Opioid Receptor-Activation: Retina Protected from Ischemic Injury

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PURPOSE. In nonocular systems, activation of opioid receptors has been shown to ameliorate tissue damage induced by ischemic stress. The current study was an investigation of whether opioid receptors activated by endogenous or exogenous agonists can ameliorate ischemic retinal injury.

METHODS. In an investigation of whether endogenous opioid receptor-activation reduces ischemic injury, the effects of the opioid antagonist naloxone (3 mg/kg; IP) on retinal neuroprotection induced by ischemic preconditioning (IPC) were evaluated. Whether exogenous opioid administration can reduce ischemic retinal injury was determined by pretreating rats with morphine (0.01–10 mg/kg) before injury. Morphometric and electroretinogram (ERG) analyses were used to assess the differences in retinal structure and function. The expression of opioid receptor subtypes was evaluated by Western blot and immunohistochemical analyses.

RESULTS. In control animals, 7 days after ischemic retinal injury, ERG a- and b-wave amplitudes were significantly reduced (23% and 41%, respectively). In addition, degeneration of the inner retina resulted in a 54% reduction in overall retina thickness. In animals receiving IPC before ischemic injury, ERG wave forms and retinal morphology were preserved. Pretreatment with naloxone reversed both the functional and structural retinal protection induced by IPC. In animals treated with morphine 24-hours before ischemic injury, ERG waveforms were preserved in a dose-dependent fashion (ED50 = 0.68 mg/kg), and this protective response was reversed by naloxone pretreatment. Immunohistochemical and Western blot data demonstrated that the δ, κ, and μ-opioid receptor subtypes are expressed in the retina.

CONCLUSIONS. These data provide evidence that activation of one (or more) opioid receptor(s) facilitates the development of IPC within the retina and can reduce ischemic retina injury. (Invest Ophthalmol Vis Sci. 2009;50:3853–3859) DOI:10.1167/iovs.08-2907

Retinal ischemia is a common cause of visual impairment and blindness. At the cellular level, ischemic retinal injury consists of a self-re-enforcing destructive cascade involving neural depolarization, calcium influx, oxidative stress, proinflammatory cytokine release, and increased glutamatergic stimulation. Retinal ischemia is believed to play a pivotal role in several ocular diseases such as glaucoma, diabetic retinopathy, retinal vein occlusion, and retinopathy of prematurity.1 However, the extent and sequence of events by which ischemia contribute to cell death requires further investigation.

Although the molecular and cellular events involved in ischemic retinal degeneration have been studied for several years, the identification of efficacious therapies remains to be determined. To identify novel neuroprotective strategies for the retina, we and others have investigated the cytoprotective mechanisms induced by ischemic preconditioning (IPC).2,3 IPC, or ischemic tolerance, is defined as the ability of one or more brief noninjurious periods of ischemia to protect a tissue from subsequent severe ischemic insults. Cytoprotective responses to IPC have been identified in heart, brain, liver, lung, skeletal muscle, spinal cord, and retina.5–8 Studies have provided evidence that in the retina, neuroprotection induced by IPC may involve multiple mechanisms including adenosine receptor activation, HIF-1α-induced gene expression, and the upregulation of stress proteins such as heme oxygenase, HSP-27, -70, and -90.2,3,5,5

Opioids are powerful analgesics, but also have other important pharmacologic properties. For example, the opioidergic system can modulate stress responses within the immune system, cardiovascular, and gastrointestinal tissues, and the central nervous system. Pharmacologic and molecular studies have identified three opioid receptors: δ-opioid (OP1), κ-opioid (OP2), and μ-opioid (OP3) receptors.9–11 Opioid receptors are linked to a variety of G-proteins and cell-signaling pathways. Early studies demonstrated that circulating levels of endogenous opioid peptides increase during periods of stress.12–14 Furthermore, myocardial ischemia has been shown to induce synthesis and release of opioid peptides that subsequently activate opioid receptors.15,14 There is a growing body of evidence from studies of the heart and brain supporting the idea that activation of the opioid system plays a central role in IPC and that the nonselective opioid antagonist naloxone can prevent the development of preconditioning in these tissues.15–16 In other organs, activation of opioid receptors by an exogenous agonist (opioid preconditioning) has been shown to elicit a protective effect during situations of stress produced by hypoxia, ischemia, cold, or an acidic environment.15,17–21 Activation of opioid receptors reduced infarct size in stroke and myocardial ischemia models.22,23

In the eye, opioid receptors have been implicated in the regulation of iris function, accommodation, aqueous humor dynamics, corneal wound healing, and retinal development.24–30 However, the neuroprotective potential of opioid agonists in the retina and their role in the development of retinal ischemic tolerance has not been investigated. In this study, we examined the roles of opioid receptor activation in the development of retinal IPC, and the ability of the nonselective opioid agonist morphine to protect the retina from ischemic injury.
**Materials and Methods**

Adult male or female Brown Norway rats (3–5 months of age; 150–200 g; Charles River Laboratories, Inc., Wilmington, MA) were used in this study. The rats were kept under a cycle of 12-hours' light and 12-hours' dark. Animal handling was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the study protocol was approved by the Animal Care and Use Committee at the Medical University of South Carolina.

To induce retinal ischemia or measure electroretinograms (ERGs), the rats were anesthetized with intraperitoneal ketamine (75 mg/kg) and xylazine (8 mg/kg) (Ben Venue Laboratories, Bedford, OH), and local analgesia of the cornea was induced using proparacaine (0.5%; 5 μL; Akorn, Inc., Buffalo Grove, IL). Body temperature was maintained at 37°C by means of a heating pad (Harvard Apparatus, Holliston, MA).

Retinal ischemia was induced using methods previously described by Whitlock et al. Briefly, the anterior chamber was cannulated with a 30-gauge needle that was connected to a container of sterile normal saline via polyethylene tubing; subsequently, the reservoir was elevated to raise the IOP above systolic blood pressure (155–160 mm Hg). IOP was monitored by an in-line pressure transducer connected to a computer. For IPC, the IOP was elevated for 5 minutes, the needle removed, and the eye allowed to reperfuse. Twenty-four hours after the preconditioning event, ischemic retinal injury was induced by raising the IOP above systolic blood pressure (155–160 mm Hg) for 45 minutes. The contralateral eye was left untreated, serving as the control. Selected animals were treated with the opioid antagonist naloxone (3 mg/kg IP), 1 hour before the preconditioning event. The dose of naloxone was based on those in published studies. In other animals, the IPC event was replaced by the administration of the opioid receptor agonist morphine (0.01–10 mg/kg IP). In the morphine-treated animals, ischemic retinal injury was created in one eye by again raising the IOP above systolic blood pressure for 45 minutes, 24 hours after morphine administration. Selected animals were treated with naloxone (3 mg/kg) 1 hour before morphine administration. In animals receiving morphine and/or naloxone, contralateral eyes were used to assess the effects of these agents on normal retinal ERGs and morphology.

**Electroretinograms**

To quantitate posts ischemic retinal function, we performed ERGs as previously described. Briefly, rats were dark adapted overnight, and the following day they were anesthetized with ketamine and xylazine as described for the induction of ischemia. The pupils were dilated with a 10-μL drop of a solution containing phenylephrine HCl (2.5%) and tropicamide (1%) (Akorn, Inc., Buffalo Grove, IL). A needle ground electrode was placed subcutaneously in the back of the animal and a reference electrode on the tongue. A stimulus intensity series of ERGs was recorded in response to single-flash intensities using a 0.004-0.013 μV. The single-flash responses were an average of two flashes with an interstimulus interval of 2 minutes to ensure that ERG amplitudes at a given intensity were identical between the first and the last flashes. The single-flash responses were an average of two flashes with an interstimulus interval of 2 minutes to ensure that ERG amplitudes at a given intensity were identical between the first and the last flashes. The single-flash ERG response was measured with a contact lens electrode. The single-flash ERG response was measured with a contact lens containing a gold-ring electrode held in place by a drop of methylcellulose (UTAS-2000; LKC Technologies, Gaithersburg, MD). Corneal electrical responses to a single, 10-μs white-light flash were delivered by a Ganzfeld stimulator. Amplitudes of ERG a- and b-waves were measured and compared to contralateral control responses and corresponding ipsilateral responses from other treatment groups.

**Morphometric Analyses**

Rats were euthanatized by an overdose of sodium pentobarbital. The eyes were then enucleated, fixed for 1 hour in 4% paraformaldehyde in 0.1 M phosphate-buffered saline at 4°C, and slit open at the ora serrata, and fixation continued for 24 hours at 4°C. After fixation, the anterior segment was removed, and the posterior eye cup dehydrated and embedded in paraffin. To evaluate structural changes in the retina paraffin embedded parasagittal (4 μm) sections of posterior eye cups were processed for hematoxylin-eosin staining (Sigma-Aldrich, St. Louis, MO) and retina layers were observed, photographed, and measured (Axioplan-2; Carl Zeiss Meditec, Dublin, CA).

For immunohistochemistry, fixed posterior eye cups were cryo-preserved in a solution of 30% sucrose overnight, mounted in optimal cutting temperature mounting medium, and frozen, and parasagittal sections (10 μm) were cut and mounted on gelatin-coated slides. Sections were then processed for the detection of opioid receptors using primary antibodies against δ-, κ-, or μ-opioid receptors (1:100; Millipore, Billerica, MA) and appropriate Alexa Fluor-conjugated secondary antibodies (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Negative control slides were incubated with normal serum in place of the primary antibodies. The sections were reviewed and photographed with a confocal microscope (Leica, Heidelberg, Germany).

**Western Blot Analysis**

The rats were euthanatized, the retinas removed and the protein extracted, and 15 μg of protein was loaded onto 10% SDS-polyacylamide gels followed by transfer to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk followed by incubation with anti-δ-, anti-κ-, or anti-μ antibodies (1,000 dilutions) for 12 hours at 4°C. After they were washed, the membranes were incubated with appropriate secondary antibodies (HRP-conjugated; dilution 1:3000) for 1 hour at 20°C. Prestained molecular weight standards (Magic Mark; Invitrogen, Carlsbad, CA) were used to determine the molecular weight of the proteins. For chemiluminescent detection, the membranes were treated with enhanced chemiluminescent reagent, and the signal was monitored (Versadoc Imaging System; Bio-Rad, Hercules, CA).

**Statistical Analyses**

Dose–response curves were analyzed by nonlinear regression. Statistical comparisons of ipsilateral and contralateral responses were made with the Student’s t-test for paired data. For comparing different treatment groups, ANOVA with Dunnett posttest (GraphPad Software, Inc., San Diego, CA) was used. A P < 0.05 was considered significant.

**Results**

**Effect of Naloxone on Retinal IPC**

Shown in Figures 1 and 2 are ERG a- and b-wave responses to full intensity light flashes 7 days after 45 minutes of complete retinal ischemia. In nontreated animals, ischemic injury significantly reduced mean b-wave amplitudes by 41% when compared to contralateral control eyes (control eyes, 688 ± 30 μV versus ischemic eyes 404 ± 25 μV; P < 0.05). We also measured a small, but significant decline of 23% in a-wave amplitudes in these eyes (control eyes, 298 ± 13 μV versus ischemic eyes 229 ± 9 μV; P < 0.05). In eyes that received an IPC stimulus 24 hours before ischemic injury, the a- and b-wave amplitudes were significantly greater (P < 0.05) when compared to the eyes of animals receiving only the 45-minute ischemic injury (IPC eyes 607 ± 35 μV versus ischemic eyes 404 ± 25 μV). In contrast, in animals that received the nonselective opioid antagonist naloxone (3 mg/kg) 1 hour before IPC and then received ischemic injury the following day, the b-wave amplitudes were not significantly different from those of animals that received only 45 minutes of retinal ischemia (naloxone+IPC+ischemic eyes 393 ± 44 μV versus ischemic eyes 404 ± 25 μV). In animals that received only naloxone 24-hours before ischemic injury, a trend toward lower ERG waveforms was noted; however, mean b-wave amplitudes were not significantly different from eyes that received only
ischemic injury (naloxone + ischemic eyes 334 ± 23 μV versus ischemic eyes 404 ± 25 μV). No significant difference between contralateral ERG a- and b-wave amplitudes was measured between any of the groups tested (Figs. 1, 2).

At lower flash intensities, significant reduction in ERG wave forms were also measured in eyes 7 days after retinal ischemia. In eyes receiving IPC, waveforms were significantly greater in amplitudes when compared to eyes only receiving ischemic injury. As with full intensity flashes, pretreatment with naloxone reversed the protective actions of IPC on the ERG wave forms.

Morphologic changes induced by 45 minutes of ischemia in each group were evaluated 7 days after ischemic injury (Fig. 3). Retinas from eyes receiving ischemic injury were compared to control eyes, and to retinas subjected to preconditioning in the presence or absence of naloxone. In eyes that received only 45 minutes of ischemia, overall retina thickness was significantly decreased (by 34%; Table 1). This reduction in thickness was primarily due to significant thinning of the inner plexiform layer (IPL) which was reduced by 49% compared with control eyes. Although trends toward reduced thickness of the inner nuclear layer (INL), outer plexiform layer (OPL), and outer nuclear layer (ONL) were noted individually, these changes were not significant. In addition, retinal ischemia resulted in a significant loss of retinal ganglion cells (RGCs), and mild disorganization of the INL and ONL. In contrast, retinas that received IPC the day before the 45-minute ischemic insult, exhibited normal retina thickness, no significant change in the number of ganglion cells, and normal organization of all retinal layers. In rats pretreated with 3 mg/kg naloxone 1 hour before IPC, the ischemic damage was similar to that observed in eyes receiving only the 45-minute ischemic insult with a significant decrease in retina thicknesses and ganglion cell number, and the nuclear layers exhibiting a disorganized appearance (Fig. 3, Table 1).

**Expression of Opioid Receptors in Rat Retina**

Opioid receptor expression in the retina was determined by staining frozen sections of posterior segment with anti-δ, anti-κ, or anti-μ-opioid-receptor antibodies. As shown in Figure 4, strong immunostaining was observed for δ, κ, and μ-opioid receptors in the nerve fiber layer and retinal ganglion cell layer. Diffuse punctate staining for δ- and κ-opioids was also observed in the IPL. The expression of retinal opioid receptors was confirmed by Western blot analysis. As shown in Figure 5, analysis of retinal extracts with anti-δ, anti-κ, and anti-μ-opioid receptor antibodies recognized bands of 38, 42, and 48 kDa for δ, κ, and μ-opioid-receptors, respectively.

**Effects of Morphine on Ischemia-Induced Retinal Degeneration**

To determine whether activation of opioid receptors by exogenously administered opioid receptor agonists can protect the retina from ischemic injury, we treated the animals with the nonselective opioid agonist morphine 24 hours before the 45-minute ischemic event. As shown in Figure 6, pretreatment with morphine (0.01-10 mg/kg) significantly preserved ERG a- and b-wave amplitudes when compared to those in with nontreated animals. However, the protection seen with 1.0 or 10 mg/kg morphine was less than that observed in animals that received IPC 24 hours before ischemic retinal injury. The morphine-induced preservation of ERG amplitudes was dose related, with an ED50 of 0.18 mg/kg.
To confirm that morphine-induced preservation of ERG waveforms was mediated via opioid receptor activation, we treated the animals with naloxone (3 mg/kg) 1 hour before morphine administration. As shown in Figure 7, naloxone pretreatment reversed the morphine-induced protective effects of ERG amplitude.

DISCUSSION

Retinal ischemia, in its various guises, is a common clinical entity. However, retinal diseases involving ischemia remain a common cause of visual impairment and blindness. Ocular ischemia induced by elevated IOP in animals is frequently used as an animal model in retina research and has been described in several studies.1,2,3 This method produces severe global ischemia by obstructing both the retinal and uveal circulation, as evidenced by significant reduction in amplitude of the ERG waveforms and obvious pallor of the fundus and iris. This experimental procedure produces pathologic features that are presumed to occur in acute angle-closure glaucoma.1

Opioids have been used clinically for centuries as analgesics. However, other biological effects induced by opioids, include cytoprotection, immunomodulation, neuroendocrine regulation, and behavioral modification. Most of these biological responses are presumed to be manifested through the activation of three G-protein-coupled receptors (δ, κ, and μ). Endogenously, these receptors are stimulated by the release of opioid peptides (endorphins, dynorphins, and enkephalins). The expression of opioid receptors have been shown in virtually all major organ systems, including the central nervous system,3 heart,3 skin,34 and eye.24,27–29,35 In the eye, opioid receptors have been implicated in the regulation of iris function, accommodation, aqueous humor dynamics, corneal wound healing, and retinal development.24–30

To investigate whether opioid receptor activation by endogenous ligands can limit ischemic injury, we used an IPC model. IPC is defined as the ability of a nondamaging (transient) ischemic event to protect an organ or tissue from a subsequent damaging insult. The cytoprotective responses resulting from IPC have been identified in heart, brain, liver, lung, skeletal muscle, spinal cord, and retina; these studies have provided evidence that multiple mediators (e.g., adenosine, nitric oxide, bradykinin, erythropoietin, norepinephrine, and opioidergic peptides) can contribute to the development of the protective

<table>
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<tr>
<th>Retina (μm)</th>
<th>IPL (μm)</th>
<th>INL (μm)</th>
<th>OPL (μm)</th>
<th>ONL (μm)</th>
<th>RGCs (Cells/200 μm)</th>
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<tr>
<td>Control</td>
<td>183 ± 9.4</td>
<td>46.6 ± 3.4</td>
<td>24.5 ± 2.4</td>
<td>13.8 ± 2.4</td>
<td>45.7 ± 2.3</td>
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<td>Ischemic</td>
<td>121 ± 10.4*</td>
<td>25.9 ± 4.1*</td>
<td>17.1 ± 1.2</td>
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<td>35.7 ± 1.0</td>
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<tr>
<td>IPC</td>
<td>171 ± 17.3</td>
<td>37.3 ± 3.5</td>
<td>25.2 ± 5.2</td>
<td>12.4 ± 1.1</td>
<td>47.1 ± 4.9</td>
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<tr>
<td>IPC+naloxone</td>
<td>135 ± 8.4*</td>
<td>26.1 ± 4.7*</td>
<td>19.5 ± 2.0</td>
<td>9.63 ± 2.3</td>
<td>43.1 ± 5.2</td>
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Data are expressed as the mean ± SE; n = 3–4. Results were compared between groups by one-way ANOVA with Dunnett posttest. RGCs, retinal ganglion cells.

* Significant difference from control eye (P < 0.05).
In the retina, IPC has been shown to protect the retina from ischemic and ocular hypertensive injury. In this study, control animals subjected only to 45 minutes retinal ischemia showed a significant reduction in ERG a- and b-wave measurements. Morphometric analysis of these eyes revealed an overall reduction in retinal thickness, which was primarily due to thinning of the IPL and INL. However, mild disorganization and thinning of the ONL was also observed. As shown in earlier studies, animals that received IPC 24 hours before ischemic injury were protected from functional and structural changes induced by ischemic injury. In the present study, pretreatment with the nonselective opioid receptor antagonist naloxone before IPC, blocked the functional and morphologic protection induced by this procedure. However, ERG results obtained in this study cannot localize the site of injury beyond the inner retina. Nevertheless, these results are similar to studies in the heart and brain, where naloxone administration has also been shown to inhibit the protective effects of IPC against damage caused by hypoxia and ischemic injury.

In the present study, administration of naloxone alone 24 hours before ischemia, produced a trend toward enhanced functional deficits and structural degeneration when compared with ischemic eyes in control animals. Previous studies on examining morphologic changes induced by retinal ischemia have reported conflicting results on the retinal protective action of naloxone. Initial studies by Lam et al. found that intraperitoneal administration of naloxone just before retinal ischemia reduced the morphologic evidence of retinal injury. However, recent studies by Riazi-Esfahani et al. found that intravitreous administration of naloxone to rabbits afforded no protection to the retina from ischemic injury. Although the varying response of the ischemic retina to naloxone may reflect differences in the route of administration, dose, or species being tested, most of the data supports the idea that activation, not inhibition, of opioid receptors protects the retina from ischemic injury. However, it is possible that the protective actions of naloxone noted in initial retina studies are related to nonreceptor actions of naloxone, such as its ability to act as a free radical scavenger.

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To extend our studies, the pattern of opioid receptor expression in rat retina was examined. Results presented in Figures 4 and 5 demonstrate that all three opioid receptors (δ, κ, and μ) are expressed in the retina. Immunostaining showed that these receptors are primarily located in the inner retinal layers; however, the most intense staining was confined to the nerve fiber layer and the retinal ganglion cell layer. Although previous studies have provided evidence of the presence of retinal opioid receptors in binding assays or autoradiography, neither of these studies identified receptor subtype expression in the retina. To the best of our knowledge, this is the first study to identify the expression and location of the three opioid receptors subtypes (δ, κ, and μ) in the adult rat retina. The expression of opioid receptors within the inner retina layers is consistent with a neuroprotective role for these receptors, as the ischemic insult we used primarily affects these inner retinal layers.

Activation of opioid receptors by exogenous opioid agonists has been shown to protect the heart from ischemic injury. To determine whether the administration of exogenous opioid agonists can protect the retina against ischemic injury, the rats were treated with the nonselective opioid agonist, morphine (0.01–10 mg/kg) 24 hours before the creation of ischemic retinal injury. As shown in Figure 6, ischemia-induced retinal damage was ameliorated in the morphine-treated animals in a dose-dependent fashion (ED50 = 0.18 mg/kg), as determined by preservation of the a- and b-wave amplitudes. Furthermore, these protective actions of morphine were antagonized by pretreatment with naloxone (Fig. 7). Taken together, these data provide evidence that opioid receptor activation can protect the neural retina from subsequent ischemic injury.

Studies have shown that opioids can lower IOP or induce hyperventilation, potentially lowering blood PO2. Hence, the protection observed in this study may be related, in part, to changes in retinal perfusion and/or preconditioning associated with an acute drop in blood PO2. Studies on selected animals revealed that intraperitoneal administration of morphine (1 mg/kg) did not produce any noticeable behavioral changes, or significantly alter IOP at 2, 4, 6, or 24 hours after drug administration. In addition, no significant change in arterial blood oxygen saturation 1 hour after drug treatment was measured (data not shown). Systemic blood pressure was not measured. Hence, the responses measured in this study are consistent with the activation of retinal opioid receptors.

The protection afforded by morphine pretreatment alone was not complete, nor was the protection as robust as that measured in animals receiving IPC. Hence, the development of retinal IPC probably involves endogenous opioids working in concert with other factors, such as adenosine and HIF-1α. The timing for the development of IPC (24 hours) reflects that opioids and other factors induce specific changes in the behavior of retinal protein expression. These expression changes then could influence the level or activity of nitric oxide, inflammatory cytokines, stress proteins, extracellular glutamate, or endothelin, to create the neuroprotection observed with IPC.

A second possibility for the incomplete retinal protection induced by morphine is that the timing of the administration was not optimal. In rabbits, intravitreal morphine administration, 1 hour before the ischemic insult, has been shown to reduce the structural changes induced by retinal ischemia. In our studies, the IP administration of morphine up to 10 mg/kg 1 hour before ischemic injury did not result in any significant protection in retinal function when compared to rats receiving ischemic injury alone (data not shown). These differences in the protective actions of morphine may reflect species and/or pharmacokinetic (time-related) differences between the studies.

In summary, our study provides evidence that both endogenous and exogenous opioids initiate neuroprotective events in the ischemic retina. It is highly probable that retinal ischemia plays a central role in several ocular diseases. Therefore, our findings support the concept that enhancement of opioidergic activity in the eye during critical periods may present a viable neuroprotective strategy for the treatment of retinal diseases that exhibit an ischemic component in their etiology.

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References


