Primary Hypoxic Tolerance and Chemical Preconditioning During Estrus Cycle in Mice

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Background and Purpose—Exogenous application of estrogens or progesterone ameliorates hypoxic/ischemic cell damage. This study investigates whether values of primary and induced hypoxic tolerance vary endogenously during the estrus cycle in female mice.

Methods—Population spike amplitude (PSA) and NADH were measured during hypoxic hypoxia and recovery in hippocampal slices from untreated control animals (C slices) and slices prepared from animals pretreated in vivo with a single intraperitoneal injection of 3-nitropropionate (3NP) (3NP slices) or acetylsalicylate (ASA) (ASA slices).

Results—Posthypoxic recovery of PSA was dose dependent in 3NP slices from males, with maximal recovery on pretreatment attained with 20 mg/kg 3NP (82±32% [mean±SD]; C slices, 38±29%; P<0.01). PSA recovered to 17±12% in C slices during proestrus, 43±23% during estrus, and 63±44% during diestrus. In 3NP slices, recovery of PSA increased to 57±36% (P<0.05) during proestrus. Hypoxic tolerance was not increased in other stages of the estrus cycle. Hypoxic NADH increase during proestrus declined from 212±76% in C slices to 133±11% in 3NP slices (P<0.05). Recovery of PSA in ASA slices was 75±36% (P<0.01 versus control) in males and 48±34% during proestrus (P<0.05 versus ASA slices from males).

Conclusions—Primary and induced hypoxic tolerance are endogenously modulated during the estrus cycle. Differences in hypoxic oxidative energy metabolism mediate part of the differential tolerance. Experimental and clinical therapeutic strategies against cerebral ischemia/hypoxia need to consider sex-related dependence. (Stroke. 1999;30:1256-1262.)

Key Words: aspirin ■ gender ■ hypoxia ■ neuroprotection ■ mice

For some time it has been known that primary hypoxic tolerance, that is, the ability of cells to withstand and recover from cellular hypoxia for a limited amount of time, is sex dependent. However, in these early studies it remained unclear whether this ischemic tolerance is related to behavioral or nonbehavioral factors. Recent studies in vitro and in vivo show that estrogens and progesterone ameliorate nerve cell lesions due to hypoxia/ischemia. It has been suggested that the protective effects are mediated by antioxidative mechanisms, an increase of GLUT1 glucose transporter, or still undefined receptor-mediated pathways.

Under appropriate conditions of time interval and dosage, a mild ischemic challenge of central nervous system tissue increases primary hypoxic tolerance and induced hypoxic tolerance by ischemic preconditioning. Chemical preconditioning is a recently developed practical strategy that allows induction of hypoxic tolerance in the central nervous system with mild cellular hypoxia as a result of drug treatment, eg, by 3-nitropropionate (3NP). Increased hypoxic tolerance is associated with improved energy metabolism during hypoxia, decreased posthypoxic free radical production, improved posthypoxic morphology, and preserved posthypoxic neuronal function.

Ischemic preconditioning is already used in clinical practice and renders heart muscle cells more tolerant against ischemia during coronary angioplasty. Recently, it was suggested that chemical preconditioning also is unknowingly already in use. However, it has not yet been investigated whether ischemic or chemical preconditioning is useful to increase hypoxic tolerance in females as well.

Currently, the same strategies are in use for secondary stroke prevention in males and females. However, the most frequently used drug, acetylsalicylic acid (ASA), seems to have a differential benefit in males and females. Since inhibition of platelet aggregation by ASA is similar in males and females, inhibition of platelet aggregation cannot explain this observation.

The goal of the present study was (1) to investigate primary hypoxic tolerance in females during the estrus cycle and (2) to determine whether increased hypoxic tolerance by

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chemical preconditioning is a potential therapeutic strategy in females.

Materials and Methods

Preparation of Slices
Male and female CD-1 mice (weight, 25 to 35 g) were killed by cervical dislocation. Preparation of slices along the long axis of the hippocampus was performed as previously described for rats. Before further treatment, slices were incubated for at least 2 hours at 35°C in Ringer’s solution containing (mol/L) NaCl 126, KCl 5, KH₂PO₄ 1.3, MgSO₄ 1.3, CaCl₂ 2.4, NaHCO₃ 26, and dextrose 10, bubbled with 95% O₂ and 5% CO₂.

The estrus cycle was determined in female mice as described by Allen. In brief, the stages of the estrus cycle can be determined by analyzing the number and type of cells in the vaginal smear of the mice. Further treatment for investigation of hypoxic hypoxia and chemical hypoxia was performed as described previously. Electro-physiological measurements were performed in the recording chamber (see below). Procedures followed institutional guidelines.

Electrophysiological Recordings
Recording of population spike amplitude (PSA) was performed as previously described. In brief, slices were transferred to a recording chamber after preincubation. The recording chamber was perfused with Ringer’s solution at 6 mL/min through a peristaltic pump. Slices with PSA <2 mV were not included in the analysis. On stabilization of PSA, Ringer’s solution made hypoxic by bubbling with 95% N₂ and 5% CO₂ (PO₂ in the recording chamber <10 kPa after 5 minutes of perfusion with hypoxic Ringer’s solution; for detailed time course of PO₂ in the recording chamber, see Reference 25) superfused the slices for 15 minutes. After 15 minutes of hypoxia, slices were superfused with oxygenated Ringer’s solution until the end of the experiment.

Fluorometric NADH Recordings
A pulsed nitrogen laser (excitation wavelength = 337 nm, 30 μJ; LM 302, Laser Labor Adlershof) was used to induce fluorescence. High spatial resolution for excitation and detection was obtained by coupling the laser light into an optical quartz fiber (diameter, 200 μm) that guides the excitation light to the probe and the fluorescence light back to the detector. A dichroic mirror separates the scattered excitation light and the fluorescence light. A narrow band-pass filter, centered at 460 nm (maximum of NADH fluorescence), is used for the spectral resolution. A photomultiplier is used as detector, and an electronic gate provides the time resolution of the signal. Data acquisition is controlled by a standard PC. All results are displayed online.

Statistical Analysis
Each experiment was conducted with 4 to 6 slices from 2 to 3 animals. Statistical testing was performed by Student’s t test and ANOVA with Fisher’s least significant difference protected t test. Statistical significance was accepted at P<0.05.

Results
The PSA on hypoxia and recovery was determined in slices prepared from untreated control animals (C slices) during different stages of the estrus cycle.

Dose Response for Chemical Preconditioning in Male Mice
For a 1-hour interval between in vivo treatment and preparation of slices, a dose-response curve was obtained.
in male mice (Figure 1). In C slices from males, PSA recovered to 33±6% (mean±SD) of onset on 15 minutes of hypoxia. Posthypoxic recovery of PSA was 59±31% (P<0.05) with 5 mg/kg 3NP, 41±24% (P=NS) with 10 mg/kg, 82±32% (P<0.01) with 20 mg/kg, and 64±40% (P=NS) with 40 mg/kg.

Primary and Induced Hypoxic Tolerance During Estrus Cycle

During proestrus, posthypoxic recovery of PSA was 17±12% (mean±SD; P=NS versus C slices from males). Similarly, posthypoxic recovery of PSA was 43±23% (P=NS versus C slices from males) during estrus. In contrast, PSA recovered to 6±44% in C slices during diestrus (P<0.05 versus C slices from males) (Figure 2).

In slices prepared from animals pretreated in vivo with a single intraperitoneal injection of 3-NP (3NP slices), at a dose of 20 mg/kg an increase of posthypoxic recovery of PSA compared with controls was observed during proestrus. PSA increased to 57±36% (P<0.05 versus male 3NP slices; P<0.05 versus C slices during proestrus). In contrast, posthypoxic recovery of PSA declined during estrus to 33±12% (P<0.01 versus 3NP slices from males). During diestrus no difference was observed between recovery due to primary hypoxic tolerance and recovery after induction of hypoxic tolerance (60±50%; P=NS versus C slices during estrus; P=NS versus C slices from males) (Figure 3).

ASA Increases Hypoxic Tolerance

Increase of hypoxic tolerance by ASA was investigated in slices from male mice and slices from female mice during proestrus. With a 6-hour time interval between in vivo treatment and preparation of slices, PSA in slices prepared from animals pretreated in vivo with a single intraperitoneal injection of ASA (ASA slices) from male mice recovered to 75±36% (mean±SD; P<0.01 versus control) of onset and to 48±34% (P<0.01 versus C slices proestrus; P<0.05 versus ASA slices from males) (Figure 5).

Discussion

The present results support previous findings from in vivo and in vitro studies that primary hypoxic tolerance is sex...
Recent studies investigated the impact of exogenous application of estrogens and progesterone on nerve cell lesions due to hypoxia/ischemia and found improved outcome with both estrogens and progesterone. In the present study, the levels of endogenous estrogens or progesterone were not measured. However, it is established that estrogen and progesterone show a typical pattern during the estrus cycle. Endogenous estrogens are at a high level during proestrus and estrus and low during diestrus. Conversely, progesterone is high during estrus and diestrus and low during proestrus. The present results show the worst recovery of PSA during proestrus and the best during diestrus. This suggests that high endogenous progesterone levels are more favorable for an improved outcome than high endogenous levels of estrogens.

One possible mechanism could be that estradiol potentiates kainate-induced currents. It is well established that glutamate plays an important role in hypoxic/ischemic brain injury. High levels of endogenous estrogens may therefore increase glutamate toxicity during estrus.

Chemical preconditioning is a recently developed experimental strategy that increases primary hypoxic tolerance. Recent reports suggest that chemical preconditioning may, like ischemic preconditioning during coronary angioplasty, be in clinical use already, although unknown. Like most other experimental strategies against cerebral hypoxia/ischemia, whether this protective strategy is sex dependent has not been tested. The present results show that hypoxic tolerance of females can be significantly increased during stages of the estrus cycle in which females are most vulnerable against hypoxia, ie, during proestrus. During other stages of the estrus cycle, however, that is, during estrus and during diestrus, preconditioning treatment resulted in no benefit.

It has been shown that estradiol antagonizes endogenous adenosine. Adenosine is a neuromodulatory peptide that repeatedly has been show to be protective against hypoxia. Recently, it has also been shown that adenosine agonists simulate preconditioning and are partial agonists at KATP channels. It might therefore be speculated that chemical preconditioning and activation of KATP channels are particularly useful in situations in which the protective effects of adenosine are antagonized by endogenous estrogen and need to be overcome by preconditioning pretreatment.

Interestingly, the increase of cellular hypoxic tolerance by ASA may help to explain that protection in secondary stroke prevention is sex dependent, although there is no sex-related difference in the effect of ASA on platelet aggregation. In the present study it was shown that the benefit of ASA is greater for females during proestrus than for males. While chemical preconditioning is of some protection in females, treatment benefit seems to be greater in males than in females.

One of the mechanisms partaking in increased hypoxic tolerance is preservation of high-energy phosphates during hypoxic energy metabolism and an attenuated increase of NADH during hypoxic hypoxia. A difference in oxidative energy metabolism also accounts for part of the
Figure 4. Top, NADH fluorescence on hypoxia and recovery in slices from untreated control animals (C slices; □, mean ± SD) and animals pretreated in vivo with a single injection of 20 mg/kg 3NP 1 hour before slice preparation (3NP slices; ■, mean ± SD) during proestrus. Hypoxic NADH increase is larger in C slices than in 3NP slices. Bottom, NADH fluorescence on hypoxia and recovery in slices from untreated control animals (C slices; ○, mean ± SD) and animals pretreated in vivo with a single injection of 20 mg/kg 3NP 1 hour before slice preparation (3NP slices; ●, mean ± SD) during estrus. Hypoxic NADH increase is smaller in C slices than in 3NP slices.
variability of primary and induced hypoxic tolerance during the estrus cycle. In fact, this study demonstrates that an improved recovery of electrophysiological function, which is a good indicator of slice integrity, correlates with a reduced hypoxic increase of NADH. In this study it is shown that the reverse is also true. When the hypoxic NADH increase becomes larger because of a preceding treatment (eg, on pretreatment during estrus), recovery of PSA becomes smaller.

We conclude that primary hypoxic tolerance depends on the estrus cycle in females. Increase of hypoxic tolerance by preconditioning treatment also depends on the estrus cycle and warrants further investigation of the detailed mechanisms. Part of the differential sensitivity is mediated by differences in hypoxic oxidative energy metabolism. Further investigations of experimental and clinical therapeutic strategies against cerebral ischemia/hypoxia need to consider the differences in males and females.

Acknowledgment
This work was supported by a grant from the Deutsche Forschungsgemeinschaft to Dr Riepe (Ri 583/2-2).

References

Figure 5. Recovery of population spikes on hypoxia and recovery in slices from untreated control male and female animals (C slices) and slices from animals pretreated in vivo with a single injection of 20 mg/kg body wt ASA from male and female mice (ASA slices) 6 hours before slice preparation. (Population spikes were evoked with submaximal stimulation of Schaffer collaterals in hippocampal region CA3 at 0.1 Hz and recorded in hippocampal region CA1.)
Hypoxic Tolerance During Estrus Cycle


Editorial Comment

It has long been the practice in scientific research to study only male animals on the basis of the belief that the cyclic variation in estrogen and progesterone levels in females results in experimental variables that are difficult to control. On the other hand, there has been a building body of evidence that estrogen is protective in a variety of disease states (with the notable exception of breast cancer) and against a variety of cellular insults in vitro studies. The mechanisms responsible for these protective effects are for the most part unknown, but they are extremely important. Understanding how estrogen and progesterone protect against cellular damage may not only explain male/female differences in disease susceptibility; a developing understanding of the actions of estrogen and progesterone on various cells and organs may also provide new and important understanding of ways in which to reduce general morbidity and mortality.

The article by Kassisckhe et al demonstrates that tolerance to cerebral hypoxia is gender dependent, as would be expected from previous studies. Beyond this observation the authors demonstrate 2 important new observations, both of which have potential clinical significance for treatment of stroke patients. Chemical preconditioning through use of 3-nitropropionate, an inhibitor of succinic dehydrogenase, acts similar to hypoxic preconditioning to promote increased tolerance to ischemia. In this study, the authors have shown that such chemical preconditioning works in males but in females is effective only during proestrus, not during estrus and diestrus. The latter observation is important, because proestrus is the period of greatest ischemic vulnerability. They also demonstrate that the known beneficial effects of acetylsalicylic acid on ischemia is greater in females than males.

Optimal treatment of patients with disease is complex. Drugs are differentially effective at different times of the day, depending on circadian and other rhythms. These studies, in conjunction with others, clearly show that male/female differences and differences in the stage of the menstrual cycle must be considered in design of optimal therapy for ischemic brain disease.

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Stroke. 1999;30:1256-1262
doi: 10.1161/01.STR.30.6.1256
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/30/6/1256

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