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The effects of local anesthetics on cellular hypoxia-induced gene responses mediated by hypoxia-inducible factor 1

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Abstract
Purpose. Hypoxia (reduced oxygen availability) induces a series of adaptive physiological responses. At the cellular level, the adaptation includes a switch of energy metabolism from oxidative phosphorylation to anaerobic glycolysis, increased glucose uptake, and the expression of stress proteins related to cell survival. One of the most important transcription factors that activate the expression of oxygen-regulated genes is hypoxia-inducible factor 1 (HIF-1). We previously reported that halothane inhibits the hypoxia-induced HIF-1 activation. In this study, we investigated the effect of local anesthetics on HIF-1 activation and its downstream gene expression.
Methods. The established cell line Hep3B and SK-N-MC cells were exposed to 1% O_2 with or without treatment by either lidocaine or bupivacaine. Expression of subunits of HIF-1, HIF-1α, and HIF-1β was examined by Western blot using specific antibodies. Expression of mRNA of HIF-1 and the HIF-1-dependent genes was investigated by RT-PCR and reporter assay.
Results. Neither of the local anesthetics tested affected the accumulation of HIF-1α induced by hypoxia, nor did they affect NOC18-induced HIF-1α accumulation. Moreover, they had no effects on HIF-1-mediated hypoxia-induced gene expression.
Conclusion. The local anesthetics lidocaine and bupivacaine did not affect the HIF-1-dependent cellular hypoxia-induced gene responses.

Key words Local anesthetics · Hypoxia responses · Hypoxia-inducible factor 1

Introduction

It is generally accepted that local anesthetics exert their anesthetic effects by inhibiting voltage-gated Na^+ channels [1], but they also affect various other ion channels and receptors [2]. In addition, it is appreciated that local anesthetics affect intracellular signal transduction pathways by modulating signaling intermediate molecules, including tyrosine kinases and mitogen-activated protein kinases [3].

Hypoxia induces a series of adaptive physiological responses [4]. At the cellular level, adaptation involves a switch of energy metabolism from oxidative phosphorylation to anaerobic glycolysis and increased glucose uptake. At the molecular level, the adaptation involves changes in expression of glucose transporters, glycolytic enzymes, stress proteins related to cell survival or death, and proangiogenic factors. One of the most important transcription factors that activate the expression of oxygen-regulated genes is hypoxia-inducible factor 1 (HIF-1) [5]. HIF-1 is a heterodimer composed of a constitutively expressed β-subunit (HIF-1β) and an inducibly expressed α-subunit (HIF-1α) [6].

A possible mechanism for drug-induced long-term modification of cellular functions including gene response to hypoxia is based on gene expression changes induced by the drugs. We have reported that the volatile anesthetic halothane inhibits the hypoxia-induced activation of HIF-1 in Hep3B cells in clinically relevant doses [7]. These results prompted us to investigate the effects of the local anesthetics lidocaine and bupivacaine on critical physiological responses using established cell lines derived from hepotocytes and neuronal cells. We demonstrated that neither lidocaine nor bupivacaine enhances or constitutively inhibits basal or hypoxia-inducible HIF-1 activity in Hep3B cells derived from human hepatocellular carcinoma and SH-N-MC cells with neuronal cell properties.

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Materials and methods

Cell culture and reagents

Hep3B cells derived from human hepatocellular carcinoma were maintained in minimum essential medium (MEM) with Earl's salts supplemented with 10% fetal bovine serum (FBS), essential amino acids, pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin [7]. SH-N-MC cells were maintained in Dulbecco's Eagle medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. MEM and DMEM contain phenol red as a pH indicator. pH of culture media was kept constant around 7.4. The iron chelator desferrioxamine (DFX) was obtained from Sigma (St. Louis, MO, USA). The local anesthetics lidocaine and bupivacaine were obtained from Sigma Chemical in powder form. The spontaneous nitric oxide (NO) releaser NOC18 was obtained from Dojindo (Kumamoto, Japan).

Immunoblot assays

Whole cell lysates were prepared following a protocol described previously [7–9]. In brief, lysates were prepared with ice-cold lysis buffer [0.1% sodium dodecyl sulfate (SDS), 1% NP40, 5 mM ethylene diamine tetraacetic acid (EDTA), 150 mM NaCl, 50 mM Tris-Cl at pH 8.0, 1 mM sodium orthovanadate, and complete protease inhibitor (Roche Applied Science, Tokyo, Japan)]. Samples were centrifuged at 10 000 *g* to pellet cell debris. Then, 100-μg aliquots were fractionated by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot assay using mouse monoclonal antibody against HIF-1α (BD Biosciences, San Jose, CA, USA) or HIF-1β (BD Biosciences) at 1 : 1000 dilution. As a secondary antibody, horseradish peroxidase-conjugated mouse monoclonal antibodies for mouse IgG (1 : 1000 dilution) were used (Amersham Biosciences, Piscataway, NJ, USA). Signal was developed using ECL reagent (Amersham Biosciences).

Hypoxic treatment and administration of the local anesthetics

Tissue culture dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) which was flushed with 1% O_2–5% CO_2-94%N_2, sealed, and placed at 37°C [7–9]. The local anesthetics are dissolved in MEM or DMEM and added into the culture media.

Reverse transcriptase-polymerase chain reaction

The reverse transcriptase-polymerase chain reaction (RT-PCR) protocol is described elsewhere [9,10]. Cells were harvested, and total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was subjected to first-strand cDNA synthesis using random hexamers (SuperScript II RT kit; Invitrogen). cDNAs were amplified with TaqGold polymerase (Roche, Mannheim, Germany) in a thermal cycler with the following primers: HIF-1α, GAAAGCGCAAGTCCTCAAA and CTATATGGTGATGATGTGGCACTA; vascular endothelial growth factor (VEGF), CCATGAACTTTCTGCTGTCTT and ATCGCATCAGGGGCACACAG; and 18S ribosomal RNA (rRNA), ATCCTGCCAGTAGCATATGC and ACCCGGGTTGGTTTTGATCTG. For each primer pair, PCR was optimized for cycle number to obtain linearity between the amount of input RT product and output PCR product. Thermocycling conditions were 30 s at 94°C, 60 s at 57°C, and 30 s at 72°C for 25 (VEGF), 25 (HIF-1α), or 20 (18S rRNA) cycles preceded by 10 min at 94°C. PCR products were fractionated by 3% Nusieve agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV.

Reporter gene assay

Reporter plasmid p2.1, harboring a 68-bp hypoxia response element (HRE) from the human enolase 1 (ENO1) gene inserted upstream of an SV40 promoter and *Photinus pyralis* (firefly) luciferase coding sequences, was described previously [9]. Reporter assays were performed in Hep3B cells; 5×10^4 cells were plated per well on the day before transfection. In each transfection, 200 ng of reporter gene plasmid p2.1, and 50 ng of the control plasmid pRL-SV40 (Promega, Madison, WI, USA), containing a SV40 promoter upstream of *Renilla reniformis* (sea pansy) luciferase coding sequences, were premixed with Fugene 6 transfection reagent (Roche). Cells were treated with the reagents for 6 h and incubated under 20% or 1% O_2 conditions for another 18 h. The cells were harvested and the luciferase activity was then determined using the Dual-Luciferase Reporter Assay System (Promega). The ratio of firefly to sea pansy luciferase activity was then determined. For each experiment, at least two independent transfections were performed in triplicate. The data shown are representative of those.

Results

Impact of the local anesthetics on hypoxia-induced accumulation of HIF-1α in Hep3B cells

To study the effect of the local anesthetics on HIF-1 activation induced by hypoxia, we tested two different types of the local anesthetics lidocaine and bupivacaine. Intracellular protein expression of the regulatory subunit of HIF-1, HIF-1α, was examined by Western blot-

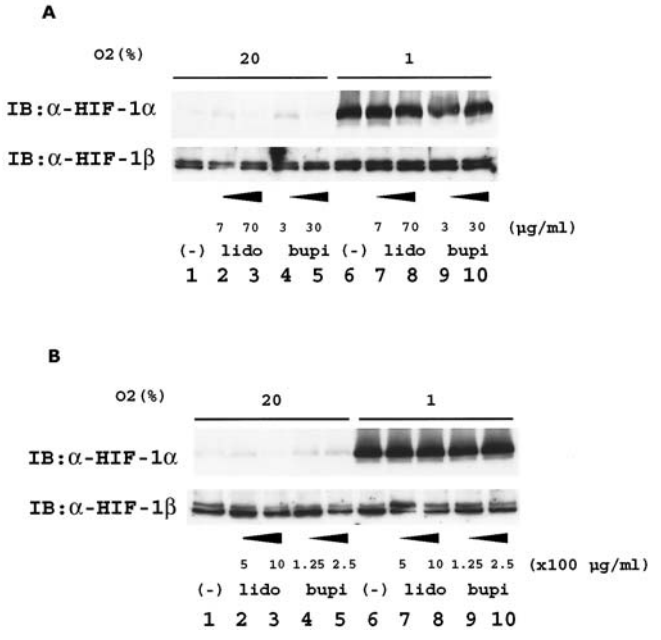

Fig. 1. Effects of lidocaine (*lido*) or bupivacaine (*bupi*) on hypoxia-induced hypoxia-inducible factor-1α (*HIF-1α*) accumulation in Hep3B cells. Hep3B cells were treated with lidocaine or bupivacaine at the doses indicated at 20% (*lanes 2–5*) or 1% O_2 conditions (*lanes 7–10*) for 4 h and harvested for immunoblot assays using anti-HIF-1α (*top panel*) and anti-HIF-1β (*bottom panel*) antibodies. **A** Doses of systemic intoxication; **B** doses of suppression of neuronal activity

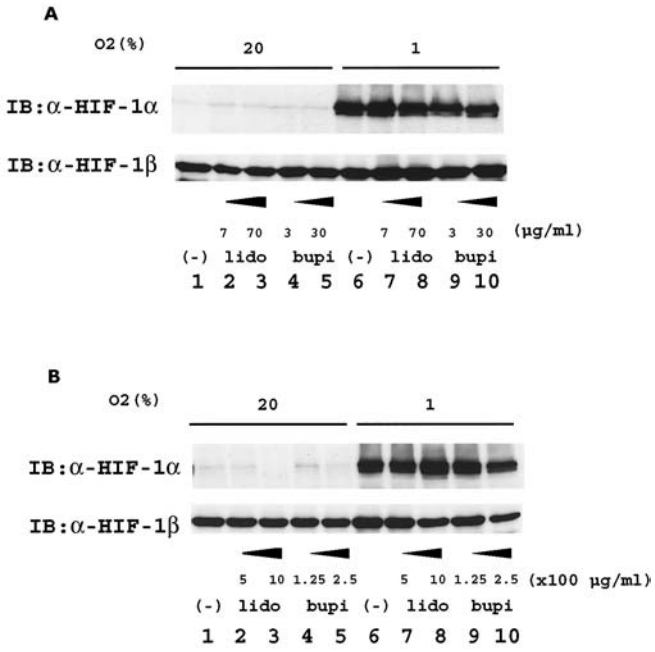

Fig. 2. Effects of lidocaine or bupivacaine on hypoxia-induced HIF-1α accumulation in SH-N-MC cells. SH-N-MC cells were treated with lidocaine or bupivacaine at the doses indicated at 20% (*lanes 2–5*) or 1% O_2 conditions (*lanes 7–10*) for 4 h and harvested for immunoblot assays using anti-HIF-1α (*top panel*) and anti-HIF-1β (*bottom panel*) antibodies. **A** Doses of systemic intoxication; **B** doses of suppression of neuronal activity

ting. Hep3B cells were exposed to 1% O_2 conditions with or without treatment by either anesthetic for 4 h, harvested, and subjected to immunoblot analysis using anti-HIF-1α (Fig. 1A, top panel) or HIF-1β (bottom panel) antibody.

First, we tested systemic toxic doses (lidocaine, 7 or 70 μg/ml; bupivacaine, 3 or 30 μg/ml). HIF-1α protein was barely expressed under 20% O_2 conditions (lane 1) and significantly induced in response to 1% O_2 (lane 6). Lidocaine did not affect expression of HIF-1α under 1% O_2 conditions at any doses tested. (lanes 7, 8). Bupivacaine also did not affect the expression of HIF-1α. Neither anesthetic affected expression under 20% O_2 conditions (lanes 2–5). Expression of HIF-1β was constant with or without hypoxia or local anesthetics.

Next, we tested higher doses that are comparable to doses of suppression of neuronal activity (lidocaine, 500 or 1000 μg/ml; bupivacaine, 125 μg/ml or 250 μg/ml) (Fig. 1B). Cells were alive, as judged by trypan blue exclusion methods, after 4 h of treatment. Expression of neither HIF-1α nor HIF-1β was affected by treatment with the local anesthetics. Although we also tried higher dosages of the anesthetics (10 mg/ml lidocaine and 2.5 mg/ml bupivacaine), the cells were detached from the culture dish and did not look healthy (data not shown). According to a document provided by the manufacturer, the osmotic pressure of DMEM is 340 mOsm/kg H_2O, and administration of the local anesthetics dissolved in DMEM affected the osmotic pressure by 10% at most (data not shown). During exposure to hypoxia and treatment with local anesthetics, significant color change of phenol red was not observed, suggesting that the pH of the medium did not drift significantly during the experiments.

Impact of the local anesthetics on hypoxia-induced accumulation of HIF-1α in SH-N-MC cells

To investigate the effects of the anesthetics on cells of neuronal origin, SH-N-MC cells were tested. SH-N-MC cells were exposed to 1% O_2 conditions with or without treatment with one of the anesthetics for 4 h , harvested, and subjected to immunoblot analysis using anti-HIF-1α (Fig. 2A,B, top panel) or HIF-1β (bottom panel) antibody. First, we tested systemic toxic doses (lidocaine, 7 or 70 μg/ml; bupivacaine, 3 or 30 μg/ml). The anesthetics did not affect expression of HIF-1α or HIF-1β (Fig. 2A). Next, we tested higher doses that are comparable to doses of suppression of neuronal activity (lidocaine, 500 or 1000 μg/ml; bupivacaine, 125 or

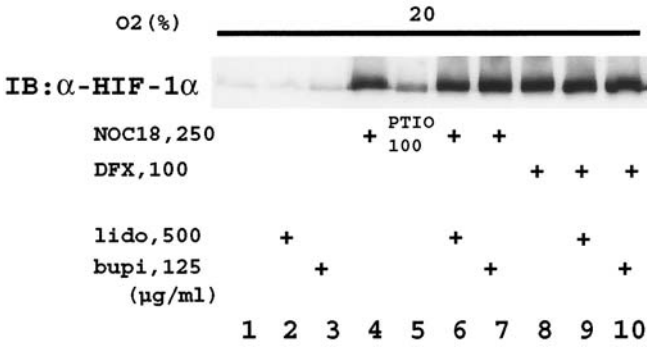

Fig. 3. Effect of lidocaine or bupivacaine on HIF-1α accumulation in Hep3B cells under normoxic conditions. Hep3B cells were exposed to *NOC18* (*lanes 4, 5, 6, 7*) or desferrioxamine (*DFX*) (*lanes 8, 9, 10*) with lidocaine (*lanes 2, 6, 9*) or bupivacaine (*lanes 3, 7, 10*) for 4 h and harvested for immunoblot assays using anti-HIF-1α

250 μg/ml). Neither anesthetic affected expression of HIF-1α induced by 1% O_2 conditions (Fig. 2B, lanes 7–10).

Impact of the local anesthetics on NO-induced accumulation of HIF-1α in Hep3B cells

We have reported that the NO donor NOC-18 significantly induces the accumulation of HIF-1α under nonhypoxic conditions via a different molecular mechanism from that of hypoxia [9]. NOC-18 stimulates translation of HIF-1α from mRNA or induces neosynthesis of HIF-1α protein so that it accumulates in the cells. On the other hand, the iron chelator DFX stabilized translated HIF-1α by inhibiting the prolyl hydroxylation of HIF-1α-like hypoxia [11]. To examine the effect of the anesthetics on HIF-1α accumulation, we investigated the impact of the local anesthetics on NO- or DFX-induced HIF-1α accumulation. As shown in Fig. 3, NOC-18 induced the accumulation of HIF-1α under 20% O_2 conditions (lane 5), and the accumulation was inhibited by the NO scavenger PTIO (lane 6). As for hypoxia-induced accumulation, neither anesthetic inhibited the NO- nor DFX-induced accumulation of HIF-1α, even in doses causing suppression of neuronal activity.

Impact of the local anesthetics on hypoxia-induced HIF-1-dependent gene expression

Next, we investigated whether the anesthetics affect gene expressions regulated by HIF-1 using RT-PCR technique in Hep3B cells (Fig. 4A). VEGF mRNA expression was induced in response to 16 h of hypoxic treatment (lane 2). Neither lidocaine nor bupivacaine affected the expression of VEGF mRNA (lanes 3, 4).

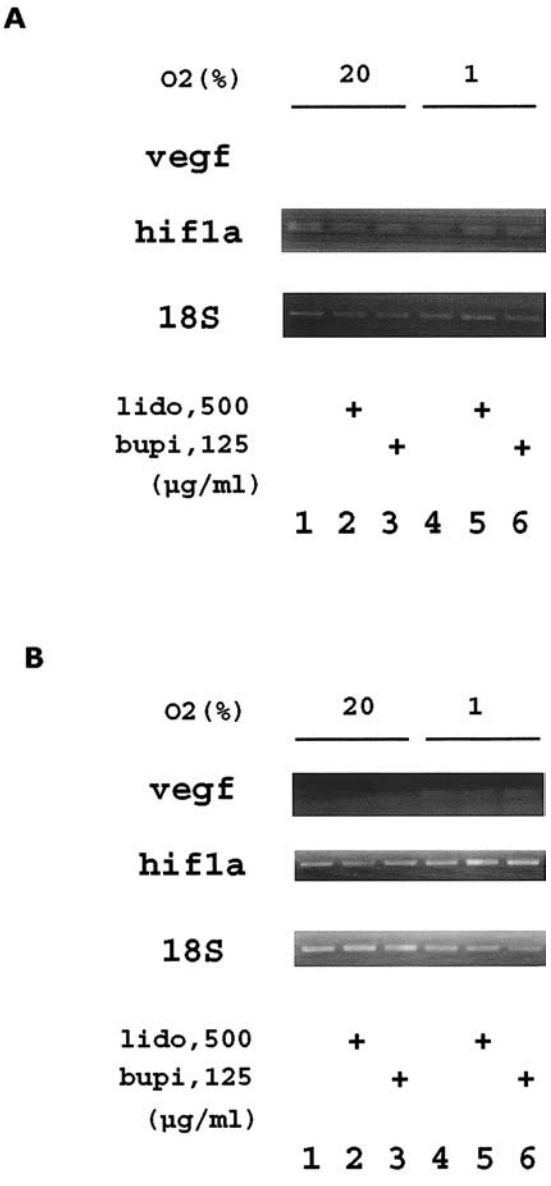

Fig. 4. Effects of lidocaine or bupivacaine on hypoxia-induced gene expression. Hep3B cells were exposed to 20% or 1% O_2 conditions in the presence or absence of each of the anesthetics for 24 h, and total RNA was isolated. Expression of vascular endothelial growth factor (*VEGF*) mRNA (*vegf*), HIF-1α mRNA (*hifla*), and 18S rRNA (*18S*) was analyzed by RT-PCR

This finding is consistent with the result of effects in HIF-1α protein expression demonstrated in Fig. 1. HIF-1α mRNA expression by itself was not affected by anesthetic treatment. No significant difference in expression of 18S rRNA was detected, indicating that the anesthetics did not affect either the reverse-transcriptase reaction or the PCR process.

Finally, Hep3B cells were transfected with the reporter p2.1 containing HIF-1-dependent HREs and

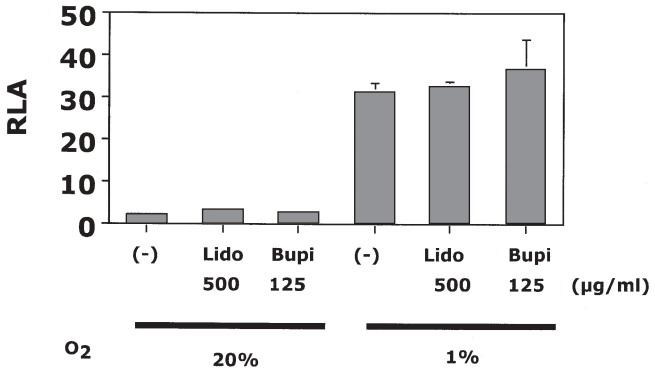

Fig. 5. Effects of lidocaine (*Lido*) or bupivacaine (*Bupi*) of hypoxia-induced HRE-dependent gene expression. Hep3B cells were transfected with pRL-SV40 (control reporter encoding *Renilla* luciferase) and p2.1 (HRE-driven reporter encoding firefly luciferase). After 6 h incubation, cells were treated with the local anesthetics under 20% (*left*) and 1% conditions (*right*) O_2 for 18 h and harvested for luciferase assays. The ratio of firefly:*Renilla* luciferase activity was determined and normalized to the value obtained from nonhypoxic untreated (−) cells to obtain relative luciferase activity (*RLA*). Results shown represent mean ± SD of three independent transfections

pSV40-RL encoding *Renilla* luciferase, treated with the local anesthetics, and then subjected to the reporter assay, which is the most sensitive assay to examine HIF-1 activation (Fig. 5). Hypoxic treatment induced HRE-dependent gene expression by about 30 fold. Neither of the local anesthetics affected hypoxia-induced HRE-dependent gene expression. Under 20% O_2 as well as under 1% O_2 conditions, neither anesthetic inhibited the activity.

Discussion

In this study, we have demonstrated, using molecular biological methods, that local anesthetics do not affect hypoxia-induced HIF-1 activation. Neither lidocaine nor bupivacaine affected the accumulation of HIF-1α protein induced by hypoxia, and neither changed the expression of the downstream genes of HIF-1. Taken together, these data clearly demonstrate that lidocaine and bupivacaine do not affect hypoxia-inducible gene responses mediated by HIF-1. Because HIF-1 plays an essential role in hypoxia-induced gene induction, our findings indicate that the cellular hypoxia-induced genetic response is not affected by local anesthetics.

Intracellular HIF-1α expression level, which is determined by the balance between protein synthesis and degradation of the protein [11], is well correlated with activity of HIF-1. Under nonhypoxic conditions, HIF-1α is so rapidly degraded by proteasome in prolyl hydroxylation and in an ubiquitination–dependent manner that HIF-1α protein barely accumulates in the cells. Under hypoxic conditions, degradation of the α-subunit is blocked, allowing HIF-1α to accumulate within the nucleus where, upon binding to HIF-1β, it recognizes HREs within the promoters of hypoxia-responsive target genes [12]. Degradation of HIF-1α under normoxic conditions is triggered by post-translational hydroxylation of the conserved proline residues [11]. Our data provide evidence that local anesthetics do not affect the processes. In good contrast to hypoxia, NOC18 induces the accumulation of HIF-1α by enhancing neosynthesis of the protein from mRNA [9]. Our results show that local anesthetics do not influence the translational process of HIF-1α.

In this study, Hep3B cells and SK-N-MC cells were tested. Hep3B cells are derived from human hepatocellular carcinoma. Although liver is not a primary target of the local anesthetics, the liver is that involved in metabolism of local anesthetics. In the case of systemic intoxication, liver can be exposed to doses comparable to those of our experiments. On the other hand, SH-N-MC cells are of neuronal origin and still have properties as neuronal cells. The local anesthetics did not have any effects on HIF-1 activation, even at doses of 1000 μg/ml of lidocaine and 250 μg/ml of bupivacaine. Considering that 1000 μg/ml of lidocaine or 250 μg/ml of bupivacaine is equivalent to 0.1% or 0.025%, respectively, our experimental setting can be considered comparable enough to the clinical setting to examine the effects of local anesthetics.

Because oxygen is an essential molecule for human beings, HIF-1 serves as a global regulator of oxygen homeostasis in a wide range of biological phenomenon [5,13]. HIF-1 is reported to govern hypoxia-induced preconditioning of the heart in mice and to regulate the expression of prosurvival factors such as VEGF, erythropoietin, and hemeoxygenase 1 [5]. When adult rats are subjected to permanent middle cerebral artery occlusion, HIF-1 mRNA is induced in the penumbra or viable tissue surrounding the infarction. The induction of HIF-1 mRNA is temporally and spatially correlated with the expression of mRNAs encoding glucose transporter 1 and the glycolytic enzymes aldolase A, lactate dehydrogenase A, phosphofructokinase L, and pyruvate kinase M, which are all known HIF-1 target genes [5]. These data suggest that induction of glycolytic metabolism may promote the survival of neurons within the penumbra. In contrast, studies of primary cortical cultures from newborn mouse brains revealed that inhibition of HIF-1 activity by molecular biological overexpression of a dominant negative form of HIF-1α is associated with reduced cell death in response to oxygen and glucose deprivation [14]. The evidence demonstrates that the involvement of HIF-1 in hypoxia-

induced cell death is still controversial. Thus, the implication of the evidence that neither lidocaine nor bupivacaine affects hypoxia-induced HIF-1 activation is largely dependent on the situation under which cells or tissues are placed. Taking account of the controversy on the implication of hypoxic induction of HIF-1, the null or neutral effect of the local anesthetics may be an "advantage."

In conclusion, we demonstrate that neither lidocaine nor bupivacaine affects hypoxia-induced cellular responses mediated by HIF-1.

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