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Regulation of dipeptidyl peptidase IV in the post-stroke rat brain and In vitro ischemia: Implications for chemokine mediated neural progenitor cell migration and angiogenesis

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Abstract

Cerebral ischemia evokes abnormal release of proteases in the brain microenvironment that spatiotemporally impact angio-neurogenesis. Dipeptidyl peptidase IV (DPPIV), a cell surface and secreted protease has been implicated in extracellular matrix re-modeling by regulating cell adhesion, migration, and angiogenesis through modifying the functions of the major chemokine stromal derived factor, SDF1. To elucidate the possible association of DPPIV in ischemic brain, we examined the expression of DPPIV in the post-stroke rat brain, and under in vitro ischemia by oxygen glucose deprivation (OGD). We further investigated the effects of DPPIV on SDF1 mediated in vitro chemotactic and angiogenic functions. DPPIV protein and mRNA levels were significantly upregulated during repair phase in the ischemic cortex of the rat brain, specifically in neurons, astrocytes, and endothelial cells. In vitro exposure of Neuro-2a neuronal cells and rat brain endothelial cells to OGD resulted in up-regulation of DPPIV. In vitro functional analysis showed that DPPIV decreases the SDF1 mediated angiogenic potential of rat brain endothelial cells, and inhibits the migration of Neuro-2a and neural progenitor cells. Western blot analyses revealed decreased levels of phosphorylated ERK1/2 and AKT in presence of DPPIV. DPPIV inhibitor restored the effects of SDF1. Proteome profile array screening further revealed that DPPIV decreases matrix metalloproteinase-9, a key downstream effector of ERK-AKT signaling pathways. Overall, delayed induction of DPPIV in response to ischemia/reperfusion suggests that DPPIV may play an important role in endogenous brain tissue remodeling and repair processes. This may be mediated through modulation of SDF1 mediated cell migration, and angiogenesis.

Keywords

Ischemic brain injury; Dipeptidyl Peptidase IV; Stromal derived factor Neural progenitor cells; Migration; angiogenesis

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Introduction

Cerebral ischemia and reperfusion injury alters the brain microenvironment including abnormal release of proteases leading to neuronal cell death and disruption of the vascular unit and [1–7]. Thus, delineating the timely and balanced regulation of proteases, cytokines, and growth factors is critical for enhancing endothelial and neural stem cell recruitment, and recovery of neuro-vascular unit [8–15]. Further understanding the cellular and molecular mechanisms that regulate these key factors is therefore important to develop strategies to prolong and improve post-stroke brain repair processes and neurological functional recovery.

Chemokines, the chemotactic cytokines, are autocrine and paracrine signaling molecules that critically modulate cellular migration, angiogenesis and neurogenesis [16–20]. The balance between the accumulation and degradation of these chemokines is integral to brain injury and repair. Particularly, the chemokine stromal-derived factor 1 (SDF1), through its receptors CXCR4 and CXCR7, elicits major control over hematopoietic and neural stem cell migration, proliferation and survival, and angiogenesis [21–26]. Of particular interest, in the ischemic brain SDF1 is released at the site of injury and is shown to enhance stem/progenitor cell recruitment and survival to induce neuro-angiogenesis [27–30]. Of note, the function of SDF1 is tightly controlled by proteolytic events. Indeed, serine proteases and matrix metallo-proteinases (MMP) are aberrantly expressed in ischemic brain causing time dependent effects. During the early acute phase of stroke, MMP9 contributes to inflammation and neuronal cell death. Paradoxically, MMP9 is beneficial during late repair phase as it promotes neuro-vascular remodeling and neuroblast migration to the injured brain region [31,2,7,32,33]. Thus, timely regulation of protease expression and activity is critical for stroke outcome.

In contrast to other proteases, much less is known about the cell surface serine protease dipeptidyl peptidase (DPPIV) in cerebral ischemia. DPPIV is a major regulator of cytokines and neuropeptides involved in inflammation, immunity, vascular, and stem cell function. DPPIV specifically cleaves amino terminal dipeptides required for receptor interaction and signaling. DPPIV is expressed by differentiated cells including neurons and is also present as a soluble form in the serum. Dysregulated DPPIV expression is associated with tumor development [34–38]. We and others have shown that DPPIV inhibits tumor cell migration and angiogenic potential through blocking the SDF1-CXCR4 signaling pathways [39,40]. Recent studies have suggested a role for DPPIV in organ-ischemia including lung, renal, cerebral, and limb ischemic injury [41–44]. Emerging studies have shown that inhibition of DPPIV improves cardiovascular outcomes after myocardial ischemia-reperfusion injury [45–47]. However, little is known about its role in cerebral-ischemia and in chemokine mediated neuroangiogenesis.

In the present study, we have identified that in response to focal ischemia, DPPIV expression is significantly upregulated during repair phase in the post-stroke rat brain, specifically in astrocytes, neuronal and endothelial cells. We further demonstrate that DPPIV also responds to in vitro ischemia, and it inhibits SDF1 mediated in vitro angiogenesis, and migration of neural progenitor and neuronal cells in association with down regulation of MAP kinase and

AKT pathways. These data point to an important role for DPPIV in regulating the processes that are highly relevant to ischemic-brain tissue remodeling.

Materials and Methods

Focal cerebral ischemic model

Animal housing and care was in accordance with the *Guide for the Care and Use of Laboratory Animals.* All surgical procedures were approved by the Animal Care and Use Committee of the University of Wisconsin-Madison. We used spontaneously hypertensive rats (SHR) that provide a consistent infarction volume with a low variability. Focal ischemia was induced in adult male SHR that weighed 270–320 g (Charles River, Wilmington, MA) by transient middle cerebral artery occlusion (MCAO) as described in our previous study [48]. Briefly, under anesthesia, a 3–0 monofilament nylon suture with a rounded tip was advanced into the left internal carotid artery (ICA) lumen to block middle cerebral artery (MCA) blood flow. After 1 hour of occlusion, the suture was withdrawn to restore the blood flow. Sham-operated rats were subjected to the same procedures without the intravascular filament occlusion, and were euthanized at day 3. The ischemic rats were euthanized at day 1, 3, 7, 14 and 21 of reperfusion. Intra-cardiac perfusion with 4% paraformaldehyde was carried out to remove brains for immunohistochemistry. Fresh brain cortex regions were obtained from ipsi and contra-lateral hemisphere.

Immunohistochemistry

Brain coronal sections were washed and incubated with blocking solution (3% normal goat serum and 0.1% Triton X-100 in TBS) for 30 min at room temperature, followed by incubation overnight with primary antibodies at 4 °C. Sections were then incubated with secondary antibodies. The following primary antibodies were used: anti-DPPIV (Sigma-Aldrich, St. Louis, MO), neurofilament 1, beta tubulin (neuronal marker), GFAP (astrocyte marker) (Cell Signaling Technology, Danvers, MA) and CD31 (a marker of endothelial cells) (Santa Cruz Biotechnology, Dallas, TX). Alexa Fluor 488 and 594 conjugated secondary antibodies (Molecular Probes, Eugene, OR) were used according to the primary antibodies. Negative controls were performed by omitting primary antibodies. Images were acquired using a Keyence BZ-9000 fluorescence microscope (Keyence, Itasca, IL, USA).

Neural progenitor cell (NPC) isolation and culture

NPC were isolated and cultured from the subventricular zone (SVZ) of 10–12 weeks SHR that underwent MCAO and 3 day reperfusion, as described previously [49]. Briefly, after euthanasia, the SVZ was dissected from rat brains, and cells were dissociated in Trypsin-EDTA for 10 min at 37 °C. Cells were then strained through a sterile mesh (BD Biosciences, Franklin Lakes, NJ) and plated on 6-well tissue culture plates. Cells were cultured in neurobasal media containing 2% B27, 100 I.U. penicillin/, 100 μg/ml streptomycin (Life Technologies, Grand Island, NY), 20 ng/ml EGF, 20 ng/ml FGF2 (Peprotech, Rocky Hill, NJ), and 5 μg/ml heparin. The first six passages of the cells were used in this study. To examine neurogenic potential, NPC were differentiated into neurons by adding 1mM dibutyryladenosine 3′,5′ -cyclic monophosphate (dbcAMP) (Sigma Aldrich, St. Louis, MO) to the culture media for 4 days.

Cell culture

Neuro-2a, a mouse neuroblastoma cell line derived from the brain (American Type Culture Collection ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM with glucose, 4.5 g/ml and L-glutamine, GIBCO-BRL) containing 10% fetal calf serum (FCS) and 100 I.U penicillin and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD). Rat brain endothelial cells (RBEC) were obtained from ScienCell Research Laboratory, Carlsbad, CA, and grown in endothelial cell media as per manufacturer's instructions. Cells were grown at 37 °C with 5% CO_2 and 21% O_2.

In vitro ischemia induction by oxygen and glucose deprivation (OGD)

To mimic in vivo ischemia, the culture medium was replaced with glucose-free medium (DMEM with L-glutamine and no glucose, GIBCO), and cells were transferred to a humidified incubator (Serico CB, Binder GmBH, Tultingen) flushed with a gas mixture of 95% N_2 and 5% CO_2 at 37 °C for 4 hours. Control cells were incubated for 4 hours in 5% CO_2 and 21% O_2 in a media identical to the OGD media except for the addition of glucose. Following OGD, cells were re-oxygenated by feeding with glucose-supplemented (4.5 g/ml) DMEM complete medium, and placing in a normoxic incubator at 37 °C in 5% CO_2 and 21% O_2 for 48 hours.

Immunofluorescence staining

Control and OGD exposed and re-oxygenated cells grown on coverslips were fixed with 3.7% paraformaldehyde for 15 min at room temperature. Immunofluorescence staining was carried out as previously described (39). Briefly, cells were fixed, blocked, permealized, and incubated with the primary anti-DPPIV (Sigma Aldrich, MO), CXCR4 (Abcam, Cambridge, MA), Nestin or MAP2 (Cell Signaling Technology, Danvers, MA) for 1 hour at 37 °C. Cells were then incubated with Alexa Fluor-conjugated secondary antibody for 1 hour at 37 °C. Subsequently, cells were washed, and stained with nuclear stain DAPI for 5 minutes, washed and mounted on microscope slides with Fluoromount (Diagnostic BioSystems, Pleasanton, CA). Stained cells were imaged using a Keyence BZ-9000 fluorescence microscope (Keyence, Itasca, IL, USA).

Total RNA Isolation and RT-polymerase chain reaction

RNA was isolated using an RNeasy Mini Kit, and cDNA was synthesized from 1 μg total RNA by using the reverse transcriptase kit from Qiagen (Valentia, CA). Quantitation of DPPIV expression levels in ischemic cortex was carried out by real time PCR using SYBR Green PCR master mix in an ABI 7000 Q-PCR machine (Applied Biosystems, Grand Island, NY, USA). Expression of β-actin was used as an internal control. Fold-changes in DPPIV expression were determined by the delta Ct (ΔΔCt) method. Semi-quantitative PCR and agarose gel electrophoresis was used to visualize and quantitate the mRNA levels of DPPIV and CXCR4 in cell culture experiments. Expression of β-actin was used as an internal control to quantitate the mRNA levels.

In vitro angiogenesis assay

In vitro angiogenesis assay was performed as described previously (39). Matrigel matrix (BD Biosciences, San Jose, CA) was distributed at 15 μl per well in a 96-well plate. RBECs suspended in 100 μl of RPMI containing 0.2% FBS were cultured in triplicate with 7×10^3 of either RBEC, RBEC+SDF1 (100ng/ml), or RBEC+SDF1 +DPPIV cells at 37°C. DPPIV was added to media after SDF1 addition. DPPIV activity was inhibited in a fourth set by pretreatment of RBEC with 5 mM diprotin A, 15 minutes before adding SDF1 and DPPIV. After 20 hours, images were captured and the total number of tube-like angiogenic structures of three randomly chosen microscopic fields were counted. Results are presented as mean values of triplicates.

Scratch induced cell migration assay

Cell monolayers were wounded with a sterile micropipette tip and fed with medium containing SDF1 (100 ng/ml) or SDF1 (100 ng/ml) and following addition of DPPIV 500 ng/ml Untreated media was used as a control. Cells were photographed at 6 and 24 hours after scratching, using a phase contrast microscope.

Transwell cell migration assay

Quantitative cell migration was assessed using matrigel-coated Biocoat cell culture inserts (BD Biosciences, Bedford, MA) with 8 μm pores, as we have described previously (38, 39). Briefly, a total of 3×10^4 cells were placed in the upper compartment and the lower compartment was filled with 500 μl growth medium alone, as a control, or media containing either SDF1 (100 ng/ml) alone, or SDF1 and following addition of DPPIV (500 ng/ml). DPPIV activity was inhibited in a fourth set by adding 5 mM diprotin A prior to adding SDF1 and DPPIV. RBEC, Neuro-2a or NPC cells were cultured in the upper compartment. After 24 h, migrated cells on the lower surface were stained with crystal violet and extracted in 200 μl of 0.2 M sodium acetate buffer. Optical density values at 540 nm correlating with cell migration were plotted. Results are presented as mean values of triplicates.

Western blot analysis

Brain tissues were homogenized in RIPA lysis buffer (Boston Bioproducts, Ashland, MA) containing protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO). Western blot analysis was performed as we have described previously [39]. Briefly, 30 μg of total protein was separated by SDS-PAGE and probed with respective antibodies, 1:1000 for phospho/total AKT, phospho/total ERK1/2 (Cell Signaling, Danvers, MA) followed by incubation with secondary antibody conjugated to horseradish peroxidase for 1 hour. Signals were developed with chemi-luminescence using a super signal ECL kit (ThermoFisher Scientific, Madison WI). Total AKT and ERK1/2 were used as loading controls. Images were obtained using an Odyssey imaging system (LI-COR Biotechnology, Lincoln, NE).

Proteomic profiling of cytokine and angiogenic factor expression in Neuro-2a cells

The effects of DPPIV on expression profiles of proteins involved in angiogenesis and migration in Neuro-2a cells were examined using the Proteome Profiler Mouse Antibody Array (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions. Briefly,

Neuro-2a cells were harvested 48 hours after the addition of DPPIV (0.5 μg/ml). Untreated cells were used as controls. Protein lysates were mixed with 1.5 ml of biotinylated detection antibodies and incubated for 1 hour at room temperature, then added to membranes, and incubated overnight at 4 °C. Next day, membranes were washed and chemiluminescent detections were carried out. Arrays were then scanned by Odyssey Infrared Imaging System, and scanned images were analyzed using Image Studio (LI-COR Biotechnology, Lincoln, NE).

In vitro MMP gelatinase assay (In situ zymography)

In situ detection of MMP9 activity was carried out by overlaying cells grown in an 8-well chamber slide with 100 μg/ml quenched fluorescein-labeled gelatinase substrate, DQ-gelatin (Molecular Probes, Eugene OR) for 2 hours at 37 °C. Slides were washed and fixed with 4% paraformaldehyde, and imaged using a flourescence microscope (EVOS, ThermoFisher Scientific, Walthem, MA).

Statistical analysis

Data are presented as the mean ± standard deviation of triplicates. Statistical significance was calculated by one-way ANOVA with Bonferroni's multiple comparisons post-test, or unpaired Student's *t*-test for comparison between 2 groups. Results were considered significant at $P \leq .05$ at 95% confidence level. The significant groups are marked with asterisks as shown in the figures.

Results

Induction of DPPIV expression in the rat brain following focal ischemia and reperfusion (I/R)

As indicated by the immunohistochemistry, following focal ischemia, we observed weak immuno-reactivity for DPPIV in the peri-infarct area of the ipsilateral hemisphere at day 1 of I/R, (Fig. 1A, panel a). The expression of DPPIV in the peri-infarct area further increased at day 3 (Fig. 1A, panel c) of I/R. Expression of DPPIV peaked at day 7 of I/R, and it remained at high to moderate levels for at least 2 weeks after reperfusion (Fig. 1A, panels e and g). The cortex region of contralateral hemisphere (Fig 1A panels b, d, f, h) and sham-operated rats (Fig. 1A, panel i**)** showed low basal levels of weak immuno-reactivity for DPPIV expression. Image of a coronal brain section indicating the infarct and the areas from which the images were taken (square boxes) is shown in Fig 1B. Real time quantitation of DPPIV mRNA showed non-significant increase in levels of DPPIV at day 1 of ischemia/reperfusion. Significant induction of DPPIV mRNA (2.5–3.8 fold) was detected at day 3, and 3.6 to 4 fold increase at day 7, that continue to remain at levels about 2.6–2.9 fold at days 14 and 21 of post ischemia-reperfusion as compared to sham animal (Fig. 1C).

Cellular localization of DPPIV protein in the rat ischemic brain

Immuno-histochemical analysis was performed using dual labeling for DPPIV, and neuron, or astrocyte or endothelial cell markers on coronal brain sections of animals subjected to 1 hour MCAO and 3 day reperfusion. Double immunofluorescence staining showed co-localization of DPPIV in β-tubulin (Fig. 2A, panel a–c) and neurofilament positive (Fig. 2A,

panels d–f) neuronal cells, and as well as in GFAP positive astrocytes (Fig. 2B, panels g–i) in the peri-infarct area of ipsilateral cortex. We also identified that DPPIV was expressed in CD31 positive cells (Fig. 2C, panels j–l) indicating the expression of DPPIV in endothelial cells following ischemia/reperfusion injury. Insets in Fig. 2C show the co-expression of DPPIV and CD31 in the endothelial cells of a larger blood vessel. These results suggest that neuronal cells, astrocytes, and endothelial cells are the primary source of DPPIV in the ischemic rat brain.

In vitro ischemia/oxygen glucose deprivation (OGD) up-regulates DPPIV expression

Given the observed effects of in vivo ischemia/reperfusion on DPPIV expression, we next determined if DPPIV is responsive to in vitro ischemic conditions of OGD in neuronal and rat brain endothelial cells (RBEC). Exposure to OGD for 4 hours followed by re-oxygenation for two days significantly up-regulated the expression of DPPIV as compared to cells grown under normoxic (Norm) condition. Immunofluorescence assay revealed increased levels of DPPIV following OGD/re-oxygenation in both Neuro-2a (Fig. 3A) and RBEC cells (Fig. 3B). RT-PCR assay indicated significantly higher levels of DPPIV mRNA, 2 days after OGD/re-oxygenation in Neuro-2a (Fig. 3C) and RBEC cells (Fig. 3D) as compared to cells grown under normoxic condition. In both cell lines, OGD increased DPPIV expression by 2–3 fold ($P < 0.05$; Fig. 3E and 3F). These results were consistent with in vivo ischemia/reperfusion effect on DPPIV expression.

DPPIV suppresses SDF1 mediated angiogenic potential, and migration of rat brain endothelial cells (RBEC) in vitro

Functional relevance of DPPIV was further examined using in vitro models. Angiogenesis is a dynamic process involving cell migration and tube formation that is essential for neurogenesis. Therefore, we determined the effect of DPPIV on the formation of pro-angiogenic structures arising from RBEC. As shown in Fig. 4A, SDF1 significantly increased the number of proangiogenic structures as compared with the untreated control group. However, addition of exogenous DPPIV diminished the effects of SDF1 as shown by decreased number of pro-angiogenic structures. Addition of DPPIV inhibitor diprotin A rescued the effects of SDF1. Quantitation revealed 25–30% increase in pro-angiogenic structures in presence of SDF1. The number of these structures in the presence of DPPIV was significantly less than those from SDF1 alone treated group. Diprotin A pretreatment inhibited the effects of DPPIV, and significantly increased the number of pro-angiogenic structures (Fig. 4B). These results indicate that SDF1 has a positive effect on induction of angiogenesis, and DPPIV diminishes this effect of SDF1. SDF1 also significantly increased the migratory potential of RBEC as compared with the untreated control group. However, in presence of DPPIV, migratory potential of RBEC decreased as shown by scratch assay (Fig. 4C). Quantitation by transwell migration assay revealed 2–3 fold increased migration in presence of SDF1, which was significantly suppressed in presence of DPPIV, and was significantly restored in presence of DPPIV inhibitor (Fig. 4D). These results indicate that SDF1 has a positive effect on induction of angiogenesis and migration, and DPPIV diminishes the effect of SDF1 on rat brain endothelial cells.

Effects of DPPIV on Neural Progenitor Cells (NPC)

SDF1 gradient regulates recruitment of CXCR4 positive NPC to an infarcted brain region, and is important for increasing neurogenesis. Hence we next examined if DPPIV modulates the SDF1 mediated effects on NPC in vitro. First, the authenticity of isolated NPC was confirmed by the expression of nestin, a neural stem/progenitor cell marker (Fig. 5A). Furthermore, the neuronal differentiation potential of isolated NPCs was demonstrated by the dbcAMP induced neuronal differentiation, as indicated by the small body and long neurite like processes, and expression of microtubule-associated protein 2 (MAP2), a neuronal marker (Fig. 5A). We also show that exposure of NPC to an in vitro ischemic condition of OGD up-regulates the receptor for SDF1, CXCR4 by about two fold as compared to untreated cells, as shown by RT-PCR analysis (Fig. 5, panels B and C). We further observed that SDF1 (100 ng/ml) significantly increased the migratory potential of NPC in vitro by about 35–40%, and this effect was significantly inhibited in presence of DPPIV (500 ng/ml). Addition of DPPIV inhibitor significantly restored the SDF1 mediated NPC migration (Fig. 5D).

DPPIV modulates in vitro migration of Neuro-2a cells through ERK1/2 and AKT pathway

Ultimately, regulation of mature neuronal cells is the key to improving post-stroke neurological recovery. Next, we focused on gaining mechanistic insight into the effects of DPPIV on mature neuronal cells using Neuro-2a as a model. Our results showed that SDF1 (100 ng/ml) enhances migration of Neuro-2a cells by about 30–40% as compared to untreated control cells. In the presence of DPPIV (500 ng/ml), SDF1 mediated migration of Neuro-2a cells was significantly decreased, and Diprotin A pretreatment significantly restored SDF1 mediated Neuro-2a cell migration (Fig. 6A). To elucidate the signaling pathway involved in SDF1 mediated Neuro-2a migration, we examined the activation of MAP kinase ERK1/2 and AKT pathways, which are shown to stimulate the expression of pro-angiogenic and migratory genes including MMP9. Exposure of Neuro-2a cells to SDF1 for 18 hours increased the phosphorylation/activation of both ERK1/2 and AKT, which was blocked in presence of 500 ng/ml DPPIV. In presence of DPPIV inhibitor Diprotin A, SDF1 mediated phosphorylation of ERK1/2 and AKT was restored (Fig. 6B). Furthermore, targeted proteome profiler array showed 2–3 fold decreased levels of MMP9 in DPPIV treated cells as indicated by the arrows (Fig. 6C) and bar graph (Fig. 6D). Decrease in MMP9 levels was confirmed by in situ zymography that detects gelatinase activity corresponding to MMP9 levels. As compared to untreated Neuro-2a cells (Fig. 6Ea), MMP activity was greatly reduced in DPPIV treated cells (Fig. 6Eb). Thus, our results show an association between decrease in migratory and angiogenic potential, and decreased activation of ERK1/2 and AKT signaling pathway in presence of DPPIV.

Discussion

The key findings of the current study is the upregulation of DPPIV under in vivo focal ischemic stroke, and in vitro ischemic conditions, and its cellular localization in rat ischemic brain. Following characterization of DPPIV expression, we next determined the functional effects DPPIV on migration and angiogenesis using in vitro cell culture models. Finally, we have identified the molecular pathway affected by DPPIV in neuronal cells.

Stroke remains a significant clinical challenge, with only a small proportion of the ischemic patients benefiting from current treatments which are limited by a narrow therapeutic time window [50–52]. Cerebral ischemic stroke results in severe neurological deficits due to massive loss of neurons and disruption of vasculature. Restoration of both neuro and vascular units is thus important for functional recovery. Although our understanding of the stroke pathology has remarkably increased, further insight into the cellular and molecular mechanisms involved in the post-stroke repair phase is still required to identify more effective drug targets with wider time window. As a self-defense mechanism, ischemic-stroke upregulates the pro-survival, pro-angiogenic chemokines including SDF1, that promote cellular migration, survival, and angiogenesis. This attempt to self-repair the post-stroke brain is short lived and has limited success. Thus, there is an urgent need to identify new molecular targets to prolong this endogenous repair process. Through mutual interactions, chemokines and proteases regulate angiogenesis and neurogenesis, and have gained recent attention due to their therapeutic potential for post stroke recovery [1,17,53–55,16,18–20]. This emphasizes the need to better understand the regulation of proteases in the ischemic brain microenvironment. In this study, we have shown that expression of a serine protease DPPIV is highly upregulated in the ischemic rat brain during delayed repair phase. DPPIV expression was also upregulated in response to *in vitro* ischemia of oxygen glucose deprivation (OGD) in neuronal and rat brain endothelial cells. Furthermore, we demonstrate that DPPIV inhibits SDF1 mediated angiogenesis, and migration of rat brain endothelial cells, neural progenitor and neuronal cells in association with decreased activation of MAP kinase and AKT pathways, the downstream effectors of chemokines. Inhibition of DPPIV restores the effects of SDF1. These data support an important role for DPPIV in regulating processes that are involved in tissue remodeling in ischemic-brain.

To maintain homeostasis, fine-tuning of the chemokine action is achieved through the regulation of both ligands and their receptors. First of all, chemokine system is modulated spatiotemporally by transcriptional regulation of the expression of these ligands and their cognate receptors. Secondly, proteolytic cleavage critically controls chemokine function by enhancing or reducing their activity and receptor selectivity [56,57,36,58,19,59]. The most studied central nervous system (CNS) serine proteases are the tissue and urokinase-type plasminogen activators and thrombin, which are thought to play a critical role in CNS homeostasis, plasticity, and stroke [60–62,6]. Although dysregulated protease expression has been recognized as an important event in the ischemic brain, little is known about the *de novo* regulation of DPPIV after focal stroke, and under ischemic conditions. Our current study has shown that DPPIV expression is induced spatiotemporally due to focal ischemia and reperfusion injury during later repair phase in the rat ischemic-brain. Delayed upregulation of DPPIV presumably promotes apoptotic cell death and impairs chemokine mediated progenitor cell recruitment, neo-angiogenesis and tissue remodeling. Indeed, we show here that DPPIV inhibits the SDF1 mediated angiogenic potential of rat brain endothelial cells, and suppresses the migratory potential of neural progenitor and as well as neuronal cells, that can be rescued by pre-treatment with DPPIV inhibitor. Of note, our previous studies have shown that DPPIV is an important player in suppressing tumor cell proliferation, migration, and angiogenesis [39,37,38,63]. These results strongly support a role for DPPIV in regulating cellular functions that are critical for post-stroke brain repair.

The prevailing model is that in response to stroke, stem cells, neurons, endothelial cells, and astrocytes produce trophic factors and chemokines that support endothelial and neuronal progenitor cell proliferation, migration, and survival in the ischemic brain. This process eventually determines the extent of angiogenesis and neuronal recovery [1,64,51,65,21,16,9,23,11,3,12]. Interestingly, we identified that DPPIV is upregulated in neurons, endothelial cells, and astrocytes in the rat brain in response to ischemia-reperfusion injury. Thus, decreased oxygen/hypoxia may be one of the key events that drives up-regulation of DPPIV. Indeed, transcription factors, such as HNF-1α and HIF-1α are shown to upregulate DPPIV expression in smooth muscle cells and Ewing sarcomas [66–68]. In support of this, we show here that similar to in vivo ischemia, exposure of RBEC and Neuro-2a cells to in vitro ischemic conditions of OGD also results in increased DPPIV expression. It has been demonstrated that chemokines, and the proteases that regulate them, are often expressed in the same microenvironment by a common stimulus facilitating the rapid regulation of chemokine activity. Although SDF1 that promotes neuro-angiogenesis is also shown to be upregulated during delayed phase, its effect on brain repair is not significant. It has been reported that specifically SDF1 is upregulated in the ischemic brain within 7 days post ischemia, where it recruits bone marrow-derived stem cells, NPC, and neuronal cells toward the damaged area after cerebral ischemia for restoration of neurons and tissue remodeling [9,23,26]. However, new neurons/neuroblasts exhibit inhibited survival and migration, possibly due to insufficient levels of active SDF1 in the injured regions. Interestingly, N-terminal end of SDF1 is sufficient and required to induce chemotaxis and proliferation of neural stem/progenitor cells [24,69]. Although, pathophysiological significance of SDF1 cleavage in ischemic brain is not clear, presumably, excessive production of DPPIV through its proteolytic cleavage of N-terminal region of SDF1 inhibits SDF1 mediated functions. Indeed, in this study, using three dimensional in vitro cell culture models, we have demonstrated that DPPIV significantly decreases the SDF1 mediated angiogenic potential of rat brain endothelial cells, and the migratory potential of neuronal and neuronal progenitor cells. This raises the interesting possibility that targeting DPPIV may be beneficial to prolong or rescue SDF1 functions including stem cell recruitment, and angio-neurogenesis processes which are briefly present after cerebral ischemia. Indeed a recent study reports that a dipeptidyl peptidase inhibitor exhibits neuroprotective properties in diabetic rats through antioxidant, anti-inflammatory, and anti-apoptotic mechanisms [70].

Ischemic stroke dysregulates molecular signaling pathways, and it is important to identify the pathways linked to protease and chemokines after stroke. Extracellular-signal-regulated kinases (ERK1/2) and PI3K/AKT signaling that are involved in upregulation of MMP9 are the downstream effectors of the SDF1/CXCR4 signaling during injury and repair processes [71–76]. Interestingly, we show here that the effects of DPPIV are accompanied by a decrease in the levels of ERK1/2 and AKT phosphorylation. Furthermore, the levels of MMP9 were also decreased in the presence of DPPIV in support of our previous work [39,38]. Although increased levels of MMP9 exacerbate early post-stroke brain injury, during the later brain repair phase MMP9 also contributes to vascular remodeling, angiogenesis, neurogenesis and axonal regeneration [2,77,32,33]. Thus, DPPIV blocking SDF1-CXCR4-MMP9 mediated intracellular signaling events might potentially contribute to

impaired tissue remodeling after stroke. Due to these effects, timely regulation of DPPIV expression after stroke to prolong SDF1 function represents a promising therapeutic strategy to improve post-stroke brain repair.

The regulatory interplay between chemokines and proteases is complex. Due to its pleiotropic nature, DPPIV might also broadly affect other cytokines including CCL2, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-α (MIP-α), and Gro-alpha, that are upregulated after brain injury, and are shown to promote neural stem survival and migration [78]. Thus, DPPIV inhibition may have additive benefits for post-stroke brain repair. It is possible that DPPIV can also affect cellular functions in a non-enzymatic way in damaged brain by acting on growth factor signaling pathways, due to its association with extracellular matrix proteins and adenosine deaminase [34,79,36]. Nevertheless, DPPIV inhibition shows a great potential for post stroke regenerative pharmacotherapy. However, further in vivo studies in pre-clinical experimental animal models are warranted to support these inferences. Overall, our present study provides lines of evidence suggesting a potential association between altered DPPIV expression due to cerebral ischemic insult, and the regulation of SDF1 mediated migration and angiogenesis, important biological processes that are highly relevant for tissue remodeling and post-stroke brain repair.

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Abbreviations

DPPIV	dipeptidyl Peptidase 4
SDF1	Stromal Derived factor
NPC	Neural progenitor cells
OGD	Oxygen Glucose Deprivation
MCAO	Middle cerebral artery occlusion
SHR	Spontaneously hypertensive rats
RBEC	Rat brain endothelial cells

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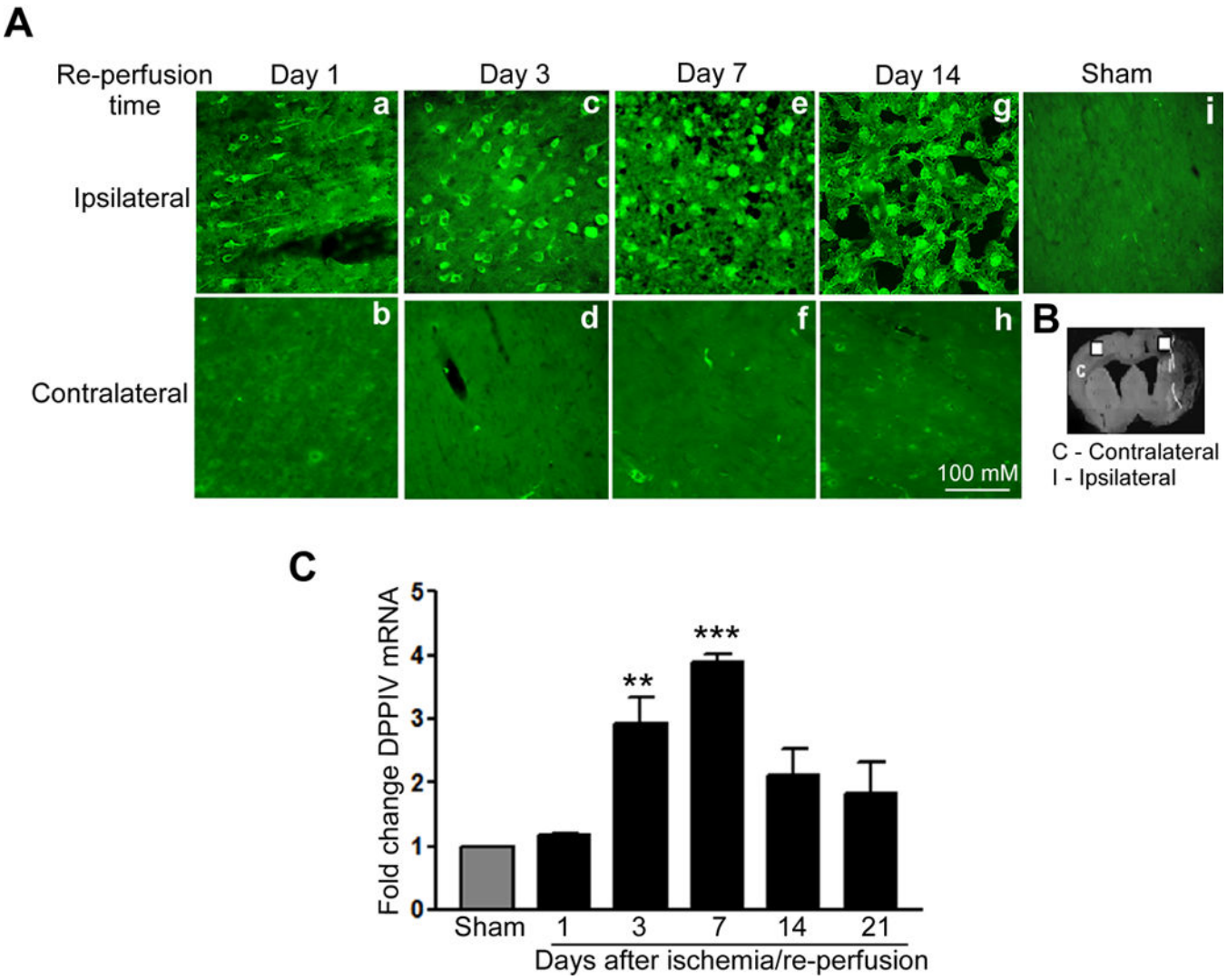

Figure 1.

Expression of DPPIV is increased in the ischemic cortex after focal ischemia/reperfusion. **A**. Representative coronal brain sections (ipsilateral and contralateral hemisphere) of SHRs subjected to MCAO and various reperfusion times are shown. The ipsilateral and contralateral cortex stained with DPPIV antibody of ischemic rats at 1 (a and b), 3 (c and d), 7 (e and f), and 14 (g and h) days of ischemia/reperfusion. Significant up-regulation of DPPIV is seen in the ipsilateral cortex after 3 days and it persisted up to day 14. Sham operated control animal showed very low levels of DPPIV expression (i). **B.** Image of a schematic coronal brain section indicating the control and infarcted area from which the images were taken (square boxes). The dotted line indicates the border of the ischemic core. **C**. Real time PCR indicating the significant levels of DPPIV mRNA induction at day 3 and 7 of ischemia/reperfusion. Mean ± SD, n = 3. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with sham control by one-way ANOVA with Bonferroni's multiple comparisons post-test.

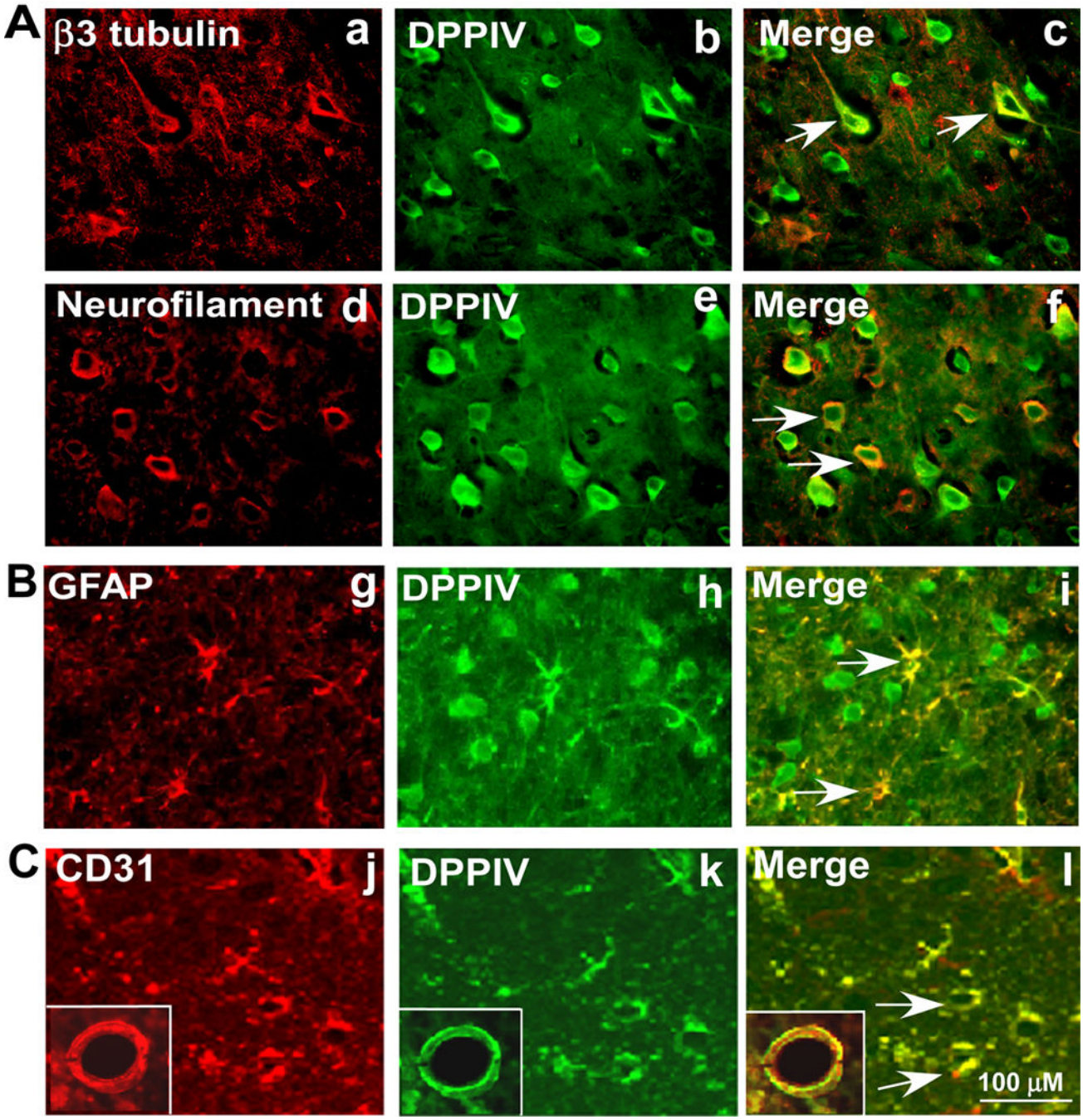

Figure 2.

Cellular distribution of DPPIV protein in the ipsilateral cortex of SHR brain neuronal cells, astrocytes, and endothelial cells. **A–C.** Double immunofluorescence staining was performed with anti-DPPIV (green) and either anti-β-tubulin, neurofilament, GFAP, or CD31 (red). DPPIV protein was coexpressed with neuronal cell markers β-tubulin (A, a–c, and neurofilament A, d–f); with astrocyte marker GFAP (B g–i); with endothelial cell marker CD31 (C j–l). Insets in panel j, k, and l show co-localization of DPPIV and CD31 expression in larger blood vessel. Co-localization are indicated with arrows in relevant panels.

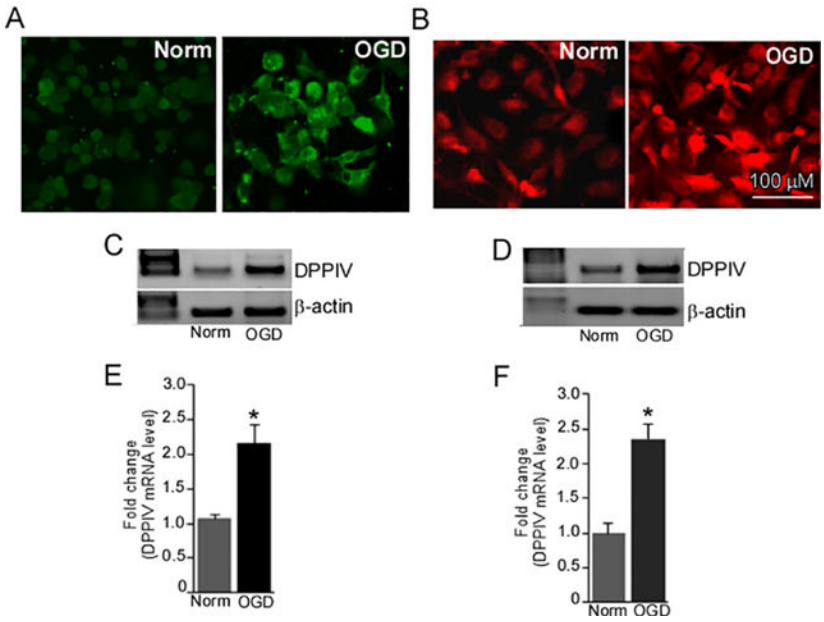

Figure 3.

In vitro oxygen glucose deprivation (OGD) upregulates DPPIV expression. **A–B**. The effects of OGD on DPPIV protein expression in Neuro-2a cells (A) and rat brain endothelial cells (RBEC) (B) were evaluated after 4 hours OGD, and 2 days of re-oxygenation by immunofluorescence staining. **C–D**. Up-regulation of DPPIV mRNA expression was confirmed by semi-quantitative PCR assay using DPPIV specific primers, and β-actin primers as internal control for RNA loading. Upregulation of DPPIV mRNA in Neuro-2a (C) and RBEC cells (D) are shown. **E–F**. Bar graphs indicate 2–3 fold increase in DPPIV mRNA levels in Neuro-2a (E), and RBEC (F) exposed to OGD/re-oxygenation. Mean ± SD, n = 3. * $p < 0.05$ compared with normoxic (Norm) controls.

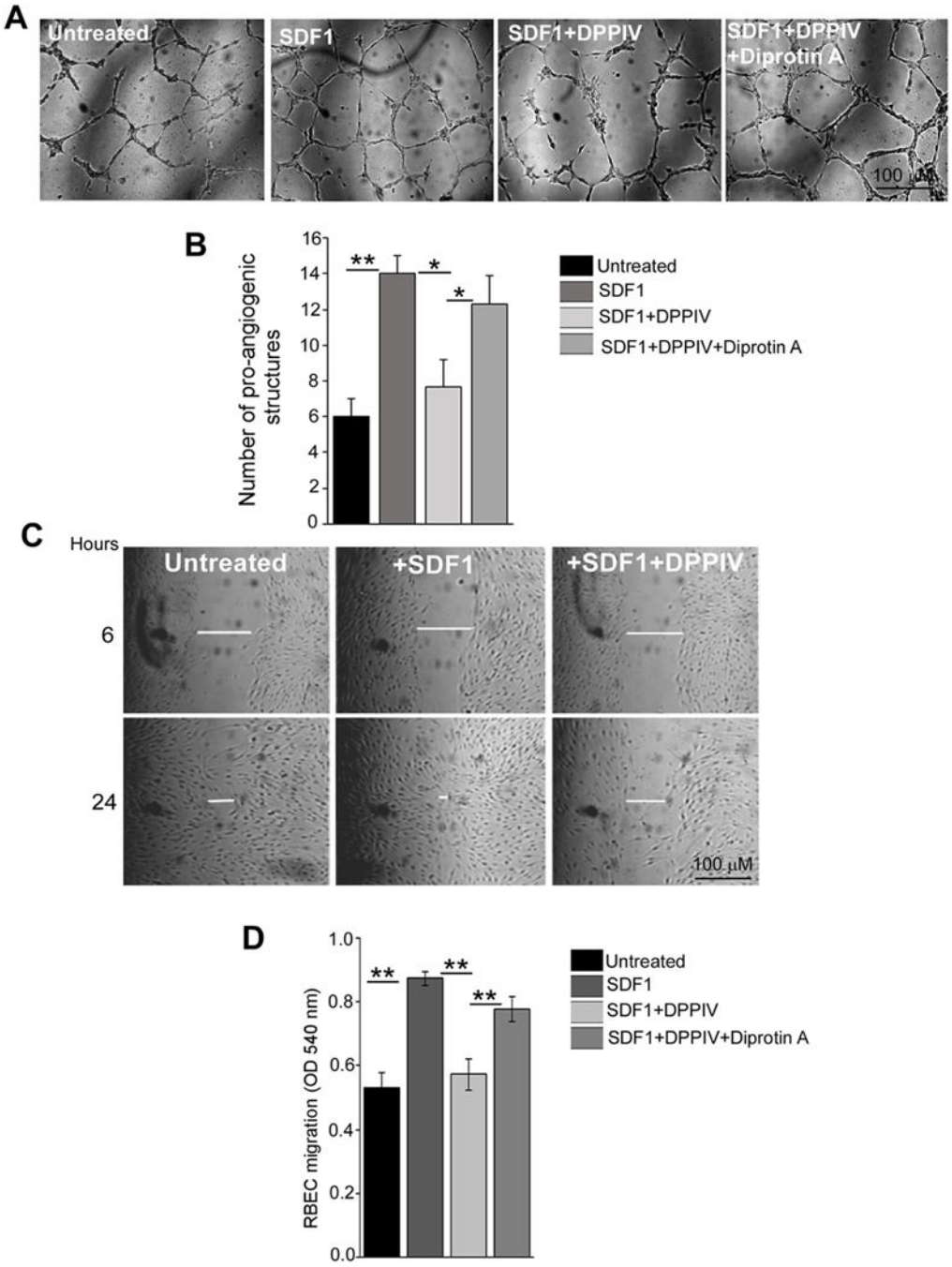

Figure 4.

DPPIV inhibits SDF1 mediated angiogenic tube formation and migration of rat brain endothelial cells (RBEC). To assess the angiogenic potential, RBEC were seeded on matrigel-coated 96-well tissue culture plates. Four experimental groups included 1) Untreated control, RBECs were cultured in media alone, 2) SDF1 (100 ng/ml), 3) SDF1 and DPPIV (500 ng/ml), and 4) 5 mM Diprotin A +SDF1+DPPIV. Pro-angiogenic structures were examined after 24 hours under a phase contrast microscope. **A.** M**o**rphology of pro-angiogenic structures formed by endothelial cells on matrigel incubated with either media

alone (untreated control), media+SDF1, or media+SDF1 and DPPIV. Representative pictures of tube formation are shown. **B.** Tube formation was scored by counting the number of pro-angiogenic rings. Five independent areas per well of culture plates were scored in each case. **C.** Bright field images of scratch induced migration at 6 and 24 hours after scratching of rat brain endothelial cells grown in media alone (untreated control), media+SDF1, or media +SDF1 and DPPIV. Representative images are shown. White line indicates the gap width. **D**. Quantitation of RBEC migration by transwell assay. RBECs were plated onto transwell inserts coated with ECM. Four experimental groups included 1) Untreated control, RBECs were cultured in media alone, 2) SDF1 (100 ng/ml), 3) SDF1 and DPPIV (500 ng/ml), and 4) 5 mM Diprotin A +SDF1+DPPIV. Optical density values at 540 nm correlating with cell migration were plotted. Results are presented as mean ± SD of triplicates. ** $p < 0.01$; * $p < 0.05$ as compared with untreated control or treated group.

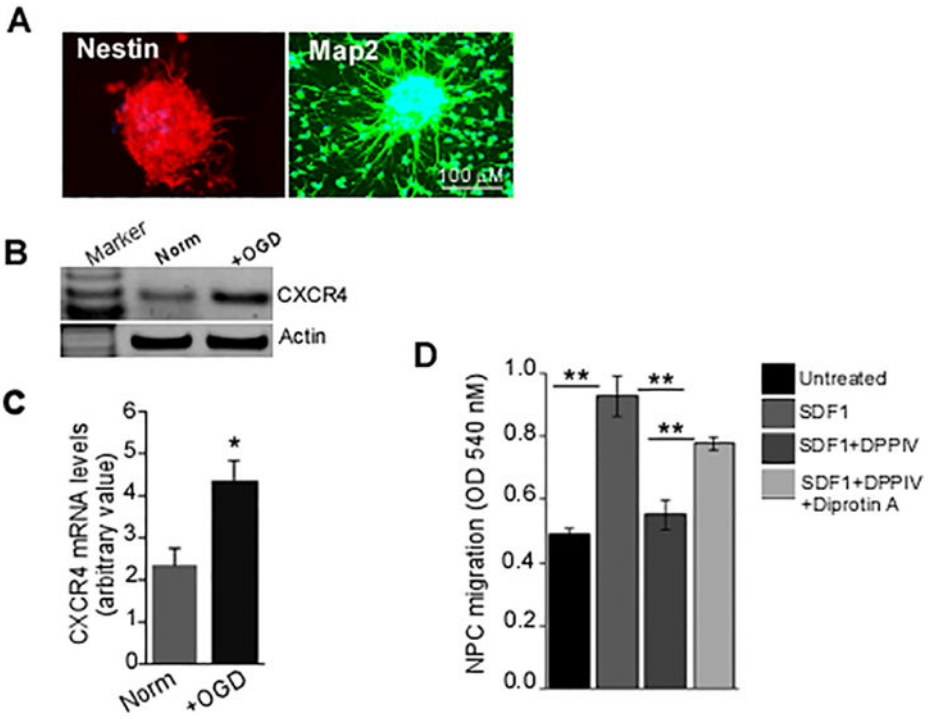

Figure 5.

Characterization of rat brain Neural Progenitor Cells (NPC), analysis of expression of CXCR4 in NPC exposed to in vitro ischemia and re-oxygenation, and effect of DPPIV on NPC migration in vitro. **A**. Photomicrographs of NPC neurospheres derived from SHR brain showing nestin positivity, and differentiated NPC showing the expression of neuronal marker MAP2 by immunofluorescence staining. **B**. Expression of CXCR4 mRNA in NPC exposed to 4 hour OGD, and 24 hours re-oxygenation, as shown by semi-quantitative PCR. **C**. Bar graph showing about 2 fold increase in CXCR4 mRNA in NPCs after OGD/re-oxygenation. Mean ± SD, n = 3. $*p < 0.05$ as compared with untreated control. **D**. Exogenous DPPIV inhibits SDF1 mediated NPC migration. Dissociated NPCs were plated on transwell inserts coated with ECM. Four experimental groups included 1) Untreated control, NPCs were cultured in media alone, 2) SDF1 (100 ng/ml), 3) SDF1 and DPPIV (500 ng/ml), and 4) 5 mM Diprotin A +SDF1+DPPIV. Cells were grown for 24 hours. Optical density values at 540 nm correlating with cell migration were plotted. Results are presented as mean ± SD of triplicates. ** $p < 0.01$ as compared with untreated control or treated group.

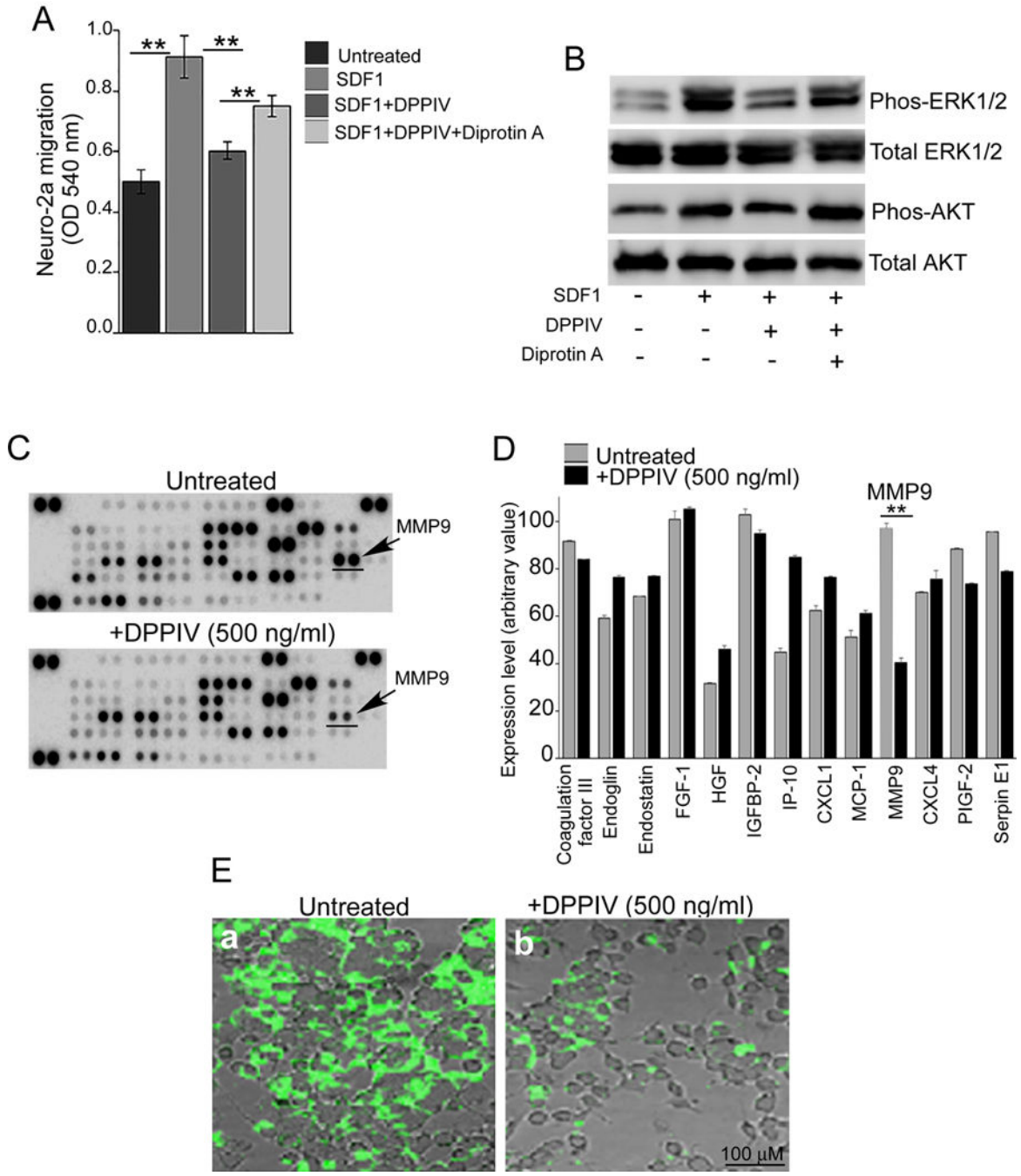

Figure 6.

DPPIV inhibits in vitro migration of Neuro-2a cells in association with decreased ERK1/2 and AKT signaling. **A**. Effect of exogenous DPPIV on SDF1 mediated Neuro-2a cell migration. Cells were cultured on transwell inserts coated with ECM. Four experimental groups included 1) Untreated control, Neuro-2a cells were cultured in media alone, 2) SDF1 (100 ng/ml), 3) SDF1 and DPPIV (500 ng/ml), and 4) 5 mM Diprotin A +SDF1+DPPIV. Cells were grown for 24 hours. Optical density values at 540 nm correlating with cell migration were plotted. Results are presented as mean ± SD of triplicates. ** $p < 0.01$ SDF1

alone compared with untreated control and ** $p < 0.01$ SDF1+DPPIV compared with SDF1 alone, ** $p < 0.01$ Diprotin A + SDF1+DPPIV compared with SDF1 and DPPIV. **B.** Western blot showing the levels of phosphorylation/activation of ERK1/2 and AKT in Neuro-2a cells treated with SDF1. Addition of exogenous DPPIV diminished the SDF1 mediated phosphorylation levels of ERK1/2 and AKT, which was restored by DPPIV inhibitor Diprotin A. Total ERK1/2 and total AKT are used as protein loading controls. **C**. The effects of DPPIV on expression profiles of pro-migratory and proangiogenic factors in neuronal cells were examined using Proteome Profiler antibody Array screening. Arrows mark MMP9 levels in antibody arrays. Decrease in MMP levels is evident in DPPIV treated cells as compared to untreated Neuro-2a cells. **D**. Histogram profiles for select proteins were generated by quantifying the mean spot pixel density from the arrays using image software analysis. Grey bars represent protein levels in lysates from untreated cells, black bars represent protein levels in lysates from DPPIV treated cells. MMP9 levels are highlighted. ** $P < 0.01$ as compared with untreated controls. **E**. Decrease in MMP9 levels was confirmed by in situ zymography that measures MMP gelatinase activity. Merged images of bright field and MMP gelatinase activity in untreated control (Ea) and DPPIV treated cells (Eb) are shown.