amino-5-phosphonopentanoic acid (AP5) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), restore the membrane to the preexposure level after ischemic exposure (Yamamoto et al. 1997).

Effects of cytochrome P-450 isozymes on the irreversible depolarization after the reintroduction of oxygen and glucose

The administration of 14,15-EET or 20-HETE (1 μM) did not alter the membrane potential before the ischemic expo-
sure and the latency of the rapid depolarization during the ischemic exposure, thus suggesting that these products catalyzed by cytochrome P-450 isozymes are not cytotoxic and did not accelerate the generation of the rapid depolarization. On the other hand, a cytochrome P-450 isozyme inhibitor, 17-ODA (10 μM), significantly restored the membrane potential toward the preexposure level. In the brain or liver, the metabolism of various substrates via cytochrome P-450 isozymes leads to the formation of oxygen radicals during the production of metabolites, such as 14,15-EET or 20-HETE (Barth et al. 1998; Mattia et al. 1993; Montoliu et al. 1994; Thompson et al. 2000). These results indicate that the oxygen radicals produced by arachidonic acid metabolism via cytochrome P-450 isozymes contribute to the generation of the irreversible depolarization after in vitro ischemia. Cytochrome P-450 isozymes are found in astrocytes and neurons, including hippocampal pyramidal neurons, in the rat, mouse, and human brains (Bylund et al. 2002; Roman 2002). It is therefore possible that the oxygen radicals produced in both astrocytes and CA1 neurons after in vitro ischemia result in the generation of the irreversible depolarization of the CA1 neurons. The 17-ODA concentration (10 μM) used in this study is comparable with the IC50 for ω-hydroxylation (0.1–7 μM) and epoxidation (0.1–5 μM).

FIG. 4. The effects of the major products of cytochrome P-450 isozymes on the potential changes produced by in vitro ischemia in rat hippocampal CA1 neurons. A: the effects of 20-hydroxyecosatetraenoic acid (20-HETE), a major product of ω-hydroxylases activity, and 14,15-epoxyeicosatrienoic acid (14,15-EET), a major product of epoxygenases activity, on the percent of neurons exhibiting recovery. In, I, and H, no, partial, and complete recovery, respectively. B: the effects of 20-HETE and 14,15-EET on the potential recovery 30 min after the reintroduction of oxygen and glucose.
via cytochrome P-450 isozymes on the rat renal function (Wang et al. 1998; Zou et al. 1994).

**Effects of free radical scavengers on the irreversible depolarization after the reintroduction**

There is ample evidence that the arachidonic acid product catalyzed by cytochrome P-450 isozymes leads to the formation of superoxyl radical in the liver and coronal artery (Bondy and Naderi 1994; Fleming et al. 2001; Puntarulo and Cederbaum 1998). Arachidonic acid is also metabolized by COX (and LOX) and thus yields eicosanoids, and a superoxyl radical is thus formed during this process (Kukreja et al. 1986). α-Tocopherol has been shown to be a scavenger of lipoperoxyl radical, superoxide, and hydroxyl radical in many mammalian organs (Burton and Ingold 1986; Fukazawa and Gebicki 1983; Nishikimi et al. 1980; Zalkin and Tappel 1960). The present study showed that the free radical scavengers, α-tocopherol (50 μM) and edaravone (300 μM), restored the membrane after the reintroduction toward the preexposure level in a concentration-dependent manner. In the rat brain homogenate, oxygen radicals produce lipid peroxidation 30 min after incubation at 37°C; the peroxidation is blocked by edaravone with IC₅₀ of 15 μM (Tanaka 2002). It is therefore possible that oxygen radicals, such as superoxyl and hydroxyl radical, mediate the irreversible depolarization after the reintroduction. Our previous report showed that in rat hippocampal CA1 neurons, the inhibitor for nitric oxide (NO) synthase and the NO scavenger restore the membrane toward the preischemic potential level after in vitro ischemia (Onitsuka et al. 1998). Because NO can react with superoxyl radical to yield peroxynitrate, which decomposes and produces a highly toxic free radical, hydroxyl radical, it is possible that NO and oxygen radicals are responsible for the generation of the irreversible depolarization after the reintroduction. It is also possible that the free radicals, including peroxynitrate and hydroxyl radical, may damage the cytoskeleton and/or induce peroxidation of the membrane lipid because large blebs and cell swelling are observed in rat CA1 pyramidal neurons during the persistent depolarization (Tanaka et al. 1999).

**Effects of LOX and COX inhibitors on the irreversible depolarization after the reintroduction**

In vivo study shows that in all forebrain regions of the gerbil, neurons exhibit dense 5-LOX immunoreactivity and that the transient occlusion of common carotid arteries induces an increase in production of leukotriene C₄, the metabolite from arachidonic acid by 5-LOX, after the reperfusion (Ohtsuki et al. 1995). A 5-LOX inhibitor reduces the neuronal death of gerbil hippocampal CA1 region after 6 days from the transient carotid arteries occlusion (Rao et al. 1999). The present results demonstrated that potent LOX isozyme inhibitors, NDGA (100 μM) and 3,4-dihydroxyphenyl ethanol (20...
In vitro ischemia induces an accumulation of extracellular Glu to activate ionotropic glutamate receptors [N-methyl-D-aspartate (NMDA)- and/or AMPA/kainate-type receptors]. The activation of ionotropic Glu receptors induces a depolarization, which increases permeability of Ca$^{2+}$ at the NMDA-type receptor and voltage-gated Ca$^{2+}$ channels. These mechanisms increase in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) and trigger the Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) from the intracellular Ca$^{2+}$ store sites (e.g., endoplasmic reticulum). Elevated [Ca$^{2+}$], triggers the activation of PLA$_2$ and nitric oxide (NO) synthase to produce arachidonic acid and NO, respectively. Cytochrome P-450 mainly metabolizes arachidonic acid to produce oxygen radicals. NO reacts with oxygen radicals and yields peroxynitrite (ONOO$^-\cdot$). ONOO$^-\cdot$ and/or oxygen radicals damage the cytoskeleton and induce peroxidation of the membrane lipid, thus causing the irreversible depolarization. Thin solid arrows: putative routes for intracellular signal transduction systems. Thick solid arrows: putative routes for intracellular signal transduction systems, which contribute relatively little to the generation of the irreversible depolarization.

\[ \text{increase in [Ca$^{2+}$]} \]

**Irreversible depolarization**

\[ \text{Ca$^{2+}$ store sites} \]

\[ \text{CiCR} \]

\[ \text{ATP} \]

\[ \text{Hypoxanthine} \]

\[ \text{Xanthine oxidase} \]

\[ \text{Cytochrome P-450} \]

\[ \text{Arachidonic acid} \]

\[ \text{LOX} \]

\[ \text{COX1} \]

\[ \text{COX2} \]

\[ \text{PLA}_2 \]

\[ \text{Oxygen radicals} \]

\[ \text{Xanthine} \]

\[ \text{HETE} \]

\[ \text{HPETE} \]

\[ \text{Prostaglandin} \]

\[ \text{Phospholipids} \]

\[ \text{CO$^-\cdot$} \]

\[ \text{NO$^-\cdot$} \]

\[ \text{NO synthase} \]

\[ \text{NO} \]

\[ \text{ONOO$^-\cdot$} \]

**FIG. 6.** Putative mechanisms contribute to the generation of the irreversible depolarization induced by in vitro ischemia. In vitro ischemia induces an accumulation of extracellular Glu to activate ionotropic glutamate receptors [N-methyl-D-aspartate (NMDA)- and/or AMPA/kainate-type receptors]. The activation of ionotropic Glu receptors induces a depolarization, which increases permeability of Ca$^{2+}$ at the NMDA-type receptor and voltage-gated Ca$^{2+}$ channels. These mechanisms increase in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) and trigger the Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) from the intracellular Ca$^{2+}$ store sites (e.g., endoplasmic reticulum). Elevated [Ca$^{2+}$], triggers the activation of PLA$_2$ and nitric oxide (NO) synthase to produce arachidonic acid and NO, respectively. Cytochrome P-450 mainly metabolizes arachidonic acid to produce oxygen radicals. NO reacts with oxygen radicals and yields peroxynitrite (ONOO$^-\cdot$). ONOO$^-\cdot$ and/or oxygen radicals damage the cytoskeleton and induce peroxidation of the membrane lipid, thus causing the irreversible depolarization. Thin solid arrows: putative routes for intracellular signal transduction systems. Thick solid arrows: putative routes for intracellular signal transduction systems, which contribute relatively little to the generation of the irreversible depolarization.

In the present study, DuP-697 (5 $\mu$M), a relatively selective COX-2 inhibitor, restored completely the membrane to preexposure level after the reintroduction in the minority of neurons tested. DuP-697 is 50-fold more selective for COX-2 than for COX-1: the IC$_{50}$ value for COX-2 is 7 nM and that for COX-1 is 260 nM in inflamed kidney (Kargman et al. 1996; Seibert et al. 1996). It is therefore possible that the activation of COX-2 may contribute in some way to the irreversible depolarization induced by in vitro ischemia. On the other hand, potent COX-1 inhibitors, resveratrol (100 $\mu$M) and indomethacin (100 $\mu$M), only partially restored the membrane toward the preexposure level. The ED$_{50}$ value of resveratrol for COX-1 (ability to inhibit the COX-1 activity) is 15 $\mu$M on cancer chemopreventive activity (Jang et al. 1997). The IC$_{50}$ value of indomethacin for COX-1 is 13 $\mu$M, whereas that for COX-2 is >1 mM on human COXs expressed in COS-1 cells (Laneuville et al. 1993). These previous findings support the idea that the activation of COX-2 rather than COX-1 may be involved in the generation of the irreversible depolarization induced by in vitro ischemia. The present results are comparable to those described in previous reports in which several COX-2 inhibitors where shown to protect the neurons against neuronal death after the transient in vivo ischemia in gerbil or rat (Candelario-Jalil et al. 2002; Govoni et al. 2001; Hara et al. 1998; Nogawa et al. 1997). In the present study, a xanthine oxidase inhibitor, allopurinol (300 $\mu$M), only partially restored the membrane toward the COX-2 mRNA and immunoreactivity (Miettinen et al. 1997; Ohtsuki et al. 1996). In the present study, DuP-697 (5 $\mu$M), a relatively selective COX-2 inhibitor, restored completely the membrane to preexposure level after the reintroduction in the minority of neurons tested. DuP-697 is 50-fold more selective for COX-2 than for COX-1: the IC$_{50}$ value for COX-2 is 7 nM and that for COX-1 is 260–500 nM in inflamed kidney (Kargman et al. 1996; Seibert et al. 1996). It is therefore possible that the activation of COX-2 may contribute in some way to the irreversible depolarization induced by in vitro ischemia. On the other hand, potent COX-1 inhibitors, resveratrol (100 $\mu$M) and indomethacin (100 $\mu$M), only partially restored the membrane toward the preexposure level. The ED$_{50}$ value of resveratrol for COX-1 (ability to inhibit the COX-1 activity) is 15 $\mu$M on cancer chemopreventive activity (Jang et al. 1997). The IC$_{50}$ value of indomethacin for COX-1 is 13 $\mu$M, whereas that for COX-2 is >1 mM on human COXs expressed in COS-1 cells (Laneuville et al. 1993). These previous findings support the idea that the activation of COX-2 rather than COX-1 may be involved in the generation of the irreversible depolarization induced by in vitro ischemia. The present results are comparable to those described in previous reports in which several COX-2 inhibitors where shown to protect the neurons against neuronal death after the transient in vivo ischemia in gerbil or rat (Candelario-Jalil et al. 2002; Govoni et al. 2001; Hara et al. 1998; Nogawa et al. 1997). In the present study, a xanthine oxidase inhibitor, allopurinol (300 $\mu$M), only partially restored the membrane toward the COX-2 mRNA and immunoreactivity (Miettinen et al. 1997; Ohtsuki et al. 1996). In the present study, DuP-697 (5 $\mu$M), a relatively selective COX-2 inhibitor, restored completely the membrane to preexposure level after the reintroduction in the minority of neurons tested. DuP-697 is 50-fold more selective for COX-2 than for COX-1: the IC$_{50}$ value for COX-2 is 7 nM and that for COX-1 is 260–500 nM in inflamed kidney (Kargman et al. 1996; Seibert et al. 1996). It is therefore possible that the activation of COX-2 may contribute in some way to the irreversible depolarization induced by in vitro ischemia. On the other hand, potent COX-1 inhibitors, resveratrol (100 $\mu$M) and indomethacin (100 $\mu$M), only partially restored the membrane toward the preexposure level. The ED$_{50}$ value of resveratrol for COX-1 (ability to inhibit the COX-1 activity) is 15 $\mu$M on cancer chemopreventive activity (Jang et al. 1997). The IC$_{50}$ value of indomethacin for COX-1 is 13 $\mu$M, whereas that for COX-2 is >1 mM on human COXs expressed in COS-1 cells (Laneuville et al. 1993). These previous findings support the idea that the activation of COX-2 rather than COX-1 may be involved in the generation of the irreversible depolarization induced by in vitro ischemia. The present results are comparable to those described in previous reports in which several COX-2 inhibitors where shown to protect the neurons against neuronal death after the transient in vivo ischemia in gerbil or rat (Candelario-Jalil et al. 2002; Govoni et al. 2001; Hara et al. 1998; Nogawa et al. 1997).
preexposure level in a few neurons. The Ki value of allopurinol for xanthine oxidase is 0.2–0.7 μM on the mouse liver (Elion 1966). Therefore the allopurinol concentration used in this study is high enough to inhibit xanthine oxidase. The contribution of xanthine oxidase to the generation of the irreversible depolarization induced by in vitro ischemia may, if present, be minimal. Based on the findings of all the above-mentioned results, it is likely that the LOX isozymes and the COX-2 may contribute in some extent to the generation of the irreversible depolarization induced by in vitro ischemia (Fig. 6).

Pathophysiological relevance

In our previous reports, changes in the membrane potential produced in CA1 neurons by in vitro ischemia are a mirror image of the DC potential changes produced by in situ ischemia (e.g., cardiac arrest) or asphyxia in the rat brain cortex (Hansen 1978, 1985; Tanaka et al. 1997, 1999; Yamamoto et al. 1997). The rapid depolarization induced by in vitro ischemia corresponds to the terminal depolarization (or phase II depolarization) produced by in situ ischemia or asphyxia. Simultaneous recordings of both intra- and extracellular recordings from rat hippocampal CA1 region have revealed that the rapid depolarization simultaneously occurs with the negativgoing DC potential, which resembles the spreading depression in the cerebral cortex observed during in vivo ischemia (Hansen 1985; Leão 1947; Rader and Lanthorn 1989; Uchikado et al. 2000). Moreover, the cell swelling and no functional recovery of the membrane in rat hippocampal CA1 neurons 3 min after the reintroduction of oxygen and glucose indicate that the process leading to the irreversible depolarization after ischemic exposure is a necrotic event (Tanaka et al. 1999). These morphological changes are similar to so-called severe cell change, which has long been recognized as the response of the neuron to hypoxia (Brierley and Graham 1984; Brown and Brierley 1966). However, 10–30 min ischemia of forebrain in vivo produced by four-vessel occlusion (permanent occlusion of vertebral arteries and transient occlusion of common carotid arteries) induces ischemic neuronal damage in only a small number of hippocampal neurons 3 h after reperfusion and in most of the neurons 72 h after (delayed neuronal damage) (Pulsinelli and Brierley 1979; Pulsinelli et al. 1982). Therefore in vitro ischemia in the present study would be a relatively strong ischemic condition in comparison to the four-vessel occlusion model in vivo.

After this strong ischemic condition, the activation of PLA2 contributes to the generation of the irreversible depolarization. The activation of PLA2 may produce arachidonic acid, which would be mainly metabolized by cytochrome P-450 isozymes. The production of oxygen radicals from arachidonic acid via cytochrome P-450 isozymes may thus contribute to the generation of the irreversible depolarization after the reintroduction of oxygen and glucose. In addition, LOX isozymes and the COX-2 may also play some role in the onset of the irreversible depolarization. Because the expression of COX-2 is induced in the rat cortex and hippocampus after spreading depression and transient focal ischemia in vivo (Miettinen et al. 1997), the COX-2 may play an important role in the delayed neuronal damage observed after in vivo ischemia.

DISCLOSURES

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