Quantitative evaluation of the neuroprotective effects of a short-acting ß-adrenoceptor antagonist at a clinical dose on forebrain ischemia in gerbils: Effects of esmolol on ischemic depolarization and histological outcome of hippocampal CA1

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Summary

Esmolol has neuroprotective effects in the acute phase of ischemia, although esmolol does not affect the duration of ischemic depolarization.

Abstract

Background: Neuroprotective effects of esmolol in laboratory and clinical settings have been reported. The present study was designed to quantitatively evaluate the neuroprotective effects of esmolol by using logistic regression curves and extracellular potentials.

Methods: In forty-two gerbils, bilateral occlusion of common carotid arteries was performed for 3, 5, or 7 min (n=7 in each group). In treated animals, esmolol (200 μg/kg/min) was administered for 90 min from 30 min before the onset of ischemia. Direct current potentials were measured in bilateral CA1 regions, in which histological evaluation was performed 5 days later. Relations of neuronal damage with ischemic duration and duration of ischemic depolarization were determined by logistic regression curves.

Results: There was no significant difference in onset time between the two groups (control group vs. esmolol group: 1.65±0.46 min vs. 1.68±0.45 min, P=0.76), and significant differences in durations of ischemic depolarization were not observed with any ischemic duration. However, logistic regression curves indicated that esmolol has a neuroprotective effect from 2.95 to 7.66 minutes of ischemic depolarization (P<0.05), and esmolol prolonged the duration of ischemic depolarization causing 50% neuronal

damage from 4.97 to 6.34 minutes (P<0.05). Logistic regression curves also indicated that esmolol has a neuroprotective effect from 3.77 to 7.74 minutes of ischemic duration (P<0.05), and esmolol prolonged the ischemic duration causing 50% neuronal damage from 4.26 to 4.91 minutes (P<0.05).

Conclusion: Esmolol has neuroprotective effects in the acute phase of ischemia by a mechanism other than shortening the duration of ischemic depolarization.

Keywords

esmolol; β-adrenoceptor; ischemic depolarization; neuroprotective effect; brain ischemia

Introduction

It has been reported that β-adrenoceptor antagonists had neuroprotective effects in experimental models including reduction in infarct size and attenuation of histological outcomes.[1-7] In a clinical setting, it has also been reported that the β-adrenoceptor had neuroprotective effects such as reduction in the incidence of postoperative neurologic complications after cardiac surgery[8] and attenuation of the severity of stroke in ischemic stroke patients.[9]

In previous studies, the neuroprotective effects of β-adrenoceptor antagonists have been evaluated in various experimental models and with various ischemic durations. However, it is difficult to compare the results of various experiments or neuroprotective effects of other drugs. Therefore, quantitative evaluation of the neuroprotective effect is important. However, to the best of our knowledge, quantitative evaluation of the neuroprotective effects of β-adrenoceptor antagonists has not been performed.

When neuronal cells lose membrane potential due to cerebral ischemia, intracellular calcium concentration is increased up to 300 times of the control level,[10] and this increase in calcium concentration triggers secondary neuronal damage. Since ischemic depolarization triggers cascades of neuronal damage, the duration of ischemic

depolarization is strongly correlated with the degree of neuronal damage.[11] However, effects of β-adrenoceptor antagonists on duration of ischemic depolarization have not been evaluated.

The purpose of the present study was to quantitatively evaluate the neuroprotective effects of a β-adrenoceptor antagonist during the acute phase of brain ischemia. We used esmolol because it is rapidly metabolized and it is suitable for observing the effects of a β-adrenoceptor antagonist in the acute phase of cerebral ischemia. We induced ischemia of different durations (3, 5, and 7 min) in gerbils and observed the degree of damage of hippocampal CA1 pyramidal neurons. Then the correlation between ischemic duration and neuronal damage was depicted by use of logistic regression curves (probit curves), and the ischemic duration that would induce 50% neuronal damage was obtained. At the same time, ischemic depolarization in the hippocampal CA1 region was observed. The effect of esmolol on duration of ischemic depolarization was then evaluated by extracellular recording in the CA1 region.

Materials and Methods

Animals

Forty-five male Mongolian gerbils (SLC, Hamamatsu, Japan), weighing 64.5 ±

5.5 g, were used. The animals had free access to water and were fed *ad libitum* before the experiments. All experiments were performed in accordance with the National Institutes of Health Animal Care Guidelines and were approved by the Animal Research Control Committee of Okayama University Medical School.

Direct current (DC) potential and histological outcome

All animals were anesthetized before surgery with halothane (1%–2%) in 30% oxygen and 70% nitrogen under spontaneous respiration. Polyethylene catheters (PE-10) were inserted into the right femoral artery for continuous monitoring of mean arterial blood pressure (mABP) and blood sampling and into the right femoral vein for administration of saline or esmolol. Arterial blood samples were obtained 20 minutes after administration of saline or esmolol. Arterial blood gas, glucose, and hemoglobin were analyzed (i-STAT 300F, i-STAT Corporation, East Windsor, NJ, USA).

The bilateral common carotid arteries were exposed, and a ring (silicon tube, 0.5 mm in diameter) was loosely placed around each artery. After securing the head in a stereotaxic apparatus (Narishige, Tokyo, Japan), a reference electrode was placed in the left ear and a laser Doppler flow probe (FLO-C1, Omegawave, Tokyo, Japan) was placed on the right parietal cortex to continuously monitor regional cerebral blood flow

(CBF). Rate of change in CBF was utilized because it has been reported that a laser-Doppler flow meter only provides accurate information on changes in CBF.[12]

Two burr holes were made in bilateral temporal bones just above the CA1 regions of the bilateral hippocampus in accordance with the brain atlas (2 mm caudal to the bregma, 1.5 mm bilateral from the sagittal line)[13]. Two borosilicate glass electrodes (tip diameter, <5 μm; filled with physiological saline) were then placed for measurement of DC potentials (MEZ-8300, Nihon Kohden, Tokyo, Japan) 1 mm below the cortical surface in the vertical direction through the burr holes by using a stereotaxic apparatus. After surgery, the halothane concentration was reduced to 1%.

The 42 animals were randomly assigned to either a saline-administered group (control group, n = 21) or esmolol-administered group (esmolol group, n = 21). Another three animals were assigned to a sham group (no ischemia). In the esmolol group, esmolol was administered at the rate of 200 μg/kg/min (0.4 ml/hr) for 90 minutes. Forebrain ischemia was initiated 30 minutes after the start of continuous infusion. In the control group and sham group, an equivalent amount of saline was administered. In both groups, 1% halothane administration was continued until closure of the incision.

In the control group and esmolol group, forebrain ischemia was initiated by bilateral occlusion of the common carotid arteries for a predetermined duration (3, 5, or

7 minutes, n = 7 for each duration in each group). Initiation of ischemia and reperfusion were confirmed by a sudden decrease and rapid increase in CBF, respectively. Changes in DC potentials and CBF were recorded with the use of an analog/digital system (PowerLab, ADInstruments, Sydney, NSW, Australia). Changes in DC potentials were assessed by measuring onset time (from the initiation of ischemia to sudden negative shift of DC potentials) and duration of ischemic depolarization (from sudden negative shift of DC potentials to 80% recovery from maximal DC deflection) (Figure 1).

Brain surface temperature was maintained at 37.0 ± 0.5°C with a gentle flow (1.6 ml/min to 3.0 ml/min) of warmed saline (38.0 ± 0.5°C) into a polyethylene cylinder (5 mm in height, 13 mm in inner diameter) that had been placed on the skull surface. Rectal temperature was maintained at 37.0 ± 0.5°C using a heated-water blanket and infrared lamp. These temperatures were continuously measured and controlled from 30 minutes prior to the initiation of ischemia until 90 minutes after initiation of reperfusion to avoid the influence of temperature on ischemia because it had been previously reported that any chance of neuronal death induced by post-ischemic hyperthermia could be eliminated by maintaining normothermia for a duration of 85 min after initiation of reperfusion[14].

After a 5-day survival period, all animals were anesthetized with 4% of halothane

in oxygen and perfused with heparinized physiologic saline (20 U/ml) and 4% formaldehyde with buffer solution (pH 7.4). At the cortical surface, needle tracks were made with a 27-gauge needle using blue ink through the burr holes before enucleating the brains, because we confirmed that the cortical surface did not shift after 5 days of ischemia by identifying cortical arteries and veins through the burr holes in a pilot study.

After brain removal and paraffin-embedding, tissue including the bilateral hippocampal CA1 regions (area marked with blue ink) was sectioned coronally (5 μm in thickness). The sections were stained with hematoxylin and eosin. The areas in which DC potential had been recorded were enlarged to 400-power magnification, and the numbers of both damaged and intact pyramidal neurons in bilateral hippocampal CA1 regions were counted (visual field: 340 × 230 μm). In the current study, pyramidal neurons showing aggregated chromatin in the nucleus, shrinkage, or eosinophilic staining in the cytoplasm were defined as damaged neurons. The number of injured pyramidal neurons in the bilateral hippocampal CA1 regions was counted by an observer who was blinded to this study. The percentages of neuronal damage in the two groups were calculated as damaged neurons/total neurons × 100 in the visual field.

Statistical analysis

Values are expressed as means ± SD.

Parameters for arterial blood gas, glucose, hemoglobin and ischemic depolarization were analyzed by Student's t-test. The changes in CBF, mABP and heart rate were analyzed by two-way analysis of variance (ANOVA). Scheffé's F test was used as a post hoc test if the results of ANOVA were significant. Parameters for neuronal damage were analyzed by the Mann-Whitney U-test. In all statistical tests, a level of $P < 0.05$ was considered to be significant.

Dose–reaction curves for evaluating acute drug toxicity in toxicology are usually expressed by the use of probit curves. In the current study, the relationships of neuronal damage with ischemic duration and duration of ischemic depolarization were determined by logistic regression curves (probit curves) as dose–reaction curves. Ischemic duration or duration of ischemic depolarization was represented on the x-axis and neuronal damage was represented on the y-axis. These regression curves were drawn by using data analysis software (Microcal Origin 8; Microcal Software, Northampton, MA, USA). A probit curve, which expresses the probability of occurrence, is used to search for the median lethal dose in toxicology. Therefore, in this study, ischemic durations and durations of ischemic depolarization necessary for

causing 50% neuronal damage in both groups were determined from logistic regression curves.

Results

As shown in Table 1, we obtained physiological values before administration of esmolol (baseline), immediately before initiation of brain ischemia, during ischemia, and 10 min after reperfusion. Arterial blood samples were obtained 20 min after administration of saline or esmolol and 10 min before initiation of ischemia. Although heart rate was significantly decreased following administration of esmolol before initiation of ischemia (esmolol vs. control, $P < 0.001$; baseline vs. before initiation of brain ischemia, $P < 0.001$) and after reperfusion (esmolol vs. control, $P < 0.001$; baseline vs. after reperfusion, $P < 0.001$), there were no statistically significant differences in other parameters between the control group and esmolol group.

The variables of DC potential in each experimental group are summarized in Table 2. No significant difference in onset time between the two groups was observed, and there was no significant difference in duration of ischemic depolarization between the two groups with any ischemic duration.

The variables of neuronal damage are also shown in Table 2. The percentage of neuronal damage with 5 min of ischemia was significantly improved in the esmolol group (58.9 ± 25.4%) compared with that in the control group (80.8 ±15.3%) ($P = 0.01$). The percentages of neuronal damage with 3 and 7 min of ischemia were not significantly different in the two groups.

As can be seen in Figure 2, logistic regression curves showed significant correlations between ischemic time and percentages of damaged neurons (control: r^2=0.86, P<0.001; esmolol: r^2=0.80, P<0.001). The 95% confidence intervals did not overlap from 3.77 to 7.74 min of ischemic duration. Ischemic durations necessary for causing 50% neuronal damage in the control and esmolol groups were estimated to be 4.26 min (95% CI, 4.01 - 4.48 min) and 4.91 min (95% CI, 4.65 - 5.16 min), respectively.

As can be seen in Figure 3, other logistic regression curves showed significant correlations between duration of ischemic depolarization and percentages of damaged neurons (control: r^2=0.66, P<0.001; esmolol: r^2=0.79, P<0.001). The 95% confidence intervals did not overlap from 2.95 to 7.66 min of duration of ischemic depolarization. Durations of ischemic depolarization necessary for causing 50% neuronal damage in the control and esmolol groups were estimated to be 4.97 min (95% CI, 4.01 - 4.48 min)

and 6.34 min (95% CI, 4.65 - 5.16 min), respectively.

Discussion

As shown in Table 2, administration of esmolol suppressed the percentage of neuronal damage in the hippocampal CA1 region with 5 min of ischemia. However, the percentages of neuronal damage with 3 and 7 min of ischemia were not different between the two groups. Therefore, if ischemia of only one intensity had been induced, there is a possibility that the difference in the effects of esmolol during cerebral ischemia would have been overlooked. These results indicated that significant neuroprotective effects were elicited with a limited range of ischemic intervals.

As can be seen in Fig 2, the 95% confidence intervals did not overlap from 3.77 to 7.74 min of ischemic duration between regression curves of the esmolol group and control group, indicating that esmolol significantly reduced neuronal damage during this period of ischemia. In addition, administration of esmolol prolonged durations of ischemia necessary for causing 50% neuronal damage by 0.65 minutes. Propofol and thiopental prolonged ischemic durations necessary for causing 50% neuronal damage by 1.4 min and 3.3 min, respectively, in the same experimental model.[15] These results suggested that the neuroprotective effect of esmolol tends to be weaker than the effects

of such agents.

In the current study, esmolol reduced neuronal damage in the hippocampal CA1 region. In previous studies, it has been shown that β-adrenoceptor antagonists had neuroprotective effects in both clinical and experimental settings. In a clinical setting, use of β-adrenoceptor antagonists was associated with a substantial reduction in the incidence of postoperative neurologic complications during cardiac surgery[8] and less severe stroke in cerebrovascular disease.[9] In animal models, Little et al.[1] reported in 1982 that propranolol reduced infarct size in a rat model of focal ischemia. Atenolol, a selective β1-adrenoceptor antagonist, had a neuroprotective effect after permanent focal cerebral ischemia in rats.[6] In addition, esmolol and landiolol, short-acting β1-adrenoceptor antagonists, have neuroprotective effects after transient focal cerebral ischemia and global ischemia in rats.[3-5,7] From these reports, it is believed that β-adrenoceptor antagonists have neuroprotective effects. However, previous studies used β-adrenoceptor antagonists with long half-lives (T1/2: propranolol, 3.2 h[16]; atenolol, 6.06 h[17]) or long administration time (24 h ~ 6 days[3-5, 7]) in use of short-acting β-adrenoceptor antagonists (T1/2: esmolol, 9.19 min[18]; landiolol, 3.05 min[19]). To the best of our knowledge, neuroprotective effects of β-adrenoceptor antagonists in the acute phase of brain ischemia have not been evaluated. Therefore, this is the first study

showing the neuroprotective effects of a short-acting β-adrenoceptor antagonist administered in a peri-ischemic period (from 30 min before to 60 min after the initiation of brain ischemia).

After the loss of membrane potential, intracellular calcium concentration increases by 300 fold,[10] resulting in secondary neuronal damage. Increasing intracellular calcium activates many enzymes such as endonuclease, protease, and phospholipase,[20] leading to induction of apoptosis,[20] collapse of cytoskeletal elements,[20,21] and accumulation of arachidonic acid.[22] Because the loss of membrane potential triggers a cascade of neuronal damage, ischemic depolarization is one of the important factors for determining the degree of neuronal damage in brain ischemia.[11] From the aspect of ischemic depolarization, the mechanism of neuroprotection can be divided into two major categories.[15] The first mechanism is reduction in duration of ischemic depolarization. As shown in Table 2, durations of ischemic depolarization with ischemic times of 3, 5 and 7 min were almost the same in the control and esmolol groups. This result indicates that esmolol does not affect the duration of ischemic depolarization; that is, the first mechanism is not the mechanism underlying the neuroprotective effect of esmolol.

The second mechanism is suppression of neuronal injury during or after

ischemic depolarization by inhibiting cascades for neuronal damage such as calcium influx and extracellular glutamate accumulation. The relations between duration of ischemic depolarization and neuronal damage are shown in Figure 3. Esmolol significantly prolonged the duration of ischemic depolarization necessary for causing 50% neuronal damage from 4.97 minutes to 6.34 minutes. This result indicated that the neuroprotective effect of esmolol is likely to be elicited by the second mechanism because esmolol was administered in only a peri-ischemic period.

Although the aim of this study was not to clarify the second mechanism underlying the neuroprotective effects of esmolol, some mechanisms are considered. It is well known that β-adrenergic stimulation activates adenylate cyclase, resulting in increased cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA).[23,24] PKA increases calcium influx by phosphorylation of the voltage-dependent calcium channel,[23,25,26] ryanodine receptor,[27,28] and Na/Ca exchanger.[29] Beta-adrenoceptor antagonists attenuate calcium influx by suppressing these receptors.[24,29-32] Moreover, β-adrenoceptor antagonists suppress the increase in extracellular glutamate level,[7,33] because cAMP facilitates glutamate release[33] and decreases glutamate uptake.[34]

In the current study, esmolol was administered at the rate of 200 μg/kg/min.

Heart rate was decreased by 23% and mean arterial blood pressure (mABP) was not decreased significantly compared with the baseline. In many studies, administration of esmolol to human subjects at the rate of 100 ~ 200 μg/kg/min, which is the clinical dose in humans, decreased heart rate by 10 ~ 25%.[35-38] At that dose, blood pressure was mildly decreased in some cases[35,38] and was not decreased in other cases.[36,37] Voltz-Zang et al. showed from the dose response curve that esmolol decrease blood pressure in a dose-dependent manner.[39] Administration of esmolol at high doses (500 ~ 750 μg/kg/min) to humans greatly decreased blood pressure.[40] Since the dose of esmolol used in the current study decreased heart rate moderately without suppressing mABP, we considered this dose to be close to the clinical dose.

There were some limitations in this study. First, cardiovascular effects of β-adrenoceptor antagonists might influence neuroprotective effects of β-adrenoceptor antagonists. In this study, we used a forebrain ischemic model. During the experiment, gerbils maintained mABP, and mABP and CBF were not significantly different between the control and esmolol groups (Table 1). It is therefore thought that esmolol has neuroprotective effects independent of improvement in cardiovascular function. Second, since total numbers of neurons with 5 and 7 min of ischemia were smaller than those in the sham group, there was a possibility that the total number of neurons was

underestimated. The percentage of damaged pyramidal cells in the hippocampal CA1 region was assessed at 5 days after ischemia. Since it has been reported that histological damage is completed within 4 days after ischemia[41] and since phagocytic cells had accumulated at 7 days after ischemia,[42] it was expected that the number of damaged neurons could be counted at 5 days after ischemia. We assumed that one of the reasons for the decrease in total number of neurons is related to brain edema. In support of this notion, the area of the hippocampus (including CA1, CA2 and CA3 regions) was increased by 7% in the group with 7 min of ischemia compared to that in the sham group (data not shown). Since not only the total number of neurons but also the number of damaged neurons would be decreased, it is thought that the ratio of neuronal damage was not greatly affected though the total number of neurons decreased. Third, in this study, the percentage of damaged pyramidal cells in the hippocampal CA1 region was assessed at 5 days after ischemia. The neurological effect and long-term effect of esmolol on brain ischemia were unclear in this study.

In summary, we quantitatively evaluated the neuroprotective effects of esmolol at a clinical dose using logistic regression curves. Logistic regression curves indicated that esmolol significantly reduced neuronal damage during the period of ischemia from 3.77 to 7.74 min and prolonged ischemic duration necessary for causing 50% neuronal

damage by 0.65 min. The onset of ischemic depolarization and duration of ischemic depolarization were not affected by esmolol in three different durations of ischemia. Other logistic regression curves indicated that severity of neuronal damage with identical duration of ischemic depolarization was significantly attenuated by esmolol compared with the control during the period of ischemic depolarization from 2.95 to 7.66 min. Therefore, it is thought that decreasing neuronal injury by inhibiting cascades for neuronal damage during and after ischemic depolarization is one of the mechanisms underlying the neuroprotective effects of esmolol.

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Table 1. Physiological values

	control group	**esmolol group**
$PaCO_2$ (mmHg)	42.4 ± 3.6	42.1 ± 2.2
PaO_2 (mmHg)	131 ±17	132 ± 9
HCO_3 (mmol/l)	24.1 ± 1.8	24.0 ± 1.5
Blood glucose (mg/dl)	142 ± 24	136 ± 19
Hemoglobin (g/dl)	15.2 ± 1.0	15.0 ± 0.7
Mean arterial blood pressure (mmHg)		
Baseline	79.9 ± 11.5	83.6 ± 8.1
Before initiation of brain ischemia	79.0 ± 13.5	79.7 ± 8.9
During ischemia	118 ± 11.1†	118 ±10.5†
10 min after reperfusion	80.3 ± 9.1	77.9 ± 6.1
Heart rate (bpm)		
Baseline	321 ± 77	329 ± 63
Before initiation of brain ischemia	309 ± 77	249 ± 40 *†
During ischemia	218 ± 44†	211 ± 35†
10 min after reperfusion	344 ± 69	264 ± 37*†

%changes in CBF (%)		
Baseline	100	100
Before initiation of brain ischemia	103 ± 11	101 ± 9
During ischemia	7.6 ± 2.4†	7.5 ± 5.1†
10 min after reperfusion	103 ± 31	105 ± 36

Values are expressed as means ± SD.

Arterial blood samples were obtained 20 min after administration of saline/esmolol.

Baseline = before administration of saline/esmolol

$PaCO_2$ = arterial carbon dioxide tension; PaO_2 = arterial oxygen tension; HCO_3 = hydrogen carbonate ions; CBF = cerebral blood flow

* $P<0.05$ compared with control group

† $P<0.05$ compared with baseline

Table 2. Variables of DC potential and neuronal damage

	control group	esmolol group
Onset time (min)	1.65 ± 0.46	1.68 ± 0.45
Duration of ischemic depolarization (min)		
3 minutes of ischemia	3.31 ± 0.60	3.60 ± 0.86
5 minutes of ischemia	6.40 ± 0.84	6.46 ± 0.81
7 minutes of ischemia	9.52 ± 2.07	9.39 ± 1.46
Neuronal damage (%)　(total number of neurons in the visual field)		
0 minutes of ischemia (sham)	1.1 ± 1.0 (79.7 ± 5.0)	
3 minutes of ischemia	12.9 ± 8.3 (81.6 ± 7.9)	9.8 ± 9.9 (79.2 ± 6.4)
5 minutes of ischemia	80.8 ± 15.3 (67.7 ± 12.0)	58.9 ± 25.4* (74.0 ± 12.0)
7 minutes of ischemia	97.2 ± 2.1 (70.1 ± 8.0)	93.1 ± 9.6 (66.5 ± 8.2)

Values are presented as means ± SD.

* $P < 0.05$ compared with the control group.

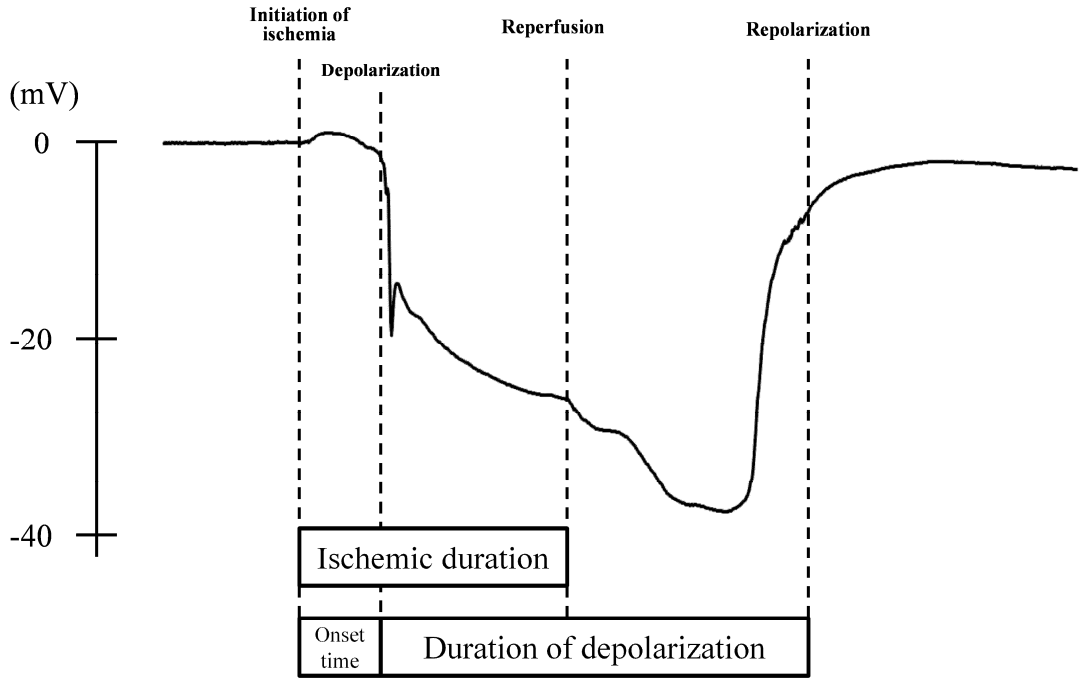

Figure 1. Onset time: from initiation of ischemia to sudden negative shift of DC potentials. Duration of ischemic depolarization: from sudden negative shift of DC potentials to 80% recovery from maximal DC deflection.

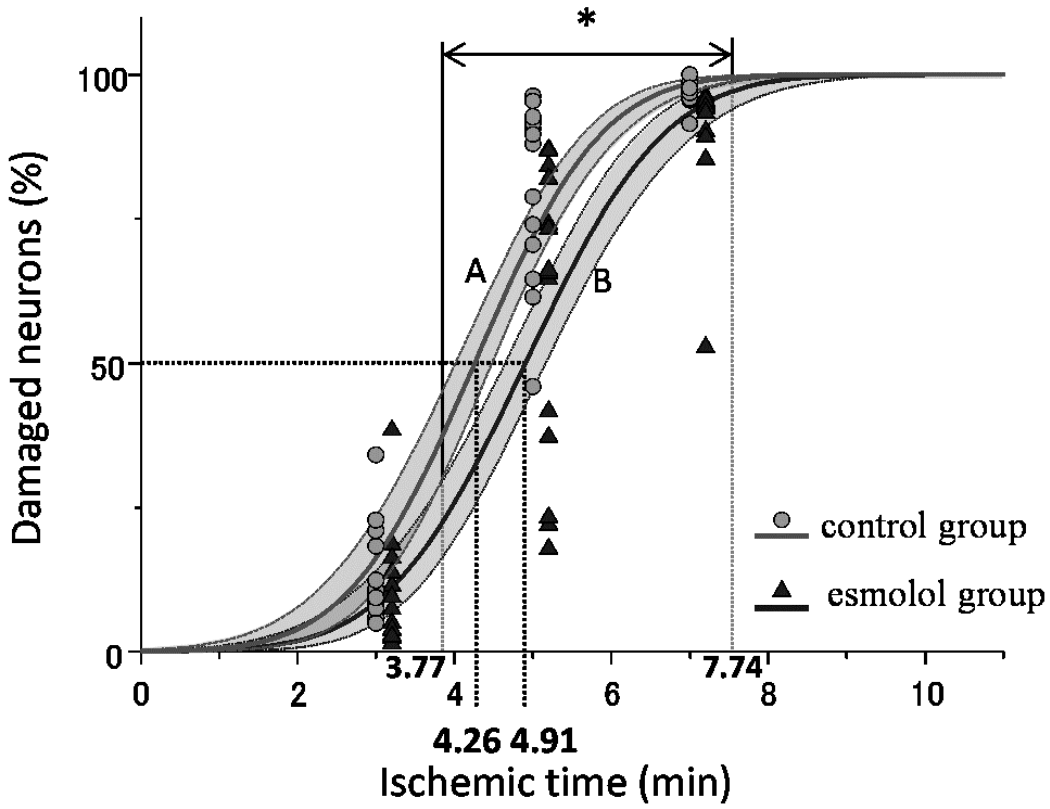

Figure 2. Relationships between ischemic duration and percentage of damaged neurons in all experimental animals. Circles, percentage of damaged neurons in the control group; triangles, those in the esmolol group. Logistic regression curves show close relationships between ischemic duration and neuronal damage (control group, line A: $r^2 = 0.86$, $P < 0.001$; esmolol group, line B: $r^2 = 0.80$, $P < 0.001$). The 95% confidence intervals (shaded areas) did not overlap from 3.77 to 7.74 min of ischemic duration (*). Ischemic durations necessary for causing 50% neuronal damage in the control group and esmolol group were 4.26 min and 4.91 min, respectively.

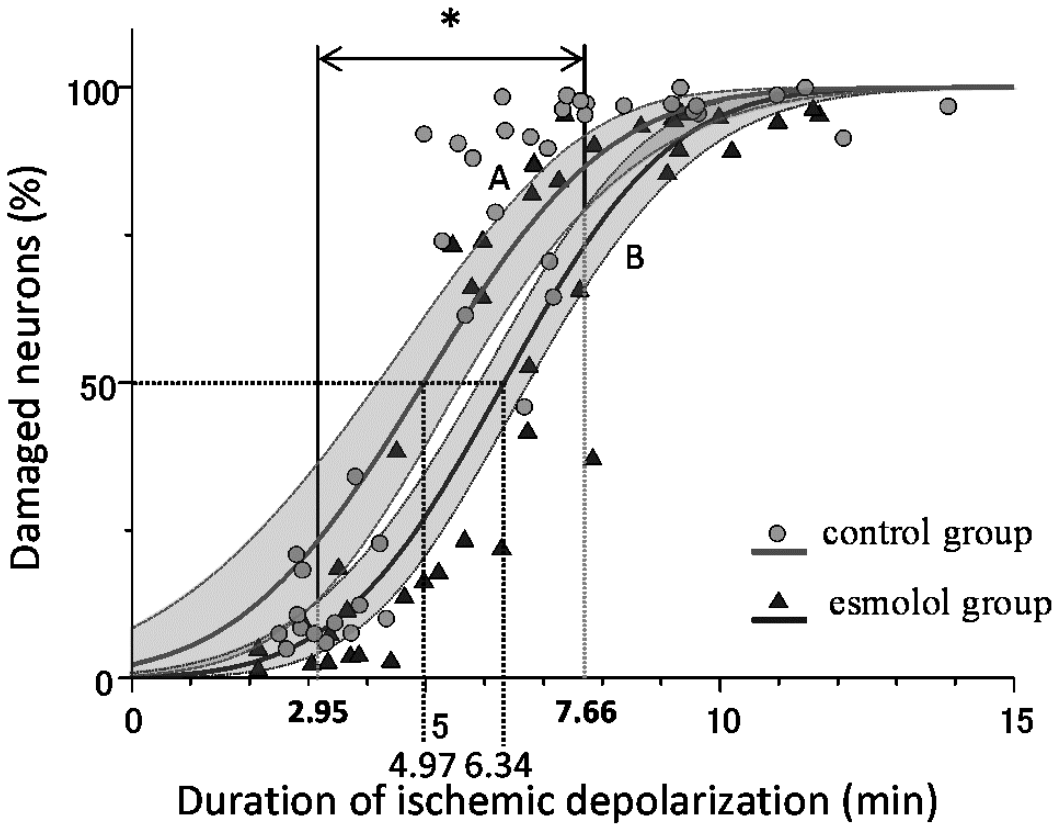

Figure 3. Relationships between duration of ischemic depolarization and percentages of damaged neurons. Percentages of damaged neurons in the control group are shown by circles and those in the esmolol group are shown by triangles. Logistic regression curves show close relationships between ischemic duration and neuronal damage (control group, line A: $r^2 = 0.66$, $P < 0.001$; esmolol group, line B: $r^2 = 0.79$, $P < 0.001$). The 95% confidence intervals (shaded areas) did not overlap from 2.95 to 7.66 min of duration of ischemic depolarization (*). Durations of ischemic depolarization necessary for causing 50% neuronal damage in the control group and esmolol group were 4.97 min and 6.34 min, respectively.