PARATHYROID HORMONE-RELATED PROTEIN INDUCTION IN FOCAL STROKE: A NEUROPROTECTIVE VASCULAR PEPTIDE

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Abstract

Parathyroid hormone-related protein (PTHrP) is a multifunctional peptide that enhances blood flow in non-CNS vascular beds by causing vasodilation. PTHrP expression is induced in non-CNS organs in response to ischemia. Experiments were therefore undertaken to determine whether PTHrP can be induced in brain in response to ischemic injury and whether PTHrP can act locally as a vasodilator in the cerebral vasculature, an effect that could be neuroprotective in the setting of stroke. PTHrP expression was examined by Northern analysis and immunohistochemical staining in male Sprague Dawley rats subjected to permanent middle cerebral artery occlusion (MCAO). Vasodilatory effects of superfused PTHrP(1-34) on pial arterioles were determined by intravital fluorescence microscopy. Effects of PTHrP(1-34) peptide administration on MCAO infarction size reduction were assessed. PTHrP expression was induced in the ischemic hemisphere as early as 4 hours after MCAO and remained elevated for up to 24 hours. Increased immunoreactive PTHrP at sites of ischemic tissue injury was located in the cerebral microvessels. Superfusion with PTHrP(1-34) peptide for up to 25 min increased pial arteriolar diameter by 30% in normal animals. In animals with permanent MCAO, PTHrP(1-34) peptide treatment significantly decreased cortical infarct size (-47%). In summary, PTHrP expression increases at sites of ischemic brain injury in the cerebrovasculature. This local increase in PTHrP could be an adaptive response that enhances blood flow to the ischemic brain, thus limiting cell injury.

Key Words: cerebrovasculature, vasodilation, infarction, brain, PTHrP
Introduction

Parathyroid hormone related protein (PTHrP) was first discovered as the causative factor in humoral hypercalcemia of malignancy (33,47). Although full length PTHrP can be post-translationally cleaved into multiple smaller peptides, most of the known biological effects of PTHrP occur via binding of N-terminal-containing peptides to the PTH receptor (PTH1R) (33). Thirty four-amino acid, N-terminal peptides of PTH and PTHrP bind with similar affinity to classic G-protein coupled PTH1R to activate two signaling pathways--adenylyl cyclase/protein kinase A (PKA) and phospholipase C/protein kinase C (PKC) (33,47). In malignancy, high circulating plasma levels of tumor-derived PTHrP bind to PTH1R in kidney and bone causing hypercalcemia (33,47). However, subsequent to the discovery of PTHrP and the cloning of PTH1R, it is now appreciated that both PTHrP and the PTH1R are expressed in a wide variety of normal tissues and cell types where PTHrP, in contrast to its systemic effects in malignancy, acts locally in a paracrine or autocrine fashion (33,47).

One such demonstrated site of paracrine or autocrine PTHrP action is the non-CNS vasculature. In vitro, cytokines or hypoxia induce PTHrP expression in vascular endothelial cells (7,8,41), while vasoconstrictive peptides induce PTHrP expression in smooth muscle cells (20). Ex vivo PTHrP(1-34) treatment causes vasodilation and increased blood flow in the perfused rat kidney, heart, aorta, or femoral artery and in the human placenta (5,6,27,30,31). Over expression of PTH1R in the vascular smooth muscle cells of transgenic mice results in a decrease in systemic blood pressure and peripheral vascular resistance (34), while intravenous bolus administration of PTHrP(1-34) can cause transient hypotension (30). To our knowledge, the effects of PTHrP on vascular tone in the CNS have not been explored.
PTHrP and PTH1R are also expressed in the brain. However, the role and regulation of PTHrP in the CNS is not well understood (33). Neurons have been identified as the main site of expression of PTHrP and its cognate receptor in normal brain (33,48,49). In vitro studies suggest that neuronal PTHrP may be induced during excitotoxic cell injury or apoptosis and prevent cellular death via autocrine or paracrine binding to the PTH1R (2,32). Transformed or reactive astrocytes (16,29,42), but not normal glial cells, also express PTHrP and PTH1R. In vitro treatment with PTHrP(1-34) induces glial expression of IL-6 (16), a cytokine with neuroprotective effects during cerebral ischemia (26). In contrast, the regulation and function of PTHrP expression in the CNS vasculature has, to our knowledge, not previously been explored.

PTHrP is a member of the cascade of cytokines induced during the inflammatory response in non-CNS organs (12,15). Recent studies by Funk et al (16) demonstrating PTHrP induction in reactive astrocytes in response to mechanical brain injury have provided the first evidence that increased PTHrP expression may also accompany CNS inflammation. Of the many factors that can incite an inflammatory response in brain, cerebral ischemia is one of the most clinically important. We have therefore postulated that cerebral ischemia might also be a stimulus for increased PTHrP expression in brain. Indeed, hypoxia has already been demonstrated to induce PTHrP expression in non-CNS tissues, such as kidney and heart (41,43), and cytokines induced during cerebral ischemia, such as TNF and IL-1, are known to stimulate PTHrP expression in vascular endothelial cells and glia (7,8,16). Although the effects of PTHrP(1-34) on cerebral blood flow are not known, we hypothesized that locally produced PTHrP could help maintain blood flow in ischemic brain by causing cerebral vasodilation.
To test these hypotheses, studies were first undertaken to determine whether PTHrP expression was indeed induced in brain in response to ischemic injury using permanent occlusion of the middle cerebral artery in rats as an experimental model. The time course of increased PTHrP expression was compared with that of other inflammatory cytokines known to be activated by ischemic stroke (1). To determine whether the cerebral microcirculation is also a potential target for PTHrP action in brain, vasodilatory effects of PTHrP on pial arterioles, vessels that mirror the vascular response of deeper cortical vessels (38), were assessed by intravital fluorescence microscopy. Lastly, to elucidate a possible protective function of PTHrP during cerebral ischemia, the effect of icv PTHrP(1-34) administration on infarct size was determined in MCA-occluded (vs. sham) animals.
Materials and Methods

Materials  PTHrP(1-34) and PTH(3-34) were obtained from Bachem (Torrance, CA); human serum albumin was obtained from Immuno-U.S., Inc (Rochester, MI); and forskolin and barium chloride were obtained from Sigma (St. Louis, MO).

Middle Cerebral Artery Occlusion  All experimental animal procedures were conducted in accordance with University of Arizona and Institute for Laboratory Animal Research guidelines using anesthetized male Sprague Dawley rats (250-350 g). Middle cerebral artery occlusion (MCAO) was induced using the intraluminal filament method, as previously described by Ritter et al. (36,40). Briefly, rats were anesthetized via a facemask with 1L N₂0, 0.5L O₂, and 0.5% to 1% halothane while maintaining a constant body temperature (37°C ± 0.5°C). Following exposure of the right common carotid and cauterization of appropriate branches, the external carotid artery was isolated and cauterized. A nylon filament (3-0) with a rounded tip was inserted into the external carotid stub and advanced 18 mm into the internal carotid artery. The filament was secured, the neck incision was sutured, and the animals were allowed to recover. Sham-operated animals underwent the same surgical procedure, excluding placement of the intraluminal filament. One hour after permanent filament placement or sham operation, neurological function was assessed using four standard tests, as previously described by Ritter et al. (40): (1) level of consciousness (LOC), (2) spontaneous circling, (3) front limb paresis, and (4) front limb symmetry. To be included in the study, MCAO animals had to demonstrate a minimum score of 1 in every test (scored 0-4/test); a total minimum score of 6; and a maximum score of 3 in the LOC test (absence of coma or seizures).
Northern Analysis Brains were removed from MCAO and sham-operated animals 2, 4, 12 or 24 h following ischemic injury and quickly dissected coronally to discard the non-injured frontal cortex and cerebellum. The remaining hemispheres were then bisected longitudinally and frozen separately in liquid nitrogen prior to storage at –70°C. Polyadenylated RNA, isolated from the hemispheres ipsilateral or contralateral to MCAO (or sham operation), was assessed by Northern analysis using methods and [32P]-labeled cDNA probes previously described by Funk et al (10,11,14,16) to determine the time course of changes in expression of mRNA for PTHrP and its cognate receptor (PTH/PTHrP receptor) vs. other CNS inflammatory cytokines known to be activated by ischemia (TNF-α, IL-1β, and IL-6) (1). Blots were exposed to film at -70°C using intensifying screens, and autoradiographic intensity was quantitated using a BioRad Model GS-700 Imaging Densitometer.

Immunohistochemistry Brains were immediately removed from euthanized animals, cut into two-mm coronal sections, and fixed in 10% buffered formalin prior to paraffin embedding. Serial tissue sections were processed for immunohistochemical staining as previously described by Funk et al (14) using an affinity-purified polyclonal primary antibody directed against PTHrP(34-53) (Oncogene, Cambridge, MA). Brain sections were processed for antigen unmasking by heating for 10 minutes at 95°C in 10 mmol/L sodium citrate (pH 6.0) prior to immunostaining. Specificity of PTHrP immunostaining was verified by the absence of staining observed when serial tissue sections were treated with PTHrP antibody that had been preincubated with a 20-fold excess by weight of PTHrP(34-53) peptide (Oncogene, Cambridge, MA). Astrocytes, endothelial cells, and smooth muscle cells were also identified in serial sections using antibodies directed against glial fibrillary acidic protein (GFAP, Zymed, So, San
Francisco, CA), Factor VIII-related antigen (BioGenex, San Ramon, CA), or smooth muscle actin (Zymed, South San Francisco, CA), respectively.

Cranial Window Placement and Intravital Microscopy Preparation  Male Sprague-Dawley rats (250 to 350 g) were anesthetized with pentobarbitol (50 mg/kg), intubated, and continuously respirated. Anesthesia was maintained with 0.1 cc (50 mg/ml) of pentobarbital per hour as needed. Tail artery and vein catheters were placed for continuous measurement of blood pressure and administration of drugs, respectively. To achieve muscle paralysis for a stable microscopic field, vecuronium bromide (2.4mg/hr) was infused continuously. Throughout each experiment, body temperature was monitored continuously with a rectal probe and maintained at 37°C with a heating pad. An open cranial window was prepared over the right cortical-parietal brain surface as previously described by Ritter et al (36). Immediately after the craniotomy, mineral oil was placed over the preparation to prevent exposure of the pial vasculature to air during removal of the dura. Subsequent to removal of the dura, the microvascular preparation was continuously superfused with a 37°C artificial cerebrospinal fluid (aCSF) solution that was monitored with respect to gas tensions and pH (Radiometer, ABL) (40). Using fluorescence videomicroscopy and iv injection of FITC-BSA (1 ml of a 5% solution) to identify arteriole margins, changes in cerebral arteriole diameter were assessed in randomly selected pial arterioles by measurement of TV monitor images using a calibrated ruler. In each experiment, peptide superfusion was preceded by superfusion with peptide vehicle alone (0.1% apyrogenic human serum albumin [HAS] in aCSF) to monitor for non-specific effects of vehicle on arteriole tone. Additionally, the vascular reactivity of all pial preparations was verified 15 minutes after the peptide superfusions by treatment with 10^6 mol/L forskolin, a cAMP stimulating agent that is a
known cerebral arteriolar vasodilator (45), followed by 2.5% barium chloride (\(\text{BaCl}_2\)), a known vasoconstrictor (39). Each pial arteriole preparation was superfused once with PTHrP(1-34), delivered at a constant rate by mixing with continuously flowing aCSF (1.7 cc/min) to achieve a final PTHrP concentration of \(1 \times 10^{-6}\) mol/L. This dose elicits a maximal dilatory and/or blood flow response in non-CNS vascular beds (5,6,27). In one set of experiments, animals were treated with \(1 \times 10^{-6}\) mol/L PTH(3-34), a peptide that does not activate the adenylyl cyclase pathway (31), prior to superfusion with PTHrP(1-34) in order to identify post-receptor pathways responsible for the vascular effects of PTHrP.

**Intracerebral ventricular infusion of PTHrP(1-34)** One week prior to MCAO, outer guide cannulas (Plastics One, Roanoke, VA) were placed in pentobarbital-anesthetized rats under stereotaxic control using coordinates and methods previously described by Chen *et al* (4,35) to allow for later placement of an inner cannula into the right lateral ventricle. At the time of the experiment, animals were prepared as described for MCAO, with the additional manipulation of placement of the inner cannula into the lateral ventricle (4,35,37). For icv PTHrP(1-34) peptide treatment, a dosing strategy similar to that successfully used for icv treatment of stroke with other short acting peptides, such as IL-6 and IL-1ra, was employed (25,26). Animals were given a bolus (5 µl/1-2 min) of 200 ng PTHrP(1-34) in apyrogenic 0.1% HSA in normal saline (NS) vs. 0.1% HSA/NS alone (vehicle) 30 minutes prior to and 90 minutes after permanent occlusion of the MCA. Because up to 50% of the total amount of icv administered peptides can appear in the peripheral circulation over 4 h (3), a dose of PTHrP (200 ng) was chosen that could not elicit a systemic hypotensive response (30). Arterial blood pressure was continuously monitored by arterial line at the times of injections. Subsequent to the last icv injection, tail catheters and inner
icv cannulas were removed. Animals were allowed to recover with neurological testing at 4h and removal of brain for infarct analysis at 24 h.

**Measurement of Cerebral Infarction Size** Cerebral infarction volume was determined as previously described by Ritter *et al* (40) using standard methods. Briefly, 24 h after MCAO, animals were euthanized by an overdose of halothane. The brains were immediately removed and sectioned into seven 2-mm coronal slices, followed by immersion in 2% triphenyl tetrazolium chloride (TTC) and subsequent fixation in 10% buffered formalin. For image analysis, each brain section was photographed, scanned (600 dpi), and the area of infarction and area of each hemisphere were measured using NIH Image software. The contribution of edema (which was not different between controls and treated animals) to infarct volume was corrected for using standard methods (by subtracting the volume of the noninfarcted ipsilateral hemisphere from the volume of the contralateral hemisphere), with infarcted volume expressed as a percent of the contralateral hemisphere (40).

**Statistical analysis** Values are presented as mean ± SEM with statistical significance determined by ANOVA with post hoc testing, Student t test, or Mann Whitney testing (Instat, Graphpad, San Diego, CA). For analysis of changes in vessel diameter over time, values for an individual vessel were compared by paired analysis.
Results

Effect of MCAO on cerebral PTHrP and cytokine mRNA levels  Twenty four h after permanent MCAO, PTHrP mRNA levels were increased 4-fold in the ischemic cortex, as compared to the non-ischemic contralateral cortex (Fig. 1A,B). In sham-operated animals, PTHrP mRNA levels were unchanged and no different than those found in the non-ischemic cortex of MCAO animals (Fig. 1B). At 24 h, mRNA levels for TNF-α, IL-1β, and IL-6 were also increased 4-8 fold in the ischemic (vs. contralateral non-ischemic) cortex (Fig. 1C). Unlike PTHrP, mRNA levels for these cytokines were not detected by Northern analysis in sham-operated animals (Fig. 1C). However, consistent with previous reports (23,24), TNF-α, IL-1β, and IL-6 mRNA levels were detectable, albeit at lower levels, in the hemisphere contralateral to the ischemic injury (Fig. 1C). The time course of mRNA induction for these cytokines was compared to PTHrP in the ischemic cortex (Fig. 2). As has been previously reported (23,24), TNF-α and IL-1β mRNA levels were increased at the earliest time point examined (2 hrs after permanent MCAO) (Fig. 2A-B). In contrast, PTHrP and IL-6 mRNA levels in the ischemic cortex were not increased until 4 h after MCAO (Fig. 2C-D). PTH/PTHrP receptor mRNA levels, which were unchanged in sham-operated animals, exhibited a small, but statistically significant, decrease in ischemic (vs. contralateral non-ischemic) brain at 24 h (Fig. 1B), but not at earlier times (data not shown).

Immunohistochemical localization of PTHrP following focal stroke  PTHrP protein was localized in normal and ischemic brain by immunohistochemical analysis (Fig. 3). Specificity of PTHrP staining was verified in all cases by the absence of staining seen in consecutive tissue sections treated with PTHrP antibody that had been preincubated with an excess of antigen (e.g. Fig. 3E vs. 3F). In non-ischemic brain, immunoreactive PTHrP in the striatum (Fig. 3A) and cortex (Fig.
3B) was located in neurons (double arrowheads), while the microvasculature (arrows) was PTHrP negative. Four h following permanent MCAO, neuronal PTHrP immunoreactivity persisted and PTHrP immunoreactivity also began to appear in the vessels of the ischemic hemisphere (data not shown). At 24 h, neuronal PTHrP staining (double arrowheads) persisted in the infarct penumbra (Fig. 3C) but decreased in the infarct (Fig. 3D [cortex]/3E[striatum]), while vascular PTHrP staining increased in large and small vessels in all areas of injury (Fig. 3C/D/E, arrows). Astrocytes showed no PTHrP immunoreactivity 4 or 24 h after MCAO. Comparison of immunostaining for vascular PTHrP (Fig 3C-E), Factor VIII-related antigen-positive endothelial cells (Fig. 3G), smooth muscle actin-positive vascular smooth muscle cells (Fig. 3H), and GFAP-positive perivascular astrocytes (Fig. 3I) in serial sections suggested that longitudinal endothelial cells lining the vasculature, and not circumferential vascular smooth muscle cells or perivascular astrocytes, were the source of immunoreactive PTHrP in ischemic vessels.

Effects of PTHrP(1-34) on pial arteriolar diameter  When the pial microcirculation of normal animals was sequentially superfused (5 min/drug with a 5-15 min aCSF washout between drugs) with vehicle (0.1% HSA), PTHrP(1-34) (10^{-6} mol/L), forskolin (10^{-6} mol/L), and BaCl_2 (2.5 %), PTHrP(1-34) and forskolin both caused a significant increase in arteriolar diameter (31% and 54%, respectively), while BaCl_2 caused arteriolar diameter to decrease (Fig. 4A). Comparison of the dilatory response of larger (> 25 µm) vs. smaller (< 25 µm), more terminal arterioles revealed a dilatory effect of PTHrP and forskolin, a cAMP stimulating agent, on vessels of both sizes (Fig. 4B). However, the smaller terminal arterioles had a greater dilatory response to both PTHrP(1-34) (42%) and forskolin (76%) (Fig. 4B). Prolonged treatment with PTHrP(1-34)
resulted in an immediate (1 min) and persistent increase in arteriolar diameter over 25 minutes of superfusion (Fig 4C). Vessels remained responsive to subsequent challenge with $10^{-6}$ mol/L forskolin (43% increase, p< 0.001) following prolonged PTHrP(1-34) treatment. Superfusion with $10^{-6}$ mol/L PTH(3-34), a peptide that binds the PTH/PTHrP receptor but does not activate the cAMP signaling pathway (31), had no effect on arteriolar diameter (Fig. 4D, open circles), while subsequent challenge with $10^{-6}$ mol/L PTHrP(1-34) elicited a typical 30% increase in arteriolar diameter (Fig. 5, closed circles). A representative example of the vasodilatory response of the pial microcirculation to PTHrP(1-34) is given in Figure 4E (baseline) and Figure 5F (during PTHrP(1-34)). Mean arterial blood pressure did not change in response to superfusion with PTHrP(1-34) peptide, even after 25 minutes of treatment (data not shown). Physiologic $P_{O_2}$ (132.2 ± 7.7 mm) and $P_{CO_2}$ (32.1 ± 1.10) values were carefully maintained throughout the course of the experiments.

**Effects of PTHrP(1-34) treatment on infarct size**  Physiologic parameters, including mean arterial blood pressure, pH, $P_{CO_2}$, temperature, and glucose, were the same in vehicle and PTHrP(1-34)-treated animals at baseline, immediately pre-MCAO, and pre- and post- treatments. No experimental animals were excluded from either treatment group based on neurologic scoring. Mean total infarct size in rats treated with PTHrP(1-34) peptide was 34% less than that in animals treated with vehicle alone (Fig. 5), but this difference was not significant (p < 0.06). However, examination of infarct volume in the cortical vs. subcortical areas (Fig. 5) demonstrated a significant reduction in cortical infarct volume in the PTHrP(1-34) treated group (-47%, p < 0.02). Subcortical infarct size was not effected by PTHrP(1-34) peptide treatment (Fig. 5).
Discussion

These experiments provide, to our knowledge, the first evidence that enhanced PTHrP gene expression is induced in the brain in response to focal ischemia. Unlike other inflammatory cytokines, PTHrP is expressed constitutively in brain, mainly by neurons, including those in the parietal cortex and striatum (48,49). Because increased CNS PTHrP expression was previously described in reactive astrocytes formed in response to stab wound injury (16), we anticipated finding increased PTHrP expression in reactive astrocytes in the infarct penumbra in response to CNS ischemia. Instead, the vasculature of the injured hemisphere, rather than reactive astrocytes, was found to be the site of increased immunoreactive PTHrP during the first 24 h following permanent MCAO.

Just as endothelial cells have been reported to be the source of increased PTHrP expression in the ischemic myocardium (41), vascular endothelial cells appeared to be the source of increased immunoreactive PTHrP in the vasculature of ischemic brain. Increased immunoreactive PTHrP was found in vascular endothelial cells as early as 4 h after MCAO and persisted for up to 24 h. This increase in vascular PTHrP protein may be the result of a local increase in gene expression, as PTHrP mRNA levels were increased in the ischemic hemisphere over the same time period. Additionally, preliminary evidence of a positive arterio-venous PTHrP gradient across the ischemic brain at 24 h (i.e. 20% lower PTHrP levels in superior sagittal sinus vs. aortic plasma; Funk et al, unpublished data) prior to breakdown of the blood brain barrier (17,22) suggests the possibility that uptake of PTHrP from the circulation may also contribute to the increase in vascular PTHrP demonstrated at later time points in ischemic brain (35,44).
PTHrP mRNA induction in the ischemic hemisphere was preceded by induction of mRNA for TNF-α and IL-1β, two cytokines that have been demonstrated to induce PTHrP expression in other in vivo and in vitro models of inflammation, including endotoxemia and cytokine stimulation of endothelial cells and astrocytes (7,8,10,16,42). This finding is therefore consistent with the postulate that TNF-α and/or IL-1β may also mediate ischemia-induced PTHrP expression in the brain. Similarly, because PTHrP can induce IL-6 expression in multiple cell types, including glia (14,16), the delayed expression of IL-6 found in ischemic brain might also be attributable to local increases in PTHrP.

At all time points examined, mRNA for the PTH/PTHrP receptor (PTH1R) was expressed in the ischemic hemisphere, although at the time of maximal induction of PTHrP (24 h), levels of receptor expression were decreased. This reciprocal regulation of PTHrP and PTH1R, which is consistent with the well-described ability of PTHrP to downregulate the expression of its receptor (9,13,43), provides further evidence of a biological effect of locally enhanced PTHrP expression in ischemic brain.

Given our finding of increased immunoreactive PTHrP in the microcirculation of the ischemic brain, we postulated that one possible protective effect of this vasoactive peptide during stroke could be to enhance cerebral blood flow. Consistent with this hypothesis and with the known vasodilatory effects of N-terminal PTHrP in non-CNS vascular beds (5,6,27,30,33), we found that superfusion of the pial microcirculation with PTHrP(1-34) significantly increased arteriole diameter by 30%. According to Poiseuille’s equation, wherein flow is proportional to the fourth power of the vessel radius, this 30% increase in arteriolar diameter could result in a 3-fold
increase in blood flow in a setting of constant pressure, as was documented in these experiments. Because the smaller terminal arterioles, such as those studied here, strongly influence cerebrovascular resistance and blood flow (18), PTHrP may therefore play a critical role in the maintenance of cerebrovascular blood flow. Moreover, because the vascular response of the pial arterioles are similar to the cerebral circulation as a whole and because changes in pial arteriole diameter parallel changes in regional blood flow (38), these findings suggest that ischemia-induced PTHrP in microvessels in areas of ischemic brain could serve to enhance cerebral blood flow to the damaged cortex.

Binding of N-terminal PTH/PTHrP peptides to the PTH1R can stimulate adenylyl cyclase/PKA and/or phospholipase C/PKC signaling pathways (33,47). A previous report by Huang et al has demonstrated a PTH(1-34)-mediated increase in cAMP formation in cerebral microvessels ex vivo (21), suggesting that the vasodilatory effects of PTHrP(1-34) demonstrated here could be mediated via an adenylyl cyclase signaling pathway. Consistent with this hypothesis and with the demonstrated role of cAMP in mediating PTHrP(1-34) vasodilation in non-CNS vascular beds (28,46), superfusion of the pial arterioles with PTH(3-34), a peptide that binds to the PTH1R but does not stimulate cAMP formation (21,31), had no effect on arteriolar diameter. Homologous desensitization to the sustained vasodilatory effects of PTH/PTHrP peptides, but not heterologous desensitization to subsequent dilation by other cAMP-stimulating agents, such as forskolin, has been reported to occur in response to PTH/PTHrP peptides in some non-CNS vascular beds (28,31). However, under the conditions of the experiments described here, neither homologous nor heterologous desensitization of the cerebral microcirculation was seen in response to PTHrP(1-34) treatment. Because parenchymal and pial arterioles respond similarly
to vasoactive stimuli (38), these findings suggest that sustained increases in PTHrP in the cerebral microcirculation, such as those occurring during ischemia, may be associated with a sustained increase in the diameter of those microvessels that regulate local blood flow.

Lastly, the demonstration of a protective effect of PTHrP(1-34) peptide treatment in limiting cortical infarct size is consistent with the hypothesis that endogenously produced PTHrP also has a protective effect in ischemic brain. In particular, because a lack of collateral blood flow and differences in microvascular structure that may allow for early plugging of terminal arterioles make the striatal region more difficult to salvage following MCAO (50), the isolated protective effect of PTHrP(1-34) treatment in decreasing cortical, but not striatal, infarct size suggests that this vasodilatory peptide is neuroprotective in those areas of the brain that can be most easily salvaged by an increase in blood flow.

In summary, the studies described here provide novel evidence demonstrating an increase in local vascular PTHrP gene expression in ischemic brain, as well as the ability of N-terminal PTHrP to act as a potent vasodilator in the pial microcirculation and to reduce cortical infarct size by almost 50%. Additional studies will be required to identify all possible CNS targets for PTHrP action during ischemia, as PTHrP, in addition to the vasodilatory effects demonstrated here, has also been reported to have direct protective effects on neurons and to induce glial expression of neuroprotective cytokines (2,16,32). However, the beneficial effect of PTHrP on cortical infarction is consistent with the hypothesis that endogenous increases in cerebral PTHrP may serve to protect the brain during ischemia by preserving cerebral blood flow. Moreover, the demonstration of a protective effect of exogenously administered PTHrP(1-34) suggests that this
peptide, which has been administered in clinical trials for the treatment of osteoporosis in doses as high as 400µg/d (19), may also be useful in acute therapeutic interventions aimed at improving clinical outcomes in patients suffering from stroke.
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References


Figure Legends

1. **Effect of 24 h permanent MCAO (vs. sham-operation) on PTHrP, PTH1R and cytokine mRNA expression.** (A) Northern analysis of PTHrP (and actin) mRNA levels in ischemic vs. nonischemic hemispheres 24 h after MCAO, performed as described in Methods. (B) PTHrP and PTH/PTHrP receptor mRNA levels in cerebral hemispheres ipsilateral (+) or contralateral (-) to MCAO (or sham operation) are expressed in arbitrary densitometry units relative to actin (n=1 sham, or mean ± SEM for n=3 MCAO animals). (C) TNF-α, IL-1β, and IL-6 mRNA levels were similarly determined in the same animals as in (B). In (B) and (C), indicated differences between ischemic and non-ischemic contralateral cortex in MCAO animals are statistically significant by Student’s t test. * p < 0.05 vs. contralateral hemisphere. ND = not detected.

2. **Time course of induction of PTHrP and cytokine mRNA expression following permanent MCAO.** TNF-α (A), IL-1β (B), PTHrP (C) and IL-6 (D) mRNA levels in ischemic vs. non-ischemic cerebral hemispheres were determined at the indicated times after permanent MCAO. Results are expressed in arbitrary densitometry units relative to actin (mean ± SEM for n=3 animals). Where indicated, differences between ischemic and non-ischemic contralateral cortex in MCAO animals are statistically significant by Student’s t test. * p < 0.05 vs. contralateral hemisphere. ** p < 0.01 vs. contralateral hemisphere.

3. **Immunohistochemical localization of PTHrP following permanent MCAO** (A) In the striatum of sham-operated animals, immunoreactive PTHrP (brown) was primarily located in neurons (double arrowheads) in a fine reticular pattern in the perikarya, while the vasculature
(arrows) was PTHrP-negative. (B) Cortical PTHrP immunoreactivity was similarly located, in a more diffuse pattern, in neuronal perikarya of sham-operated animals (double arrowheads), while the vasculature (arrows) was PTHrP-negative. (C) Following MCAO (24h), PTHrP persisted in the neurons in the infarct penumbra (double arrowhead), while vascular endothelial cells (arrows) became PTHrP-positive. (D) In the infarcted cortex, the vasculature (arrows) was similarly PTHrP-positive, while neuronal staining (double arrowheads) persisted but was somewhat decreased. (E) Within the infarcted striatum, vascular endothelial cells also became PTHrP positive (arrows), while immunoreactivity of striatal neurons was significantly decreased. (F) Specificity of PTHrP staining was verified by the absence of staining seen when PTHrP(34-53) antibody was preincubated with antigen, as indicated here in a serial section of the striatum shown in (E). (G) Factor VIII-related antigen immunostaining of endothelial cells lining ischemic vessels (arrows) revealed a staining pattern similar to that of PTHrP. (H) In contrast, smooth muscle actin-positive vascular smooth muscle cells (arrows) formed a concentric ring around the longitudinal endothelial cells (double arrowhead) in larger vessels and were absent from small, PTHrP-positive capillaries (not shown). (I) The foot processes of GFAP-positive astrocytes (arrows) circumscribe the perimeter of the vascular cells. Nuclei are counterstained with methyl green.

4. **Effect of PTHrP(1-34) on pial arteriole diameter** (A) Pial microcirculation was sequentially superfused (5 min/solution), as described in Methods, with vehicle (0.1% HSA in aCSF), 1 x 10^{-6} \text{ mol/L} PTHrP(1-34), 1 \times 10^{-6} \text{ mol/L} forskolin, or 2.5\% barium chloride, with 5-15 minute aCSF washouts between each treatment. Results are expressed as the maximal percent change in arteriole diameter (mean ± SEM for n=23 vessels observed in 6 animals) during the 5
minutes of superfusion for each solution. Statistically significant changes in arteriole diameter from baseline (baseline diameter, 26.9 ± 1.0 µm) were determined by paired Student’s t test for each treatment. *** p < 0.001 vs. baseline. (B) Effects of 5 min of sequential superfusion with 0.1% HSA, 1 x 10^{-6} mol/L PTHrP(1-34), or 1 x 10^{-6} mol/L forskolin on arteriole diameter, expressed as fold change relative to baseline, are presented for vessels (n=11-12/group) less than 25 µm (average diameter, 19.7 ± 1.2 µm) or greater than 25 µm (average diameter, 34.8 ± 1.8 µm). Statistically significant changes in arteriole diameter from baseline were determined by paired Student’s t test for each treatment. *** p < 0.001 vs. baseline. Differences between treatments on fold-change in arteriole diameter were determined by ANOVA. ** p < 0.001. (C) Pial microcirculation was continuously superfused for 25 minutes with 1 x 10^{-6} mol/L PTHrP(1-34), and effects on arteriole diameter, expressed as fold change relative to baseline, were determined every 1-2 minutes (n=20 vessels in 4 animals). Changes in arteriole diameter relative to baseline (baseline diameter 27.3 ± 2.2 µm) were determined by paired ANOVA. * p < 0.05. ** p<0.01. *** p < 0.001. (D) Pial microcirculation was sequentially superfused (5 min/solution) with 1 x 10^{-6} mol/L PTH(3-34), followed by 1 x 10^{-6} mol/L PTHrP(1-34), with an intervening aCSF washout. Effects of treatment on changes in arteriole diameter (mean ± SEM, n=25 vessels in 4 animals) relative to baseline were determined by paired ANOVA. *** p < 0.001. (E) Representative pial arterioles at baseline before PTHrP(1-34) infusion. (F) Same pial arterioles as in (E) 17 minutes after start of PTHrP(1-34) infusion.

5. Effect of icv PTHrP(1-34)peptide on infarct volume following permanent MCAO Animals were treated icv with PTHrP(1-34) peptide (vs. vehicle alone) 30 min prior and 90 min post
MCAO. Brains were harvested 24 h after permanent MCAO for analysis of infarct volume, as described in Methods. Total infarct volume, cortical infarct volume, or subcortical infarct volume are expressed as % of the contralateral hemisphere (mean ± SEM, n = 16/group). Effect of PTHrP(1-34) on infarct volume was determined by 2-tailed Mann Whitney testing. * p < 0.02.
Fig 1A
B

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FIGURE 2

A. **TNF-α**

- mRNA levels (arbitrary units relative to actin)
- **non-ischemic** vs **ischemic cortex**
- 2H, 4H, 12H Time points after MCAO

B. **IL-1β**

- mRNA levels (arbitrary units relative to actin)
- **non-ischemic** vs **ischemic cortex**
- 2H, 4H, 12H Time points after MCAO

C. **PTHrP**

- mRNA levels (arbitrary units relative to actin)
- **non-ischemic** vs **ischemic cortex**
- 2H, 4H, 12H Time points after MCAO

D. **IL-6**

- mRNA levels (arbitrary units relative to actin)
- **non-ischemic** vs **ischemic cortex**
- 2H, 4H, 12H Time points after MCAO
FIGURE 5

Infarct Volume (% of hemisphere)

- HSA
- PTHrP(1-34)

ns (p < 0.06)

(p < 0.02)

ns