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RAGE signaling mediates post-injury arterial neointima formation by suppression of liver kinase B1 and AMPK activity

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Abstract

Objective—Intima formation involves smooth muscle cell (SMC) proliferation and migration that ultimately drives arterial stenosis, thrombosis, and ischemia in atherosclerosis, hypertension, and arterial revascularization. Receptor for advanced glycation endproducts (RAGE) is a transmembrane signaling receptor implicated in diabetic renal and vascular complications, and post-injury intima formation, partly via Signal transducer and activator of transcription 3 (STAT3) activation. The metabolic super-regulator Adenosine monophosphate kinase (AMPK) inhibits SMC proliferation and intima formation. AMPK activation is promoted by liver kinase B1 (LKB1), and LKB1 inhibits STAT3 activation. Here, we tested the hypothesis that RAGE promotes arterial intima formation by modulating both LKB1 and AMPK.

Methods and Results—RAGE ligands (the calgranulin S100A11, and glycated albumin) suppressed AMPK activation in conjunction with increased proliferation and migration of cultured SMCs. These effects were inhibited both by RAGE deficiency and by prior AMPK activation. In SMCs, RAGE ligands decreased LKB1 activity. Moreover, knockdown of both LKB1 and AMPK were associated with increased STAT3 phosphorylation levels. In response to murine carotid artery ligation, expression of RAGE and S100A11 increased, whereas AMPK and LKB1 activity decreased *in situ*. Conversely, LKB1 and AMPK activity increased *in situ*, and neointima formation was attenuated in *Rage*^{-/-} mice.

Conclusion—The linkage of decreased LKB1 and AMPK activity with increased STAT3 in SMCs mediates the capacity of RAGE ligand-induced signaling to promote neointima formation in response to arterial injury.

Keywords

RAGE; AMPK; LKB1; Arterial restenosis; Neointimal hyperplasia

Neointima formation occurs in arterial responses to a variety of insults, including revascularization, and is characterized by vascular smooth muscle cell (SMC) proliferation, migration from the media, and increased MMP activity¹. RAGE, the receptor for advanced glycation endproducts (AGE), is a multiligand transmembrane signaling receptor, originally

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described as having a pivotal role in vascular, renal, and peripheral neurologic lesion development and progression in diabetes ². RAGE signaling mediates arterial disease in diabetes, atherosclerosis, diabetes, and after vessel injury, mediated by accumulation of AGE and other RAGE ligands^{3–6}. In this context, RAGE drives full phenotypic expression of neointima formation following vascular injury^{4, 7}. Moreover, when a transgenic dominant negative RAGE cytosolic tail deletion mutant was specifically expressed in SMCs and tested in the post-endothelial denudation arterial injury model, the specific role of SMC-expressed RAGE in neointima formation was established⁴.

Ligands of RAGE, other than AGE, include high mobility group box protein 1 (HMGB1) and many members of the S100 calgranulin family of low molecular weight calcium-binding proteins that form homodimers and heterodimers ⁸. SMCs, other resident arterial cells, and phagocytes express S100 calgranulins ^{5, 9}. Physiologic functions of intracellular S100 proteins include intercompartmental calcium shuttling, and regulation of cell growth ¹⁰. Several of the S100 proteins also are secreted by activated cells. These include S100A11 ¹¹ and S100A8/A9, a dual RAGE and toll-like receptor 4 (TLR4) ligand released by phagocytes ¹². Extracellular S100 protein binding to RAGE, like that of other RAGE ligands, induces several signaling pathways that promote inflammation, exemplified by mitogen activated protein kinase, NF- κ B, and STAT signaling¹³.

Arterial SMCs must adapt to changing metabolic demands in response to inflammation and biomechanical stress. We studied the role of the serine/threonine protein kinase AMPK, an evolutionarily conserved metabolic “super-regulator”. Activated AMPK phosphorylates downstream targets that inhibit ATP-consuming pathways and activate ATP-producing pathways, thereby allowing cells to balance cellular energy supply and demands ¹⁴. AMPK activation also enhances endurance within skeletal muscle, partly by promoting mitochondrial biogenesis ¹⁵. Activated AMPK inhibits proliferative signaling of cultured SMCs and inhibits VRD *in vivo* ¹⁶. Furthermore, AMPK has anti-inflammatory effects, mediated partly by suppression of NF- κ B activation¹⁷.

AMPK is normally activated by nutrient deprivation, hypoxia, and exercise, which act by increasing the cellular AMP: ATP ratio ¹⁴. Regulation of AMPK activity also involves altered phosphorylation by protein phosphatases, such as Protein phosphatase 2A (PP2A), which dephosphorylates and thereby inactivates AMPK. Conversely, the tumor suppressor LKB1, a serine/ threonine protein kinase, is the major upstream kinase that phosphorylates and activates AMPK. LKB1 also has been reported to suppress STAT3 activation, and the molecular mechanisms by which RAGE mediates vascular intima formation involves induction of STAT3 signaling ¹⁸. Hence, this study tested the hypothesis that RAGE mediates injury-induced intima formation in a manner modulated by linked regulation of LKB1 and AMPK activity in SMCs.

Methods

Mice

We studied previously described *Rage*^{-/-} mice on a C57BL/6 background ¹⁹, and control congenic wild-type (WT) C57BL/6 (from Jackson Laboratories). All animal procedures were humanely performed, with institutionally approved protocols.

Primary aortic SMC Isolation and Culture

Primary SMCs were isolated from 6–8 weeks old mice aortae or mature bovine aortas by collagenase and elastase digestion ²⁰. Digested cells were seeded in 0.1% gelatin precoated 6-well plates and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin. SMC

purity was confirmed using immunocytochemistry for smooth muscle -actin (>95% positive) and VWF (<1% positive). Unless otherwise indicated, SMCs were treated for 18 h, following 8 h serum starvation in DMEM, containing 0.1% FCS using cells up to passage 6.

SMC Proliferation, Migration, and Transfection In Vitro

To measure SMC proliferation, aliquots of 5,000 cells were seeded in a 96-well plate and cultured overnight to allow adherence. After serum starvation, and then stimulation, 20 μ l of CellTiter 96 Aqueous One Solution (Promega) was pipetted into each well containing the samples in 100 μ l of culture medium. Plates were incubated at 37°C for 1–4 hours and absorbance was measured at 490 nm. To measure SMC migration, cells were plated in the upper chamber of a 24-well Costar Transwell (1×10^5 cells/well), and allowed to adhere for 18 hr. After 48 h of stimulation, SMCs migrated to the lower face of the membrane were fixed with methanol and stained with 1% Toluidine blue O and 1% sodium bicarbonate. The Micron (EVOS) system was used to take pictures at 20X magnification. Cell numbers are indicated per 1 field of view, with 5 fields of view examined in each experiment. For transfection of bovine SMCs, we used Nucleofection (Amaxa), with the manufacturer protocol optimized for Primary SMCs yielding a transfection efficiency of 60–80%.

Carotid Artery Ligation

Left carotid artery ligation surgery was performed as reported previously²¹. In brief, 8 weeks old mice were anesthetized by IP injection of a mixture of ketamine (15 mg/kg/0.1 ml), xylazine (1.6 mg/kg/0.1 ml) and acepromazine (1.2 mg/kg/0.1 ml). Left carotid arteries were dissected and ligated proximal to the carotid bifurcation, using 6-0 nylon silk. After surgery, mice received IP injection of buprenorphine (0.1 mg/kg, 0.03 mg/ml concentration). All animals recovered well and showed no signs of stroke. Animals were euthanized at 3, 7, 14 and 21 days after carotid ligation. Carotid arteries were fixed with 4% paraformaldehyde (PFA) in PBS for morphological analysis²² or frozen for protein extraction.

Immunohistochemistry

Slides of mice carotid arteries were deparaffinized in citrisolve and rehydrated through serial dilutions of alcohol. Endogenous peroxide activity was blocked with 0.3% H₂O₂ for 20 min at room temperature (RT). After washing in PBS, non-specific antibody binding was blocked by incubation in PBS supplemented with 5% normal goat serum for 20 min at 22°C prior to incubation with primary antibodies overnight at 4°C. Incubation in secondary antibody for 30 minutes at 22°C, was followed by treatment for 30 min using the Histostain Plus kit (Invitrogen, Carlsbad, CA). Sections were then washed, and incubated with 3,3'-diaminobenzidine (DAB) substrate for 2–5 min, and then counterstained with Hematoxylin. The slides were mounted for examination, and images captured by the Olympus BX51 microscopic DP71 Digital Camera System.

Statistical analyses

Results were expressed as mean $\pm$ SEM. Statistical differences between group means were determined by one-way ANOVA with post hoc Tukey testing, using GraphPad PRISM 5. P values < 0.05 were considered statistically significant.

Additional Description of Reagents and Methods

Additional detailed descriptions of reagents and methods for SDS/PAGE and Western blotting are provided in the Online Supplemental Material.

Results

Effects of RAGE ligands and RAGE deficiency on activation of AMPK and on proliferation and migration in cultured SMCs

In comparison to WT SMCs, cultured aortic SMCs from *Rage*^{-/-} mice demonstrated elevated constitutive AMPK threonine phosphorylation, a standard marker of AMPK activation, (Figure 1A). Phosphorylation of the downstream AMPK target acetyl-CoA carboxylase (ACC) Ser79 was increased in *Rage* knockout compared to WT SMCs (Figure 1A). The RAGE ligands S100A11 and human glycated albumin (HGA) did not suppress AMPK activity (assessed by phosphorylation of AMPK and ACC), and did not promote proliferation and migration in *Rage*^{-/-} SMCs (Figure 1B, C, D, E). Basal and PDGF-induced proliferation and migration also were reduced in cultured *Rage*^{-/-} mouse SMCs under these conditions (Figure 1 D, E). Conversely, S100A11 and HGA induced decreased bovine SMC p-AMPK levels, and stimulated proliferation and migration that both were suppressed by pharmacologic AMPK activation (Supplemental Figures 1–2).

Neointima formation in response to carotid ligation is inhibited in *Rage*^{-/-} mice in association with preserved AMPK phosphorylation

Permanent ligation of the left common carotid artery was performed in WT and *Rage*^{-/-} mice. Carotid artery ligation induced increased lesion expression of S100A11 and decreased AMPK phosphorylation, and increased expression of the proliferation marker Ki-67 in WT mice, but less so for these responses to arterial injury in *Rage*^{-/-} mice (Figure 2A–D). At 21 days post-injury, marked neointima formation was observed in WT mice in the area 500 μ m to 1 mm proximal to the ligation, but this neointimal response was inhibited in *Rage*^{-/-} mice (Figure 3A, B, D), with reduction of the intima/media ratio of the injured arteries by 38% associated with *Rage* knockout ($P < 0.001$, 0.58 ± 0.093 vs 0.20 ± 0.044) (Figure 3E, Supplemental Table 1)

AMPK activity-dependence of RAGE ligand-induced SMC activation *in vitro*

We tested the role of AMPK activity in RAGE-mediated SMC functions by transfecting constitutively active (CA) myc-tagged AMPK α 1 (CA-AMPK α 1), or empty vector control, into bovine aortic SMCs. CA-AMPK α 1 transfection prevented suppression of p-AMPK levels in response to S100A11 or HGA, promoted downstream ACC phosphorylation, and reversed the decrease in p-AMPK to total AMPK ratios seen in control cells in response to RAGE ligands (Figure 4A, B). Under these conditions, transfection of CA-AMPK α 1 induced a loss of SMC proliferative responses to S100A11 and HGA, as well as to PDGF (Figure 4C). Conversely, AMPK α siRNA, which was confirmed to suppress total AMPK expression, and AMPK and ACC phosphorylation (Figure 4D), significantly increased proliferation in *Rage*^{-/-} SMCs (Figure 4E).

Effects of LKB1 and PP2A in RAGE-mediated modulation of AMPK and STAT3 activity in SMCs *in vitro*

AMPK phosphorylation is increased by LKB1, and is decreased by the phosphatase PP2A. Phosphorylation at Tyr307 in the conserved PP2A C-terminal domain inactivates PP2A. Here, we observed that decreased LKB1 and PP2A expression, induced by siRNA, stimulated increased and decreased p-AMPK levels, respectively in *Rage*^{-/-} SMCs (Figure 5A). Moreover, RAGE ligands induced decreased p-AMPK and decreased tyrosine-phosphorylated PP2A (p-PP2A) in cultured SMCs (Supplemental Figure 3). Conversely, constitutive phosphorylation of LKB1 was increased in *Rage*^{-/-} SMCs (Figure 5B). In *Rage*^{-/-} SMCs p-PP2A was increased, whereas total PP2A expression was not grossly altered relative to WT cells (Figure 5B).

Levels of STAT3 expression and phosphorylation both were markedly decreased in *Rage*^{-/-} SMCs relative to control SMCs, but these changes were reversed by siRNA knockdown of AMPK (Figure 5C, Supplemental Figure 4A). Though LKB1 siRNA knockdown did not appreciably alter STAT3 levels, it increased the level of STAT3 phosphorylation in SMCs (Figure 5D, Supplemental Figure 4B). In contrast, PP2A knockdown did not appreciably modulate either STAT3 expression or phosphorylation in SMCs (Figure 5D, Supplemental Figure 4B).

RAGE deficiency *in vivo* is associated with increased activation of AMPK linked to regulation of LKB1 and PP2A

Carotid artery ligation induced decreased AMPK activation and increased RAGE expression *in situ* (Figure 6A,C). Both uninjured and injured arteries from *Rage*^{-/-} mice demonstrated higher *in situ* levels of p-AMPK than did carotid arteries in WT mice (Figure 6C), in association with decreased expression and phosphorylation of STAT3 (Figure 6B). Last, carotid ligation induced decreased levels of p-LKB1 and p-PP2A in WT mice *in situ*, changes that were inhibited by RAGE knockout (Figure 6D).

Discussion

AMPK activity suppresses SMC proliferation *in vitro* and inhibits neointima formation *in vivo*¹⁶. This study revealed that RAGE ligand-induced signaling by S100A11 and HGA induced decreased AMPK activity, which was central to RAGE-mediated SMC proliferation and migration *in vitro*. Moreover, this study was the first to identify increased expression of S100A11 in injured carotid arteries.

AMPK activity is partly regulated by threonine phosphorylation at the catalytic domain of the α subunit, modulated by the balance between activities of the serine/threonine kinase LKB1 and the protein phosphatase PP2A. In this study, distinct RAGE ligands (S100A11, HGA) induced not only decreased AMPK phosphorylation, but also decreased LKB1 phosphorylation and decreased PP2A phosphorylation *in vitro* in bovine SMCs. Furthermore, LKB1 and PP2A played a major role in regulation of AMPK activity in cultured SMCs, as demonstrated by siRNA studies. However, *in vivo* results after carotid ligation suggested a more prominent role of altered LKB1 phosphorylation, which was increased *in situ* in resting and injured carotid arteries in *Rage*^{-/-} compared to WT mice. In contrast, changes in PP2A phosphorylation were minor *in situ*. These *in situ* results, limited to a single time point, do not rule out a significant role of modulation of PP2A activity in regulating AMPK activity in earlier phases of vascular intima formation after injury. It also is possible that the capacity of PP2A to promote NF- κ B activity in vascular SMC activation²³ modulates neointima formation. Moreover, AMPK suppresses NF- κ B activation and exerts other anti-inflammatory effects¹⁷ likely significant in neointima formation.

LKB1 is a tumor suppressor gene, known to inhibit cell proliferation by modulation of c-Myc expression²⁴, and by binding to STAT3 and limiting STAT3-induced transactivation of multiple genes²⁵. LKB1 acts in part by suppressing tyrosine phosphorylation of STAT3. Importantly, AGE-induced RAGE signaling drives a STAT3/serine/threonine protein kinase Provirus integration site for Moloney murine leukemia virus (Pim1)/NFATc2 signaling axis centrally involved in cultured SMC proliferation and activation²⁶. RAGE signaling is essential for STAT3/Pim1/NFAT activation *in vivo*, and for neointima formation *in vivo*, in response to carotid artery angioplasty¹⁸. Here, we determined that LKB1 and AMPK activity both inhibited levels of STAT3 activity in cultured SMCs. LKB1 effects were principally on LKB1 phosphorylation, whereas AMPK suppressed the level of AMPK expression. Studies to further assess relationships between LKB1 and AMPK activity, and

the STAT3/Pim/NFATc2 signaling axis, would be of interest in SMCs. Other mediators potentially involved in RAGE-mediated responses to arterial injury include SIRT1, an NAD-dependent histone/protein deacetylase that activates AMPK via stimulation of LKB1 activity²⁷. Expression of SIRT1 is decreased in injured arteries²⁸. Furthermore, the cyclin-dependent kinase inhibitor p27 (Kip1), S-phase kinase-associated protein 2 (Skp2) (a known E3 ubiquitin ligase for p27), and increased activation of p52 NF- κ B²⁹, mediate AMPK effects on SMCs.

There are limitations of this study, and other questions to be addressed in the future, based on the results herein. For example, it would be of interest to examine deficiency states for individual RAGE ligands such as S100A11 *in vivo*, though the multiplicity of RAGE ligands argues against a dominant effect of a single RAGE ligand over others in carotid artery injury. This study also did not examine SMC-specific deficiency of RAGE or AMPK *in vivo*. Murine LKB1 homozygous knockout is embryonic lethal due to multiple developmental abnormalities, which include dysregulated blood vessel formation in the yolk sac and placenta, modulated by altered angiogenesis and expression of VEGF³⁰. Hence, the possibility that LKB1 modulates neointima formation, merits further investigation, but requires highly tissue-selective modulation of LKB1 expression. Last, the carotid artery ligation model results in dramatic reduction of vessel diameter and decreased blood flow that results in altered shear stress, leading to the formation of an extensive smooth muscle cell-rich neointima after 2–4 weeks, with advantage of its reproducibility due to the ease of operation. However, carotid artery ligation is only one of the commonly used procedures to induce artery injury in mice, and is only a model of human disease. One alternative, the mechanically-induced endothelial denudation model, resembles the physiological setting of vascular intervention, but there are technical challenges to this procedure, due to potential penetration of the guide wire through the vessel wall, and elastic lamina tearing and disruption.

Recently, Song et al, studying differential knockout mice for AMPK subunits, demonstrated that heterotrimeric AMPK containing the 2 subunit (AMPK α 2), but not AMPK α 1, is anti-proliferative in cultured mouse aortic SMCs²⁹. AMPK α 2^{-/-} (but not AMPK α 1^{-/-}) mice developed increased neointima formation after mechanical arterial injury²⁹. Hence, future studies to flesh out the effects of RAGE signaling on different AMPK heterotrimers will be of interest.

We conclude that RAGE ligand-induced signaling promotes decreased LKB1 and AMPK activity that modulate STAT3 phosphorylation in SMCs. These effects promote neointima formation after arterial injury.

Highlights

RAGE deficiency inhibits post-injury vascular remodeling by activating AMPK pathway.
Carotid ligation decreases p-AMPK and increases RAGE and ligands, which promotes SMCs proliferation and migration.
RAGE/ligands mediates vascular remodeling by regulation of AMPK activity via LKB1 and STAT3

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Effects of RAGE deficiency on AMPK activation and SMC function

A, SMC lysates from WT and *Rage*^{-/-} mice were analyzed by Western blotting using antibodies for phospho-ACC (Ser79)/ACC and p-AMPK/AMPK. Representative blots of 3 independent experiments are shown. **B**, SMCs were treated with vehicle, S100A11, or HGA for 16 h, and p-AMPK, total AMPK, and β-Actin assessed by Western blotting. **C**, SMCs were seeded in Trans-well plates for 18 h and then treated with stimuli as indicated and migration assay results shown, * $p < 0.05$ vs. WT, † $p < 0.05$ *Rage*^{-/-} vs. WT. Scale bar: 100 μm. **D**, Quantitative proliferation assay of WT and *Rage*^{-/-} SMCs stimulated as indicated. * $p < 0.05$ vs. WT Ctrl, † $p < 0.05$ *Rage*^{-/-} vs. WT.

Figure 2. Effects of RAGE deletion on lesion p-AMPK, S100A11 and Ki-67 after carotid artery ligation

A, Sections from the right and left common carotid arteries of WT and *Rage*^{-/-} mice 21 days after left carotid artery ligation, stained for S100A11, p-AMPK, AMPK and the proliferation marker Ki-67. Representative images shown. Scale bar: 200 μ m. **B**, Quantification of S100A11 lesion area. * $p < 0.01$ vs. WT right arteries, † $p < 0.001$ vs. WT left arteries. **C**, Quantification of p-AMPK/AMPK ratio. * $p < 0.01$, ** $p < 0.001$ vs. WT right arteries, † $p < 0.001$ vs. WT left arteries. **D**, Quantification of Ki-67-positive nuclei. * $p < 0.001$ vs. WT right arteries, † $p < 0.001$ vs. WT left arteries.

Figure 3. Effects of RAGE deletion on vascular neointima formation following left carotid artery ligation

A, Representative cross-sections of common carotid arteries obtained from WT (n=9) and *Rage*^{-/-} (n=7) mice 21 days after the ligation, stained with hematoxylin and eosin. **B**, Representative paraffin sections of Verhoeff-van Gieson Elastin staining of common carotid arteries 21 days after ligation. **C**, Cross-section of mouse carotid artery; arrows point to the *external* and *internal elastic laminae*; EEL and IEL, respectively. Radius R1, R2 and R3 were used to compute the different areas. EEL Area= $\pi R1^2$; IEL Area = $\pi R2^2$; Lumen Area = $\pi R3^2$; Media Area = EEL Area - IEL Area; Intimal Area = IEL Area - Lumen Area. **D**

and E, Morphologic measurements of left mice carotid arteries. I/M Ratio: Intimal Area divided by Media Area. * $p < 0.05$, ** $p < 0.01$ vs. WT.

Figure 4. S100A11 and HGA induce AMPK-dependent SMC proliferation

A, Bovine aortic SMCs were transfected with CA-AMPK α 1 or empty vector; 48 h after transfection, cells were stimulated with S100A11 or HGA for 16 h. Cell lysates were studied by Western blotting. **B**, Quantification of Western blots. * $p < 0.05$. ** $p < 0.01$. **C**, 48 h after transfection, cells were treated with S100A11, HGA or PDGF for 24 h and proliferation quantified. * $p < 0.05$, * * $p < 0.01$ vs. control. **D**, *Rage*^{-/-} SMCs were transfected with control siRNA or AMPK siRNA for 48 h. Representative Western blots are shown. **E**, Quantitative proliferation assay of *Rage*^{-/-} SMCs transfected with control siRNA or AMPK siRNA. ** $p < 0.01$ vs. WT Ctrl siRNA control group. † $p < 0.05$ vs. AMPK siRNA control group.

Figure 5. RAGE regulates AMPK activity by modulating LKB1 and PP2A activity *in vitro*
A, *Rage*^{-/-} SMCs were transfected with control siRNA, LKB1 siRNA, or PP2A siRNA. Representative Western blots are shown. **B**, Representative Western blots of SMC lysates from WT and *Rage*^{-/-} mice. **C**, WT SMCs were transfected with empty vector, whereas *Rage*^{-/-} SMCs were transfected with control siRNA or AMPK siRNA for 48 h. Cell lysates were studied by Western blotting. **D**, *Rage*^{-/-} SMCs were transfected with control siRNA, LKB1 siRNA, or PP2A siRNA. The efficiency of LKB1 knockdown was $81 \pm 4\%$, and PP2A knockdown $0.59 \pm 5\%$ assessed by densitometry in these studies. Representative Western blots for p-STAT3/STAT3 expression are shown.

Figure 6. RAGE deficiency modulates in situ AMPK and STAT3 activity following carotid ligation

A, Carotid tissue Western blots of WT and *Rage*^{-/-} mice (0, 3, 7, 14 days after left carotid artery ligation) for RAGE, p-AMPK, total AMPK, and anti-β-Actin. **B**, Western blots for p-STAT3/STAT3 expression in carotids of WT and *Rage*^{-/-} after 21 days ligation. **C**, Carotid lesion p-AMPK expression 21 days after ligation. **D**, Western blots using kinase-specific and phosphokinase-specific antibodies for AMPK pathway mediators LKB1, PP2A, and ACC 21 days after left carotid artery ligation.