Relaxin Peptide Hormones Are Protective During the Early Stages of Ischemic Stroke in Male Rats

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The pregnancy hormone relaxin protects tissue from ischemic damage. The ability of relaxin-3, a relaxin paralog, to do so has not been explored. The cerebral expression levels of these peptides and their receptors make them logical targets for study in the ischemic brain. We assessed relaxin peptide-mediated protection, relative relaxin family peptide receptor (RXFP) involvement, and protective mechanisms. Sprague-Dawley rats receiving permanent (pMCAO) or transient middle cerebral artery occlusions (tMCAO) were treated with relaxin peptides, and brains were collected for infarct analysis. Activation of the endothelial nitric oxide synthase pathway was evaluated as a potential protective mechanism. Primary cortical rat astrocytes were exposed to oxygen glucose deprivation and treated with relaxin peptides, and viability was examined. Receptor involvement was explored using RXFP3 antagonist or agonist treatment and real-time PCR. Relaxin and relaxin-3 reduced infarct size after pMCAO. Both peptides activated endothelial nitric oxide synthase. Because relaxin-3 has not previously been associated with this pathway and displays promiscuous RXFP binding, we explored the receptor contribution. Expression of rxfp1 was greater than that of rxfp3 in rat brain, although peptide binding at either receptor resulted in similar overall protection after pMCAO. Only RXFP3 activation reduced infarct size after tMCAO. In astrocytes, rxfp3 gene expression was greater than that of rxfp1. Selective activation of RXFP3 maintained astrocyte viability after oxygen glucose deprivation. Relaxin peptides are protective during the early stages of ischemic stroke. Differential responses among treatments and models suggest that RXFP1 and RXFP3 initiate different protective mechanisms. This preliminary work is a pivotal first step in identifying the clinical implications of relaxin peptides in ischemic stroke. (Endocrinology 156: 638–646, 2015)

Stroke is the second leading cause of adult disability in the world (1). To date, there have been numerous advances toward a better understanding of the pathophysiology of ischemic stroke, leading to a number of new potential therapeutic targets. However, there is currently only one US Food and Drug Administration–approved agent for the treatment of ischemic stroke (2). The present study provides evidence to suggest that relaxin peptides have potential clinical applications in ischemic stroke.

Relaxin (H2 relaxin in humans and relaxin-1 in rats; traditionally referred to as simply “relaxin”) was discovered by Frederick Hisaw in 1926 when he injected serum from pregnant guinea pigs or rabbits into virgin guinea pigs and observed a dramatic relaxation of the pelvic ligament (3). For many years after its discovery, relaxin was studied solely as a reproductive hormone (4, 5). However, diverse roles in multiple tissues have since been identified. Various studies indicate that relaxin may be involved in

Abbreviations: eNOS, endothelial nitric oxide synthase; ic, intracortically; iNOS, inducible nitric oxide synthase; MCA, middle cerebral artery; nNOS, neuronal nitric oxide synthase; OGD, oxygen glucose deprivation; pMCAO, permanent middle artery occlusion; RXFP, relaxin family peptide receptor; tMCAO, transient middle artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride; WST, water-soluble tetrazolium salt.
protecting tissues, such as the lungs (6), pancreas (7), intestines (8), heart (9), and brain (10, 11) from the detrimental effects of ischemia. The protective effects of relaxin are numerous and include effects on vascular tone (12, 13) and cellular protection (14). The ability of relaxin to initiate multiple protective mechanisms upon administration may modulate numerous pathophysiological processes that are initiated after ischemic stroke. The cognate receptor for H2 relaxin is relaxin family peptide receptor (RXFP) 1 (15).

In addition to the main circulating relaxin peptide, a relaxin paralog called relaxin-3 (H3 relaxin in humans) was discovered in 2002 (16). Primarily found in the central nervous system, relaxin-3 has been shown to modulate neuroendocrine responses to stress (17), regulate feeding (18) and drinking (19) behaviors, stimulate gonadotropin release (20), and mediate sleep/wake rhythms (21) in rodents. The ability of relaxin-3 to provide protection to tissues under ischemic stress has not yet been explored, although the abundant cerebral expression of this peptide (16, 22) and its native receptor (23), RXFP3, make it a logical target for study. Because relaxin-3 demonstrates promiscuous binding among RXFPs (23, 24), development of an RXFP3 agonist (25) and antagonist (26) has allowed for assessment of selective receptor-mediated effects.

The objectives of the current study were to determine the effects of relaxin peptide administration on infarct size after ischemic stroke, elucidate receptor involvement, and explore mechanisms responsible for observed protection. Both permanent (pMCAO) and transient middle cerebral artery occlusion (tMCAO) rat stroke models were used, because differences in the pathophysiologic of each model provide unique insight into protective mechanisms. To allow for exploration of protective mechanisms in a cell-specific manner, an in vitro oxygen glucose deprivation (OGD) model using rat primary cortical astrocytes was used.

Materials and Methods

Male Sprague-Dawley rats (250–330 g; Charles River Laboratories) were used for the in vivo experiments, which were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the Animal Care Committees at the University of Guelph (Guelph, ON, Canada), Acadia University (Wolfville, NS, Canada), and the University of Prince Edward Island (Charlottetown, PEI, Canada). Animals were housed on a 12-h light/dark photoperiod and received food and water ad libitum. The relaxin peptides used, including recombinant H2 relaxin (H2), recombinant H3 relaxin (H3), and the selective RXFP3 agonist R3/I5 (23), were purchased from the Howard Florey Institute (Melbourne, Victoria, Australia). Dr Ross Bathgate of the Howard Florey Institute generously donated the RXFP3 antagonist R3 B1-22R (26).

Rats receiving pMCAO (performed at The University of Guelph and Acadia University) were anesthetized (sodium pentobarbital, 50 mg/kg ip; Ceva Animal Health), and left femoral veins were cannulated for iv administration of supplementary anesthetic (sodium pentobarbital, 15–30 mg/kg/h; Ceva Animal Health), which provided a stable plane of anesthesia for the full duration of the experimental time period. Body temperature was monitored and maintained at 37°C ± 1°C using a heating pad regulated by a temperature controller (TR200; Fine Science Tools). A burr hole was made in the skull overlying the position of the primary somatosensory (S1) cortex. Treatments were administered intracortically (ic) into the medial margin of the S1 cortex dorsal to the corpus callosum (coordinates: 0.8 mm caudal to bregma, 4.9 mm lateral to midline, and 5.0 mm ventral to the cortical surface) (27) with a microsyringe (30 gauge needle; Hamilton Company).

pMCAO was performed as described previously (10, 11). In brief, a burr hole (2 mm) was drilled in the squamous bone caudal to the orbit of the eye. The squamous bone was removed using bone rongeurs (Fine Science Tools) until the middle cerebral artery (MCA) was exposed. The meninges were then reflected, and the MCA was occluded using bipolar coagulation (Geiger 150-I) at 2 branch points: proximally ventral to the bifurcation to the frontal and parietal cortices and proximal to the bifurcation to the parietal cortex. Interruption of blood flow was visually confirmed.

Four hours after pMCAO, rats were humanely euthanized and perfused transcardially (with heparinized saline), followed by brain removal. Coronal brain sections (1 mm) were made using a Harvard brain matrix (Harvard Apparatus), stained using 2% 3,3',5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich) for 1.5 minutes and fixed in 10% formalin (Fisher Scientific). Digital images of each slice were analyzed using ImageJ software (National Institutes of Health) to measure the area of the infarct and the area of the ipsilateral hemisphere. Measurements were made from stained brain slices at the level of the anterior commissure, fornix, optical chiasm, and S1 cortex. Data are reported as the infarct area/ipsilateral hemisphere area ratio (infarct size).

In a first set of experiments, rats treated with H2 (n = 5) or H3 (n = 5) were compared with saline-treated (n = 5) rats. Treatments were administered ic 30 minutes before pMCAO. A separate set of rats (n = 5/group) received H2, H3, or saline ic 30 minutes after pMCAO. Activation of the endothelial nitric oxide synthase (eNOS) signaling pathway was then assessed as a potential relaxin peptide-mediated protective mechanism. A potent inhibitor of eNOS, L-NIO (9 mg in 200 mL of saline; Tocris Bioscience), was injected into the medial margin of the S1 cortex at a dose previously demonstrated to block the protective effects of relaxin (11). Injections were administered 15 minutes before iv treatment (n = 4/group) with either H2 (5 μg in 0.1 mL), H3 (5 μg in 0.1 mL), or saline (0.1 mL) 30 minutes before pMCAO.

In a second set of experiments, we assessed the relative gene expression of rxfp1 and rxfp3 in the S1 cortex of rats receiving (n = 4/group) MCAO or no MCAO (control) using real-time PCR. Rats were euthanized, and brains were removed as described above 30 minutes after pMCAO. The S1 cortex of each animal was rapidly dissected and flash frozen in liquid nitrogen. Total RNA from the collected tissue was isolated using TRIzol reagent (Life Technologies). The quantity and quality of isolated RNA were then assessed using the NanoDrop ND1000 spectrophotometer (Thermo Scientific) and Agilent 2100 bioanalyzer.
The relative expression of relaxin receptors between control and series spanning 5 points. The primers used are shown in Table 1. The efficiency was determined for all primers using a 10-fold dilution because splice variants have been reported (28). Next, primer room temperature. First, primers were designed to prevent amplification of genomic DNA. Moreover, rxfp1 primers were designed to only amplify the full-length receptor mRNA sequence, because splice variants have been reported (28). Next, primer efficiency was determined for all primers using a 10-fold dilution series spanning 5 points. The primers used are shown in Table 1. The relative expression of relaxin receptors between control and MCAO was calculated using the ΔΔCt method with β-actin as the housekeeping gene. We next assessed infarct size after activation of RXP1 and RXFP3 through administration (n = 5/group) of either H3 (10 ng in 200 nL) administered together with R3 B1-22R (RXFP3 agonist; 100 ng in 200 nL), or R3/I5 (selective RXFP3 agonist; 10 ng in 200 nL) to 30 minutes before pMCAO.

To allow for thorough exploration of protective mechanisms, a third set of experiments assessed relaxin peptide efficacy in a tMCAO model (performed at The University of Prince Edward Island). Rats were anesthetized with sodium thiobutabarbital (10 mg/kg ip; Sigma-Aldridge), which provided a stable plane of anesthesia for the full duration of the experimental time period. An endotracheal tube was inserted to facilitate breathing. Body temperature was monitored and maintained at 37°C using a temperature controller system (Physitemp Instruments). All treatments were administered into the medial margin of the S1 cortex dorsal to the corpus callosum as described above. Treatment was administered 10 minutes before tMCAO, followed by a 30-minute occlusion and 5.5 hours of reperfusion. Rats were treated (n = 6/group) with either H2 alone (10 ng in 200 nL), H3 alone (10 ng in 200 nL), R3 B1-22R alone (100 ng in 200 nL), R3 H2 (100 ng in 200 nL), R2/I5 (10 ng in 200 nL). All treatment groups were compared with saline-treated rats (200 nL of saline).

tMCAO was performed as described previously (29). In brief, animals were placed in a stereotaxic frame (David Kopf), and the right MCA was approached through a rostralcaudal incision of the skin and frontalis muscle at the approximate level of bregma. Blood flow through the MCA was impeded by the placement of a surgical suture behind the MCA at 3 designated positions along the exposed vessel. The ends of the sutures were positioned so that the middle of the each suture applied an upward pressure to the MCA and impeded blood flow. This 3-point placement of surgical sutures produced a highly reproducible and consistent focal ischemic lesion restricted to the ipsilateral cerebral cortex. To facilitate removal of the sutures at the end of the occlusion period (30 minutes), a few drops of warm PBS (37°C) were first applied to the areas where the MCA was in contact with the sutures. Blood was allowed to reperfuse the area for an additional 5.5 hours postocclusion.

At the end of each experiment, animals were perfused transcardially (with heparinized saline), and brains were removed, sliced, and stained as described above. Infarct volumes were calculated through use of scanned digital images of each brain section. The infarct areas for both sides of each brain section were calculated using a computer-assisted imaging system (Scion Corporation). The infarct areas for each side for each individual section were averaged and multiplied by the width of each section (1 mm) to give the infarct volume for each section. The sum of all the individual infarct volumes provided the infarct volume for each rat.

Astrocytes play a pivotal role in the pathogenesis of neuronal death in ischemic strokes, making them a promising therapeutic target (30). Although many of the supporting functions of astrocytes are functions that relaxin peptide administration affects (eg, providing cellular protection [14] and regulating blood flow [12, 13]), the presence of relaxin receptors on astrocytes has yet to be explored. To further investigate the mechanism(s) by which RXFP activation protects the brain from the detrimental effects of ischemia, primary rat cortical astrocytes (Life Technologies) were used in a fourth set of experiments. Astrocytes were cultured in astrocyte growth medium consisting of 85% DMEM (high glucose; Life Technologies), 14% fetal bovine serum (Life Technologies), and 1% penicillin-streptomycin (Life Technologies). Cultures were treated (n = 9/group) either 30 minutes before or after OGD initiation with 10 mL of glucose-free DMEM alone (control), low H3 (0.1 μg in 10 mL), high H2 (1 μg in 10 mL), low H3 (0.1 μg in 10 mL), high H3 (1 μg in 10 mL), high R3/I5 (0.1 μg in 10 mL), or high R3/I5 (1 μg in 10 mL). Ischemic conditions were induced in vitro with an OGD model, as described previously (31). In brief, glucose-free DMEM replaced the culture medium, and culture dishes were transferred to a hypoxia incubator chamber (Stemcell Technologies), which was purged with gas (95% N2 and 5% CO2) at a rate of 20 L/min for 5 minutes. The hypoxia incubator chamber was sealed and placed into a temperature-controlled (37 ± 1°C) incubator for 24 hours of OGD.

After 24 hours of OGD, culture dishes were removed from the hypoxia incubator chamber and washed once with PBS. A master mix of water-soluble tetratozolium salt (WST)-1 reagent (Roche Applied Science) was prepared in glucose-free DMEM at a dilution of 1:10 according to the manufacturer’s instructions. Primary cortical rat astrocytes were incubated with the master mix. After 2 hours of incubation, the absorbance was measured at 450 nm on a microplate reader. Empty wells containing WST-1 plus glucose-free DMEM were used as background controls. Data are reported as mean background corrected absorbance ± SEM. To assess the effect of relaxin peptide treatment on primary cortical astrocytes not exposed to OGD (no OGD), the same treatment groups as above were administered in high-glucose DMEM for 24 hours.

To assess relative gene expression of rxfp1 and rxfp3, total RNA was collected from rat primary cortical astrocytes receiving (n = 4/group) OGD and no OGD using TRizol reagent. The quantity and quality of isolated RNA were then assessed as described above for rat brain tissue. Amplification was performed as described above using the same primers.

**Table 1. Sequence of PCR Primers**

| Primer | Primer Sequence |
|--------|-----------------|
| RXP1   | 5'-TGGGCAGAATTCGGGATG-3' |
|        | 5'-TCCATTGCAGGGGACATTAC-3' |
| RXFP3  | 5'-TTGTGCTAAACCTGGCC-3' |
|        | 5'-AAATCTAGTGCCCCTCACCC-3' |
| β-Actin | 5'-CTAGGCAACCGTGAAMAAGAT-3' |
|        | 5'-AGAGGCTACAGGGACAACACA-3' |

(Agilent Technologies). Amplification was performed using one-step PCR on a ViiA7 real-time PCR system (Life Technologies) using the Power SYBR Green RNA-to-Ct 1 Step Kit (Life Technologies) as follows: reverse transcription at 48°C for 30 minutes for 1 cycle, Taq polymerase activation at 95°C for 10 minutes, denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute, both for 40 cycles, concluding with a hold at room temperature. First, primers were designed to prevent amplification of genomic DNA. Moreover, rxfp1 primers were designed to only amplify the full-length receptor mRNA sequence, because splice variants have been reported (28). Next, primer efficiency was determined for all primers using a 10-fold dilution series spanning 5 points. The primers used are shown in Table 1. The relative expression of relaxin receptors between control and MCAO was calculated using the ΔΔCt method with β-actin as the housekeeping gene. We next assessed infarct size after activation of RXP1 and RXFP3 through administration (n = 5/group) of either H3 (10 ng in 200 nL) administered together with R3 B1-22R (RXFP3 agonist; 100 ng in 200 nL), or R3/I5 (selective RXFP3 agonist; 10 ng in 200 nL) to 30 minutes before pMCAO.

To allow for thorough exploration of protective mechanisms, a third set of experiments assessed relaxin peptide efficacy in a tMCAO model (performed at The University of Prince Edward Island). Rats were anesthetized with sodium thiobutabarbital (10 mg/kg ip; Sigma-Aldridge), which provided a stable plane of anesthesia for the full duration of the experimental time period. An endotracheal tube was inserted to facilitate breathing. Body temperature was monitored and maintained at 37 ± 1°C using a temperature controller system (Physitemp Instruments). All treatments were administered into the medial margin of the S1 cortex dorsal to the corpus callosum as described above. Treatment was administered 10 minutes before tMCAO, followed by a 30-minute occlusion and 5.5 hours of reperfusion. Rats were treated (n = 6/group) with either H2 alone (10 ng in 200 nL), H3 alone (10 ng in 200 nL), R3 B1-22R alone (100 ng in 200 nL), H3 (10 ng in 200 nL) administered together with R3 B1-22R (100 ng in 200 nL), or R3/I5 (10 ng in 200 nL). All treatment groups were compared with saline-treated rats (200 nL of saline).

tMCAO was performed as described previously (29). In brief, animals were placed in a stereotaxic frame (David Kopf), and the right MCA was approached through a rostralcaudal incision of the skin and frontalis muscle at the approximate level of bregma. Blood flow through the MCA was impeded by the placement of a surgical suture behind the MCA at 3 designated positions along the exposed vessel. The ends of the sutures were positioned so that the middle of the each suture applied an upward pressure to the MCA and impeded blood flow. This 3-point placement of surgical sutures produced a highly reproducible and consistent
All rats and culture plates were randomized to receive treatments. In addition, all investigators were blinded to treatments during experimentation and analysis. In each experiment, means were compared between treatment groups by one-way ANOVA and the Tukey post hoc mean comparison test with GraphPad Prism (version 5.0 for Mac OS X; GraphPad Software). Means were considered significantly different at \( P < 0.05 \). Data are reported as means ± SEM.

**Results**

Pretreatment ic with H2 (0.05 ± 0.01) or H3 (0.04 ± 0.01) reduced infarct size by 55% or 64%, respectively, compared with that for saline-pretreated rats (0.11 ± 0.01) (Figure 1A). Post-treatment ic with H2 (0.05 ± 0.01) or H3 (0.02 ± 0.01) reduced infarct size by 62% or 84%, respectively, compared with that of saline post-treated rats (0.13 ± 0.04) (Figure 1B). There were no differences in infarct size between H2 treatment before or after MCAO and the respective H3 treatment before or after MCAO. Permanent occlusions of the MCA produced infarcts in the S1 cortex consistent in size with those of previous reports (10, 11).

Elucidation of eNOS involvement in mediating relaxin-induced protection was assessed with administration of relaxin peptides iv and L-NIO ic. Treatment with H2 iv (0.05 ± 0.01) or H3 iv (0.04 ± 0.01) reduced infarct size compared with that of saline-treated rats (0.11 ± 0.01) (Figure 1C). As anticipated, treatment with H2 and local inhibition of eNOS with L-NIO (H2 + L-NIO; 0.11 ± 0.02) abolished H2-mediated protection, and infarct size was not different from that for saline + L-NIO–treated rats (0.13 ± 0.01). Surprisingly, coadministration of H3 + L-NIO (0.12 ± 0.01) also abolished H3-mediated effects and infarct size was not different from that of salinel-treated rats (Figure 1C).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Relaxin peptide treatment 30 minutes before and after pMCAO decreased infarct size: implication of eNOS activation. A and B, Analysis of infarct size 4 hours after pMCAO in rats (n = 5/group) receiving ic pretreatment (A, left panel) or posttreatment (B, left panel) with saline (200 nL), H2 (10 ng in 200 nL), or H3 (10 ng in 200 nL) and representative TTC-stained slices from saline and relaxin peptide pretreated (A, right panel) or posttreated (B, right panel) rats. C, Analysis of infarct size 4 hours after MCAO in rats (n = 4/group) receiving iv pretreatment with saline (0.1 mL), H2 (5 μg in 0.1 mL), or H3 (5 μg in 0.1 mL) alone or in combination with ic L-NIO (9 mg in 200 nL of saline) (left panel) and representative TTC-stained slices (right panel). Values are means ± SEM. Means without a common superscript differ (\( P < .05 \)) by one-way ANOVA with the Tukey post hoc test.

We next investigated relative receptor contribution to the protective effects of relaxin peptides. To do so, we first assessed relative expression levels of both receptors in RNA isolated from the S1 cortex (Figure 2A) of rats receiving MCAO or no MCAO (control). Expression of *rxfp3* was 13.45 ± 1.59-fold less than that of *rxfp1* in control animals (Figure 2B). At 30 minutes after MCAO, *rxfp1* expression (15.44 ± 2.41; fold change from control) remained significantly greater than *rxfp3* expression (0.96 ± 0.09; fold change from control). However, the expression of *rxfp1* and *rxfp3* in control rats did not differ from the respective expression levels in rats receiving MCAO (Figure 2B). Together these results indicate greater relative expression of *rxfp1* in the S1 cortex and no change in receptor expression by 30 minutes after MCAO. We also assessed receptor selective activation through use of...
an RXFP3 antagonist and agonist. Both H3 + R3 B1-22R (0.03 ± 0.00) and R3/I5 (0.05 ± 0.02) treatments reduced infarct size compared with that for saline-treated rats (0.11 ± 0.01) (Figure 2C). The protection provided by H3 + R3 B1-22R and R3/I5 was similar to the protection provided by H3 alone (0.04 ± 0.01).

tMCAO was pursued to evaluate relaxin peptide-mediated protection in a model where blood flow was restored. Treatment with H2 (10.90 ± 2.25) did not reduce infarct size, whereas H3 (4.42 ± 0.78) reduced infarct size compared with that in saline-treated rats (13.07 ± 2.61) (Figure 3). To elucidate specific receptor activation and to further explore potential protective mechanisms either H3 + R3 B1-22R or R3/I5 was administered to rats receiving tMCAO. Whereas H3 reduced infarct volume compared with that for saline-treated rats, when the cognate receptor for H3, RXFP3, was antagonized through coadministration of H3 + R3 B1-22R (13.50 ± 3.40), infarct volume was not different from that of saline-treated rats (13.07 ± 2.61) (Figure 3). Administration of R3/I5 (4.90 ± 0.30) resulted in reduced infarct volume compared with that for saline-treated rats (13.07 ± 2.61) (Figure 3). Infarct volume was not different between rats treated with H3 and those treated with R3/I5. Transient occlusions of the MCA produced infarcts in the S1 cortex consistent in size with those of previous reports (29).

Lastly, we explored a potential RXFP3-mediated mechanism at the cellular level through use of an in vitro OGD model applied to cultured rat primary cortical astrocytes. Only pretreatment with high H3 (2.20 × 10^{-2} ± 4.81 × 10^{-3}), low R3/I5 (2.04 × 10^{-2} ± 4.67 × 10^{-3}), and high R3/I5 (2.65 × 10^{-2} ± 7.06 × 10^{-3}) increased WST-1 absorbance compared with that of controls (0.00 ± 0.00) (Figure 4A). Only posttreatment with high R3/I5 (5.50 × 10^{-3} ± 1.38 × 10^{-4}) increased absorbance compared with that of controls (0.00 ± 0.00) (Figure 4B). Absorbance was also assessed in astrocytes exposed to no OGD for 24 hours to assess the physiological effect of relaxin peptide treatment. Treatment with only low R3/I5 (1.71 × 10^{-1} ± 2.29 × 10^{-2}) and high R3/I5 (1.97 × 10^{-1} ± 1.25 × 10^{-2}) increased absorbance compared with that of controls (1.42 × 10^{-1} ± 3.51 × 10^{-3}) (Figure 4C). RNA isolated from rat primary cortical astrocytes receiving OGD and no OGD (control) was then assessed for rxfp1 and rxfp3 gene expression. Expression of rxfp3 was greater than expression of rxfp1 in control astrocytes (16.38 ± 2.31-fold change) (Figure 4D). Expression of rxfp3 (19.10 ± 1.55) was also greater than expression of rxfp1 (1.10 ± 0.22) in astrocytes receiving OGD (Figure 4D). The expression of rxfp1 and rxfp3 in control astrocytes did not differ from the respective expression levels in astrocytes receiving OGD.

**Discussion**

The pMCAO and tMCAO models each provide unique insight into protective mechanisms and specific receptor activation upon relaxin peptide administration. We demonstrated using infarct size analysis that both H2 and H3 treatment attenuated infarct development (Figure 1, A and B) in male rats receiving pMCAO. Although each relaxin
receptor has a native ligand, studies have revealed promiscuous binding between relaxin peptides and receptors. In particular, although the native receptor for relaxin-3 is RXFP3 (23), this peptide is also able to bind and activate RXFP1 with high affinity (24). A previous study comparing the localization of RXFP1 mRNA expression and the distribution of 33P human relaxin binding sites in the rat brain provides strong evidence to suggest that RXFP1 is the preferred, high-affinity H2 receptor in the brain (32).

Real-time PCR performed on the S1 cortex (Figure 2A) demonstrated that rxp1 is more abundantly expressed (Figure 2B). Supported by these findings, it is unlikely that H2 is binding RXFP3 to produce protective effects and more likely that H3 also binds RXFP1 to produce protective effects. To elucidate the contribution of each receptor to the observed reduction in infarct size, animals were treated with an RXFP3 antagonist or agonist. Both treatments demonstrated a reduction in infarct size (Figure 2C), implicating both RXFP1 and RXFP3 as targets in mediating the overall protective actions of relaxin peptides.

The relaxin-mediated reduction in infarct size was abolished upon local inhibition of eNOS (with L-NIO) (Figure 1C). Endothelium-derived nitric oxide (NO) is pivotal in maintaining vascular homeostasis (33). For example, eNOS knockout mice demonstrate a greater extent of compromised cerebral blood flow after MCAO (34), suggesting that eNOS-derived NO is protective after ischemic stroke. Activation of RXFP1 with relaxin has a widely documented vasodilatory effect on vasculature, exerted through endogenous NO released from endothelial cells, catalyzed by eNOS (35). In the tMCAO model, H3 and R3/I5 administration significantly reduced infarct size compared with that for saline-treated rats; yet when RXFP3 was antagonized, H3-mediated protection was completely abolished (Figure 3). In addition, H2 failed to reduce infarct size in this transient stroke model. If RXFP1 activation results in protection through eNOS signaling and increased blood flow, targeting this receptor may be less effective in tMCAO, where blood flow is restored. Furthermore, excessive NO production during ischemia/reperfusion may result in hyperemia and blood-brain barrier breakdown (36). Although we have demonstrated relaxin-mediated protection through activation of eNOS, in healthy neural tissue neuronal NOS (nNOS) is also a principal synthase involved in NO production. In addition, inducible NOS (iNOS) is produced by microglia and astrocytes in response to proinflammatory stimuli. Generally, it appears that nNOS induces neuronal damage, iNOS may be either protective or detrimental, and eNOS is protective (through vasodilation and angiogenesis) in cerebral ischemia (for a review, see Ref. 37). It will thus be important to study the effects of relaxin peptide administration on both nNOS and iNOS in the future.

Unlike RXFP1-mediated protective mechanisms, mechanisms activated by RXFP3 appear to play a larger role in achieving protection from ischemia in the tMCAO model. Considering differences between the two models apart from blood flow, the apparent different modes of ischemia-induced cell death between each rat stroke model supported pursuit of cellular viability experiments; the permanent model is primarily necrotic cell death, whereas the transient model is a combination of necrotic (within the core) and apoptotic (within the penumbra) cell death. The in vitro OGD model allowed for insight into the ability of specific receptor activation to maintain cellular viability,
although more focused experiments are required to thoroughly explore this mechanism in the future.

Based on the evidence above, protective mechanisms initiated by RXFP1 activation differ from those initiated by RXFP3 activation. The current study demonstrated that R3/I5 (an RXFP3 agonist) administration most effectively maintained cellular viability in cultured astrocytes exposed to 24 hours of OGD (Figure 4A), suggesting that protection at the cellular level may be mediated via RXFP3 activation. Administration of R3/I5 to astrocytes exposed to no OGD also resulted in increased absorbance readings (Figure 4C). Because WST-1 identifies metabolically active cells through mitochondrial respiratory activity and because astrocyte mitochondrial metabolism is an important target for the maintenance of neuronal survival (38), it is likely that activation of RXFP3 maintains cellular viability through an increase in cellular metabolism. We demonstrated a significantly greater rxfp1 relative to rxfp3 gene expression in the cerebral cortex (Figure 2B), yet we identified an unexpected greater relative expression of rxfp3 to rxfp1 in cortical astrocytes (Figure 3D). There is abundant expression of rxfp1 transcripts in neurons within the somatosensory cortex (32), perhaps accounting for the high expression levels observed in rat brain tissue in the current study. Injury after ischemic stroke is primarily a result of the inability of astrocytes to provide metabolic support to neurons, which they are highly dependent on (30). Targeting neuronal protection alone, which has been a large focus of drug development, is insufficient to promote improvement after ischemic stroke. A recent focus has thus been to develop agents that protect astrocytes under ischemic conditions (39). Taken together, the ability of RXFP3 activation to maintain astrocyte viability is probably an important contributor to the overall neuroprotection.

The current study also demonstrated that administration time (30 minutes before or after MCAO) did not alter the effectiveness of either peptide to significantly reduce infarct size in vivo. Whereas pretreatment with relaxin peptides in vitro was able to significantly increase WST-1 absorbance, posttreatment did not compared with that of controls. The discrepancy between treatment 30 minutes after ischemia in vivo and 30 minutes after ischemia in vitro suggests that protection of astrocytes from ischemia is contributing to overall defenses but that other protective mechanisms are also involved. Previous identification of specific relaxin binding sites in circumventricular organs (40, 41) suggested a physiological role of relaxin in the central control of vascular volume, blood pressure and fluid balance: evidence that has since been supported by several studies (42–44). Although the current study explored cellular protection and the involvement of the NO pathway in mediating cerebral protection, supporting the respective findings that relaxin peptides may provide protection from ischemia through activation of mitochondrial respiratory pathways and the eNOS pathway, it will be important and intriguing to further elucidate the ability of relaxin peptides to alter cerebral vascular volume, blood pressure, vasoactivity, and fluid balance after ischemic stroke.

Figure 4. Relaxin peptide treatment 30 minutes before and after OGD maintained primary cortical rat astrocyte WST-1 absorbance. A and B, Analysis of cellular absorbance with WST-1 assay after 24 hours of OGD in primary cortical astrocytes (n = 9/group) receiving pretreatment (A) or posttreatment (B) with glucose-free media alone (10 mL; control), low H2 (0.1 μg in 10 mL), high H2 (1 μg in 10 mL), low H3 (0.1 μg in 10 mL), high H3 (1 μg in 10 mL), low R3/I5 (RXFP3 agonist; 0.1 μg in 10 mL), or high R3/I5 (1 μg in 10 mL). C, The same treatments were administered in high-glucose media to astrocytes not exposed to OGD for 24 hours (no OGD). D, Real-time PCR for RNA isolated from primary cortical astrocytes, demonstrating relative gene expression (normalized to β-actin) of rxfp3 and rxfp1 in cells receiving either OGD or no OGD (control). Values are means ± SEM. Means without a common superscript differ (P < .05) by one-way ANOVA with the Tukey post hoc test.
In summary, collectively, data suggest that relaxin peptides are effective in protecting the brain from the deleterious effects of ischemic stroke and that this protection is mediated by activation of both RXFP1 and RXFP3. Differential responses observed among treatment groups and ischemic models suggest that RXFP1 and RXFP3 activation initiate different protective mechanisms. This research furthers our understanding of the role that relaxin peptides play in limiting damage to brain tissue during ischemia. Our findings follow the recent exploration of a recombinant form of relaxin (called serelaxin) as a treatment for human heart failure (45). Considering that agents frequently fail to successfully translate from basic research to clinical trials, it is valuable to have evaluated the efficacy of relaxin administration in multiple models, performed in multiple laboratories and using multiple administration routes (46). Our findings are a pivotal first step in identifying potential clinical implications of relaxin peptides in ischemic stroke.

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