We recently demonstrated that limb remote ischemic preconditioning (LRP)—clamping and subsequent reperfusion of the femoral artery of the limb—protects against focal stroke, however, there is almost no research on mechanisms by which LRP distally offers cerebral protection. This study addresses how LRP affects the Tim-3/galectin-9 pathway and free radicals. Tim-3, a member of the T cell immunoglobulin and mucin domain (Tim) family, triggered by its ligand galectin-9, induces Th1 cell death and is expressed in neurons; whether it is an inducer of neuronal death is not known. Inducible nitric oxide synthase (iNOS), nitrotyrosine, and cyclooxygenase 2 (COX-2) are well-known free radical markers that contribute to ischemic injury. We assessed the hypothesis that LRP reduces Tim-3, galectin-9, iNOS, nitrotyrosine, and COX-2 protein expression. Stroke was generated by permanent occlusion of the left distal middle cerebral artery with 30 min occlusion of the bilateral common carotid arteries in male rats. LRP was generated by 5 or 15 min occlusion followed with the same period of reperfusion of the left hind femoral artery. Western blot showed that both Tim-3 and galectin-9 increased at 24h after stroke (p<0.001). LRP blocked increases in both Tim-3 and galectin-9 at 24h (p<0.001). iNOS expression increased at 1h, 5h and 24h in control (p<0.001, 0.01, 0.05, respectively); with LRP inhibiting these increases at all time points (p<0.001). Similar inhibition was found for nitrotyrosine, but interestingly COX-2 overexpression was not inhibited. Immunostaining showed similar overexpression and inhibition. LRP may block brain injury by attenuating activities of the Tim-3/galectin-9 pathway, iNOS, and nitrotyrosine, while overexpression of COX-2 may not be a critical factor for ischemic injury.

Introduction

Inflammatory response is involved in the mechanisms of neuronal death after stroke; however, how inflammation after stroke is regulated is not clear. Many factors, including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and Tim-3, are implicated in inflammation. COX-2 and iNOS are well-known free radical generators. iNOS overexpression produces nitric oxide leading to products of nitrotyrosine, which is also a marker for inflammation. COX2 is responsible for production of prostaglandins.
Figure 2 Tim-3 expression was increased after stroke, which was inhibited by remote preconditioning. Representative protein bands of Tim-3 and beta-actin are presented for rats receiving control ischemia alone (A) and ischemia plus remote preconditioning (B). The bar graph indicates that Tim-3 was immediately increased as early as 1h, reached a peak at 24h after stroke (C), which was inhibited by remote preconditioning (D). ***, vs sham, P<0.001. N=6/group. The results were further confirmed by the study of immunofluorescent confocal microscopy in control ischemia and remote preconditioning 24h after stroke (E), whereby green fluorescence is Tim-3 staining.

which causes inflammation. Tim-3, a member of the T cell immunoglobulin and mucin domain (Tim) family, has double roles in regulating inflammatory response (Anderson et al. 2007). When it is expressed on CD4+T help 1 (TH1) cells, it is triggered and activated by its ligand galectin-9, which causes calcium influx and cell aggregation inducing TH1 cell death, therefore, it inhibits inflammatory response by eliminating Th1 cells (Su et al. 2008). However, when Tim-3 is expressed on macrophage, microglia and dendritic cells, it promotes inflammatory response (Anderson et al. 2007). Tim-3 is also expressed neurons in the brain (Gielen et al. 2005), but its role in the brain is not known.

Limb remote preconditioning, which refers to repetitive ischemia/reperfusion induced in a hind limb, reduces brain injury induced by stroke. Thus, a surgery done distally in the limb protects the brain after stroke. Such a surgical technique has promising clinical applicability, for example in surgeries where the risk of brain ischemia is high. LRP research started in protection from myocardial infarction, and has been found to be clinically effective in protection from myocardial infarction during cardiac surgery, increasing patient post-operative outcomes (Venugopal et al., 2008; Kharbanda et al, 2009). The exact nature of signal transduction from limb to organ is not well studied, but evidence suggests remote preconditioning is multimodal acting on several pathways such as afferent signals from the remote organ and reducing inflammation (Schoemaker et al., 2000). LRP is also a good model for testing mechanisms of stroke injury. We have shown that remote preconditioning with limb ischemia reduces infarction after focal ischemia (Ren et al, 2008); the first goal of this project was to confirm these results. We then studied changes in galectin-9 and Tim-3 after stroke, and the protective effects of LRP on the galectin-9/Tim-3 pathway and on the production of COX-2, iNOS and nitrotyrosine. This study will address the translation of protection from limb to brain in terms of inflammation, and mechanisms of injury in stroke.

Materials and Methods

Focal cerebral ischemia

This is the experimental parallel to an ‘induced stroke’. Focal cerebral ischemia was generated in male Sprague–Dawley rats as previously described (Zhao et al., 2006), approved by the Stanford University Administrative Panel on Laboratory Animal Care. Anesthesia was induced by 5% isoflurane and maintained with 2% to 3% isoflurane during surgery and early reperfusion. Core body temperatures were maintained at 36.2–37.2 °C using a heating pad and light; blood pressure, heart rate, respiratory rate, blood glucose and pH, and PaO2, PaCO2 were monitored throughout surgery.

A ventral midline incision was made and the two CCAs were isolated. Snares were placed around the CCAs and the animal was placed on its right side. A 2-cm vertical scalp incision was made midway between the left eye and ear. The temporalis muscle
was bisected and a 2-mm burr hole was made at the junction of the zygomatic arch and squamous bone. The distal MCA was exposed and cauterized above the rhinal fissure. The CCA snares were tightened to occlude the CCAs for 30 min and then released, while the distal MCA remained occluded.

**Limb remote preconditioning**

This surgical ‘treatment’ was performed before focal ischemia surgery. Male Sprague–Dawley rats were anesthetized by isoflurane. Rats had the left femoral artery separated below the left groin ligament for induction of femoral artery occlusion. Rats were subjected to three cycles of 15 min occlusion/reperfusion of the femoral artery before the induction of stroke. After preconditioning in the limb, the rats were immediately subjected to the brain ischemia.

**Immunofluorescence staining and confocal microscopy**

Rats were perfused transcardially with normal saline followed with 4% paraformaldehyde. Sections were blocked in and incubated in primary antibodies overnight; antibodies were against Tim-3 (1:100; Santa Cruz Biotechnology; sc-30326), Galectin-9 (1:50; Santa Cruz Biotechnology; sc-19292), Cox-2 (1:400; Cayman; 160106), Nitrotyrosine (1:50; Millipore (Chemcon); 92590). Neurons were stained with MAP-2 (1:200; Sigma; M4403); astrocytes were stained with GFAP (1:1000; Sigma). Sections were washed and incubated for 2h at RT in secondary antibodies, mounted, coverslipped and examined under a LSM510 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY). Negative controls, in which the primary antibodies were omitted were run in parallel, and an isotype-matched negative control for staining was conducted.

**Western blots**

To determine protein expression of Tim-3, galectin-9, iNOS, Nitrotyrosine, Cox-2 at 1h, 5h, and 24h after stroke onset, brain tissues corresponding to the ischemic penumbra were used. Samples were lysed with RIPA buffer, extracts homogenized and insoluble debris removed by centrifugation. Protein concentration in the resulting supernatants was calculated using a Pierce protein assay kit according to the manufactures instructions (Pierce, IL, U.S.A.). Protein samples were loaded and separated using 4-15% SDS-polyacrylamide gel electrophoresis (Invitrogen, CA, U.S.A.) and transferred to nitrocellulose membrane. Membranes were scanned using Typhoon trio (GE Healthcare). We used primary antibodies against Tim-3 (1:500; Santa Cruz Biotechnology; sc-30326), Cox-2 (1:1000; Cayman; 160106), Nitrotyrosine (1:500; Millipore (Chemcon); 92590), iNOS (1:10000; BD Biosciences 610431), β-actin (1:10000; SIGMA; A3854-200μL). Band measurements were taken using ImageQuant software measurements of optical density.

**Statistical analysis**

Two-way ANOVA was used to compare the protective effect of LRP on protein bands from Western blots. Differences in protein bands within the same condition were analyzed using one-way ANOVA followed by Fisher LSD post-hoc test. All tests were considered statistically significant for P values <0.05. Data is presented as means ± SEM.

**Figure 3** Remote preconditioning inhibited galectin-9 expression 24h after stroke. Western blot indicates that galectin-9 was increased after stroke; remote preconditioning did not block its expression before 5h, but inhibited it at 24h. The results of confocal microscopy indicate that galectin-9 was increased, and such expression was inhibited by remote preconditioning. Results presented as Fig. 2.
Results

Rapid limb preconditioning reduced infarct size after stroke

Remote preconditioning in the femoral artery of the limb of rat reduced infarct size after stroke. (Fig. 1)

Tim-3/galectin-9 pathway was inhibited at 24h by remote preconditioning

From western blot analysis, both Tim-3 and galectin-9 expression showed similar increases in control ischemia, and decreases in LRP tissue. Tim-3 expression significantly increased at 24h vs. sham (p<0.001) in control ischemia (Fig. 2A,C), levels of Tim-3 increased at 5h compared to sham, but not at significant levels. Galectin-9 increased at 5h and 24h in rats receiving control ischemia (p<0.001) (Fig. 3A,C). LRP blocked the increase in both Tim-3 (Fig. 2B,D) and galectin-9 at 24h (p<0.001) (Fig. 3B,D). Reductions in both Tim-3 and galectin-9 were also seen in immunostaining results (Fig. 2E, 3E)

iNOS and Nitrotyrosine were inhibited by remote preconditioning compared to control

iNOS expression increased at 1h, 5h and 24h in control (p<0.001, 0.01, 0.05, respectively), reaching a peak at 1h (Fig. 4A). Western blot of iNOS clearly showed an increase in control ischemia compared to sham. Remote preconditioning decreased iNOS expression to 20% of sham at 1h, 5h, and 24h (p<0.001), thus attenuating the increase found in control ischemia (Fig. 4B). Nitrotyrosine increased in control ischemia (Fig. 5A,C), and like iNOS, nitrotyrosine decreased to 10-20% of sham at 1h, 5h, and 24h (P<0.001) with remote preconditioning (Fig. 5B,D). The increase in nitrotyrosine expression, thus, was also significantly attenuated with remote surgery.

**COX-2 expression did not change significantly with remote preconditioning**

COX-2 showed an increase at 24h in control (p<0.001), (Fig. 6A,C) however, LRP did not block this increase at 24h (Fig. 6B,D). As seen in Figure 6, there is a distinct maximum of expression at 24h in both preconditioned and control rats. Confocal showed similar results (Fig. 6E) with no attenuation of COX-2 represented by the blue fluorescence between control and remote brain tissue.
Discussion and Conclusions

This study begins to address how limb remote preconditioning is protecting the brain from ischemia. Though well established that inflammation is a primary cause of neuronal death (Brown & Nehar 2010), it is not known if remote preconditioning reduces inflammation in the brain, and if so, by which specific inflammatory agents. The simultaneous increase and decrease of both players at similar timepoints suggest that the Tim-3/galectin-9 pathway contributes to damage after focal ischemia, and thus contributes to the protective effects of distal remote preconditioning. Similarly, iNOS and nitrotyrosine are two players in a similar pathway, both being reduced simultaneously in remote preconditioning. Nitric oxide (NO) from iNOS expression strongly synergizes with hypoxia to induce neuronal death; NO inhibits cytochrome oxidase in competition with oxygen, resulting in glumate release and excitotoxicity, calling for inflammatory activated microglia and astrocytes to kill these neurons (Brown 2007). Nitrotyrosine is a known marker for damage caused by nitric oxide (2007). Despite COX-2’s known major role in neuroinflammation and its implicated roles in neurodegenerative diseases like multiple sclerosis, Parkinson’s, and Alzheimer’s, and traumatic brain injury (Yang et al. 2008), this major inflammatory inducer was not reduced after remote preconditioning. Our results confirm that COX-2 is elevated after stroke, but does not contribute to the protective effects of remote preconditioning. This suggests that COX-2 does not play a major role in the brain damage after stroke, and/or LRP simply does not act on COX-2. Keeping the results of this study limited, there are three conclusions that can be drawn:

1. Remote preconditioning reduces infarct size after focal ischemia, confirming prior results. These surgeries were conducted using the same protocol as Ren et al. 2008, with different hands doing the surgery—however, the results were replicated.

2. Protein expression of Tim-3, galectin-9, COX-2, iNOS, Nitrotyrosine was increased after focal ischemia. Remote preconditioning blocked increases in Tim-3, galectin-9, iNOS and nitrotyrosine, but it did not affect expression of COX-2.

3. The Tim-3/galectin-9 pathway is involved in brain injury after stroke. Reduction in galectin-9, Tim-3, iNOS and nitrotyrosine may contribute to the protective effect of remote preconditioning.

The next step in this study would be to suppress the genes for specific pathways, like the Tim-3/galectin-9 pathway, using siRNA, and test if infarct is reduced post focal cerebral ischemia.

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