Insulin-like growth factor I is required for vessel remodeling in the adult brain

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Although vascular dysfunction is a major suspect in the etiology of several important neurodegenerative diseases, the signals involved in vessel homeostasis in the brain are still poorly understood. We have determined whether insulin-like growth factor I (IGF-I), a wide-spectrum growth factor with angiogenic actions, participates in vascular remodeling in the adult brain. IGF-I induces the growth of cultured brain endothelial cells through hypoxia-inducible factor 1α and vascular endothelial growth factor, a canonical angiogenic pathway. Furthermore, the systemic injection of IGF-I in adult mice increases brain vessel density. Physical exercise that stimulates widespread brain vessel growth in normal mice fails to do so in mice with low serum IGF-I. Brain injury that stimulates angiogenesis at the injury site also requires IGF-I to promote perilesion vessel growth, because blockade of IGF-I input by an anti-IGF-I abrogates vascular growth at the injury site. Thus, IGF-I participates in vessel remodeling in the adult brain. Low serum/brain IGF-I levels that are associated with old age and with several neurodegenerative diseases may be related to an increased risk of vascular dysfunction.

After vessel formation has been completed during development, brain angiogenesis is maintained mostly to match functional demands. Vascular remodeling may also take place in the adult brain in response to specific stimuli such as injury or physical exercise (1, 2). In the case of brain injury, vessel sprouting usually leads to distorted neovascularization, probably because of a disturbed tissue architecture and scar formation; in response to physical exercise, new vessels develop within an unperturbed environment and appear normal. Because similar angiogenic mediators are present during development and in the adult, it is considered that, at the molecular level, angiogenesis in the adult brain is similar to that seen during development. However, this remains to be shown.

A potential candidate signal in this regard is insulin-like growth factor I (IGF-I) a well known angiogenic factor (3). Studies suggest that IGF-I modulates vessel formation during brain development (4), and that IGF-I may be involved in diabetic retinal neovascularization (5) and possibly in age-related changes in brain vasculature (6). It has recently been shown that both brain-derived and circulating IGF-I act as a neuroprotective signal in the adult brain (7). For instance, both local (8) and serum (9) IGF-I protect against brain injury. Serum IGF-I also is important for mediating the protective effects of physical exercise on the brain (10). Because reactive vessel remodeling occurs after both injury and exercise, an additional neuroprotective action of IGF-I might be to favor brain angiogenesis in response to injury and/or exercise. In this study, we have explored these possibilities.

Materials and Methods

Animals. Wistar rats and C57BL/6 mice from our inbred colony were used in the study. Mutant mice with low serum IGF-I were generated by disrupting the liver IGF-I gene (liver IGF-I-deleted, or LID) mice with the albumin-Cre/Lox system, as described (11). Lack of liver IGF-I results in a 60% decrease of serum IGF-I levels, whereas in the rest of the body, including the brain, serum IGF-I levels are normal (11). Mice that are deficient in serum IGF-I do not exhibit developmental defects, probably because the levels of IGF-I in serum start to decline when development is already completed (11, 12). $Lox^{+/+}Cre^{-}$ littermates were used as controls. Male mice 2–3 months old were used throughout the study. Wild-type mice were included as additional controls because they are congenic to the original FVB/N LID breeders. The animals were kept under standard laboratory conditions in accordance with European Communities Council guidelines (directive 86/609/EEC).

***In Vitro* Assays.** The brain endothelial cells were cultured as described (13), with minor modifications. The purified endothelial cells (over 95% $CD31^{+}$) from P7-8 rat pups were cultured in DMEM-F12 + 10% FCS + EGF (10 ng/ml, Sigma). Before treatments, the cells were placed in serum-free medium (14). Cell proliferation was determined by using a commercial 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide assay (Roche Diagnostics), nine wells per group. Tubulization of the endothelial cells was monitored with a 3D collagen culture system (Chemicon), and the cells were processed for lectin immunocytochemistry. In transfection assays using FuGENE reagent (Roche Diagnostics), the cells (5×10^5 cells per well in 12-well plates) were transfected by using a dominant negative mutant hypoxia-inducible factor 1α (DN-HIF-1α) with a hemagglutinin epitope tag cloned in the pcDNA3 vector (15) and maintained in DMEM-F12 + 10% FCS. An empty vector was used as a control for mock transfections. IGF-I (GroPep, Adelaide, Australia) and vascular endothelial growth factor (VEGF) (Sigma) were used at 10^{-7} M.

***In Vivo* Procedures.** To determine the effects of exercise on endothelial cell proliferation, LID mice and control littermates running daily in a treadmill (10) received i.p. injections of BrdUrd (50 mg/kg, 3 days per week). Mice ran for 1 h at 12 m/min for 4 weeks, whereas control animals remained in the treadmill without running. In a second type of experiment, we administered IGF-1 s.c. either by using an Alzet 2001 osmotic minipump (Alzet, Palo Alto, CA) (50 μg/kg per day for 2 weeks) or through injection of IGF-I microspheres (100 μg/kg per day, once every week for 1 month) as described (16). Control animals received saline.

To assess the effects of brain injury on neovascularization, cortical stab wounds were performed in deeply anesthetized LID and control mice. A 26-gauge needle was inserted 3 mm from the surface of the brain (1.3 to 2–7 mm caudal to bregma, 1.5 mm lateral) (17). Animals were allowed to survive for 1, 3, 14, or 28 days. In a separate set of experiments, brain-injured LID mice received a s.c. infusion of a blocking anti-IGF-I over 28 days, as described elsewhere (9). Control groups of injured LID mice were infused with either nonimmune rabbit serum (NRS) or vehicle (saline). To determine perilesional changes in angiogenic markers and vessel

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Abbreviations: IGF-I, insulin-like growth factor I; HIF-1α, hypoxia-inducible factor 1α; DN-HIF-1α, dominant negative HIF-1α; VEGF, vascular endothelial growth factor; VEGFR1 and -2, VEGFR receptors 1 and 2; LID, liver IGF-I-deleted; NRS, nonimmune rabbit serum.

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density, a block of brain parechyma surrounding the stab wound (2 mm wide, 4 mm high) was dissected from each animal.

Immunoassays. The animals were perfused transcardially with saline. The right brain hemisphere and hemicerebellum were removed and fixed for 24 h at 4°C by using 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Fifty-micrometer coronal sections were cut rostrocaudally by using a vibratome (Leica, Cambridge, U.K.) and immersed in 0.1 M PB. The left hipoccampi and the other half of the cerebellum were kept at −80°C for immunoassays. Two series of sections per animal were used for performing cell counts. The other series were used for immunocytochemistry. Antibody incubations were performed by using 0.1 M PB/0.5% Triton X-100/0.3% BSA. For BrdUrd detection, DNA was denatured by using 2 M HCl for 30 min at room temperature. A rat anti-BrdUrd antibody (1:5,000; Developmental Studies Hybridoma Bank, Iowa City) and biotinylated tomato lectin (1:300; Vector Laboratories) were used for staining of DNA-synthesizing cells and endothelial cells, respectively. A monoclonal anti-HA tag (1:1,000; Cell Signaling Technology, Beverly, MA) was used to detect transfected cells. The secondary antibody was a biotinylated rabbit anti-rat IgG (1:1,000; Sigma) followed by the VECTASTAIN ABC kit (Vector Laboratories). For double BrdUrd-lectin staining, we used goat anti-rat IgG-Alexa Fluor 488 (1:1,000; Molecular Probes), anti-rabbit IgG-Alexa 594, anti-mouse IgG-Alexa Fluor 594 (1:1,000; Molecular Probes), or rhodamine-labeled tomato lectin (1:200; Vector Laboratories).

A Western blot analysis was performed by using the tissue and cell culture extracts, as described (18). Anti-VEGF (Oncogene Science); anti-VEGF receptor 1 (VEGFR1) and 2 (VEGFR2), anti-angiopoietin-1/2 (all from Santa Cruz Biotechnology); anti-HIF-1α (Stressgen Biotechnologies, Victoria, Canada); and anti-CD31 (Pharmingen) were used. An anti-PI3K was used to re-blot the membranes to ensure equal protein load. The protein levels were expressed relative to protein load in each lane. Densitometric analysis was performed by using SCION IMAGE software (Scion, Frederick, MD). A commercial VEGF ELISA (R & D Systems) was used to quantify brain homogenates according to the manufacturer's instructions.

Morphometry. Morphometrical analyses were performed as described (19). In brief, BrdUrd$^+$ cells were quantified by using an unbiased stereological method with the optical dissector (20). Parallel 50-μm sections were obtained. Only nuclei that were completely filled or that showed patches of immunostaining of variable intensity were considered as BrdUrd$^+$. To determine the

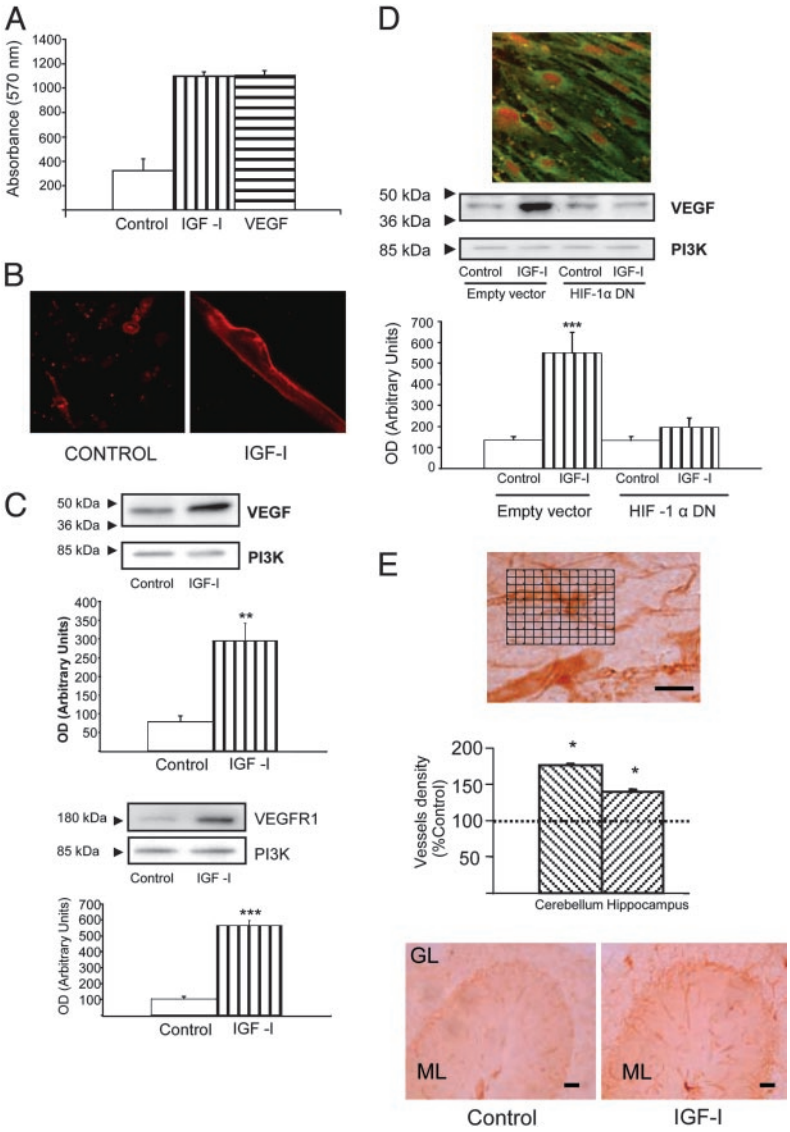

Fig. 1. IGF-I promotes brain angiogenesis. (*A*) IGF-I (10^{-7} M) stimulates proliferation of cultured brain endothelial cells as determined with the 3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide assay. VEGF (10^{-7} M) was used as a positive control [$n = 3$ independent experiments (nine replicates per experiment)]. (*B*) In the presence of IGF-I (10^{-7} M) endothelial cells plated on collagen form tubular structures, a prerequisite in the angiogenic process. Endothelial cells were labeled with tomato lectin (red). Note the absence of tubes under control conditions. (*C*) IGF-I stimulates both VEGF (upper gel) and VEGFR1 (lower gel) in endothelial cell cultures. Representative Western blots are shown. Lower gels show blots that were re-assayed with anti-PI3K antibody to control for protein load. Histograms show densitometric quantitation of the blots. **, $P < 0.01$; ***, $P < 0.001$ vs. control, $n = 5$. (*D*) The transcriptional modulator HIF-1α is required for IGF-I-induced activation of VEGF synthesis. (*Top*) Representative microphotograph of an endothelial cell monolayer transfected with HA-tagged DN-HIF-1α. Cells were stained with anti-lectin (green) and anti-HA tag (red). Note the nuclear location of mutant HIF-1α. (*Middle*) Representative VEGF blot of cultures transfected with empty pcDNA3 vector (control) and DN-HIF-1α. IGF-I increases VEGF only in mock-transfected cultures. Re-blotting with anti-PI3K shows an equal protein load in all lanes. (*Bottom*) Densitometric quantitation of five independent experiments. ***, $P < 0.001$ vs. all other groups. (*E*) A 2-week s.c. infusion of IGF-I (50 μg/kg per day) elicits a significant increase in vascularization of the adult mouse brain. (*Top*) Vessels were identified with biotynilated-tomato lectin (arrow) and the percent surface of brain parenchyma covered with vessels estimated by scoring vessel contacts with the points of the grid (a 36-point grid is shown). (Scale bar = 25 μm.) (*Middle*) The histograms indicate vessel density in the cerebellum and the hippocampus as percent of control (100%). *, $P < 0.05$ vs. saline-treated controls ($n = 5$ animals per group). (*Bottom*) Representative cerebellar section with lectin-positive vessels of saline and IGF-I-treated mice. Vessel density is increased after IGF-I treatment. ML, molecular layer; GL, granule cell layer. (Scale bar = 50 μm.)

Table 1. Levels of angiogenic-related proteins in different brain regions of serum-IGF-I-deficient (LID) mice

	VEGFR1	VEGFR2	CD31	Tie-2	Ang-1	Ang-2	VEGF	HIF-1α
Cortex	54 ± 22	86 ± 16	170 ± 51	81 ± 23	71 ± 8	107 ± 17	146 ± 17	89 ± 15
Cerebellum	132 ± 38	120 ± 23	107 ± 23	90 ± 41	101 ± 21	94 ± 12	77 ± 6	101 ± 15
Hippocampus	133 ± 38	94 ± 19	108 ± 23	143 ± 50	62 ± 15	103 ± 17	65 ± 17	95 ± 19

Values are expressed as percent ± SEM of levels found in control littermates. No significant differences were found for any of the proteins evaluated.

cell density, the counting area was drawn by using a camera lucida. The area was then estimated by using the point-counting method of Wiebel (21). Double immunohistochemistry was performed in the adjacent sections of each animal. We counted three to four areas per section and five to six sections in each animal.

Similarly, for our analysis of brain vasculature, photographs were taken and a semiquantitative analysis of vessel density was performed according to the point-counting method (21) on sections that were immunostained with lectin. The number of point intersections of lectin-positive profiles were scored in a 100-point grid used for the hippocampus and the cortex and in a 36-point grid used for the cerebellum. The grid covered the entire surface of the microscopic field so that surface area was calculated according to the magnification used (×100 for the hippocampus and cortex and ×40 for the cerebellum). In an example shown in Fig. 1*E*, the grid covers only part of the microscopic field for the sake of clarity. Vessel density is expressed as the percent of brain surface covered with vessels. The measurements were performed in 20 fields per section, with eight sections per animal (five to six when using cortical sections).

Statistical differences between groups were determined by using the two-tailed Student *t* test.

Results

IGF-I Promotes Angiogenesis in the Brain. We first determined whether IGF-I increases brain vessel growth. As reported for endothelial cells in other areas (22, 23), brain endothelial cells increase their proliferation and tubulize in response to IGF-I (Fig.

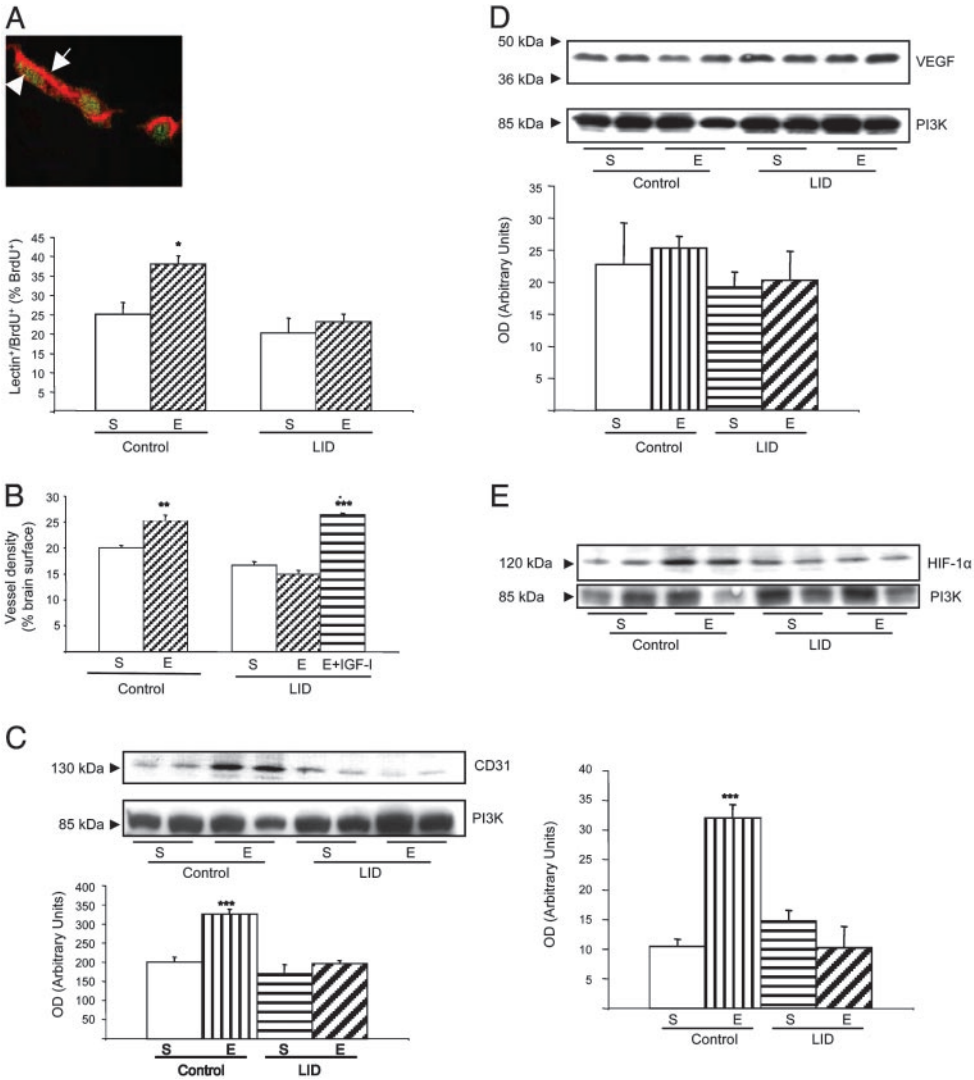

Fig. 2. Serum IGF-I is needed for exercise-induced vessel growth in the brain. (*A*) Exercise induces proliferation of endothelial cells only when serum IGF-I levels are normal. (*Upper*) Fluorescently labeled microphotograph of a newly formed brain vessel with $BrdUrd^+$ nuclei co-labeled with tomato lectin in the cytoplasm. (*Lower*) Exercise (E) significantly increased the number of double-labeled $BrdUrd^+/lectin^+$ cells, compared with sedentary (S) mice in the cerebellum of control littermates but not in LID mice with low serum IGF-I (*, $P < 0.05$ vs. sedentary; $n = 4$ animals per group). (*B*) Vessel density in the cerebellum increases after exercise only in control mice or in LID mice treated with IGF-I, but not in LID mice treated with saline while running (**, $P < 0.01$ vs. sedentary mice; ***, $P < 0.001$ vs. exercised LID mice). (*C*) Similarly, levels of CD31, an endothelial marker, increased only in exercised control mice. The upper gel shows a representative blot of CD31. The lower gel shows protein load per lane assessed by re-blotting with anti-PI3K. The histogram shows quantitation of CD31 blots after correcting for protein load (***, $P < 0.001$ vs. sedentary controls; $n = 4$ per group). (*D*) Brain levels of VEGF were not modified by exercise. The upper gel shows representative VEGF blots after 1 month of exercise. The lower gel shows the control for protein load. Quantitation is shown in the histogram ($n = 4$). (*E*) On the contrary, brain levels of HIF-1α were increased after exercise only when serum IGF-I levels are normal. The upper gel shows a representative HIF-1α blot after 1 month of exercise. The lower gel shows the PI3K control re-blot. Histograms illustrate significant increases in exercised control mice (***, $P < 0.001$; $n = 4$).

1 *A* and *B*). In addition, VEGF and VEGFR1 are up-regulated by IGF-I (Fig. 1*C*). Stimulation of VEGF by IGF-I requires the transcriptional modulator HIF-1α, which is involved in angiogenesis (24). The transfection of cultured endothelial cells with a DN-HIF-1α mutant inhibits the increase in VEGF induced by IGF-I (Fig. 1*D*). The levels of other proteins related to angiogenesis such as VEGFR2, angiopoietin-1/-2, or Tie-2 were not modified by IGF-I (data not shown).

Because IGF-I receptors are localized in the luminal side of brain vessels (25), circulating IGF-I can directly signal onto the brain endothelial cells and modulate their growth *in vivo* also. Indeed, the systemic administration of IGF-I promotes brain vessel formation (Fig. 1*E*) and increases brain VEGF levels: from 170 ng/g protein in controls to 360 ng/g after IGF-I in the cerebellum, and from 133 ng/g to 310 ng/g in the hippocampus ($P < 0.05$, $n = 5$). After a 2-week s.c. infusion of IGF-I in adult mice, the density of the vasculature was markedly augmented ($P < 0.05$, Fig. 1*E*) in two different brain areas, the cerebellar molecular layer (vessel density in controls is 18.2 $\pm$ 2%) and the hippocampal granular and subgranular layers (controls: 27 $\pm$ 1%). Therefore, IGF-I is a brain angiogenesis promoter.

IGF-I in Exercise-Induced Brain Vessel Growth. The beneficial effects of exercise on the brain are blocked when the serum IGF-I input to the brain is reduced (9, 10, 19). To determine whether the angiogenic response to exercise is also modulated by circulating IGF-I, we examined exercise-induced angiogenesis in mice that had normal levels of IGF-I in the brain (95% of the levels of control littermates, $n = 6$), but greatly reduced levels in serum [LID mice, (11)]. Importantly, although LID mice have increased brain β amyloid and moderate gliosis (26), these alterations do not affect the brain levels of proangiogenic mediators such as VEGF and their receptors, of endothelial markers such as CD31, and of other proteins related to vascular remodeling including the angiopoietins and their receptors, or HIF-1α (Table 1).

Normal mice showed enhanced proliferation of brain endothelial cells after physical exercise (Fig. 2*A*). For instance, in the cerebellar cortex, the total number of BrdUrd$^+$ cells increased from 199 $\pm$ 19 to 285 $\pm$ 17 cells per mm^3 in response to 1 month of exercise. Of these, $\approx$38% were associated with the endothelial marker lectin. Other BrdUrd$^+$ cells were associated with the pluripotent marker nestin but not with glial (glial fibrillary acidic protein and OX42) or neuronal antigens (β_3 tubulin and γ-aminobutyric acid type A

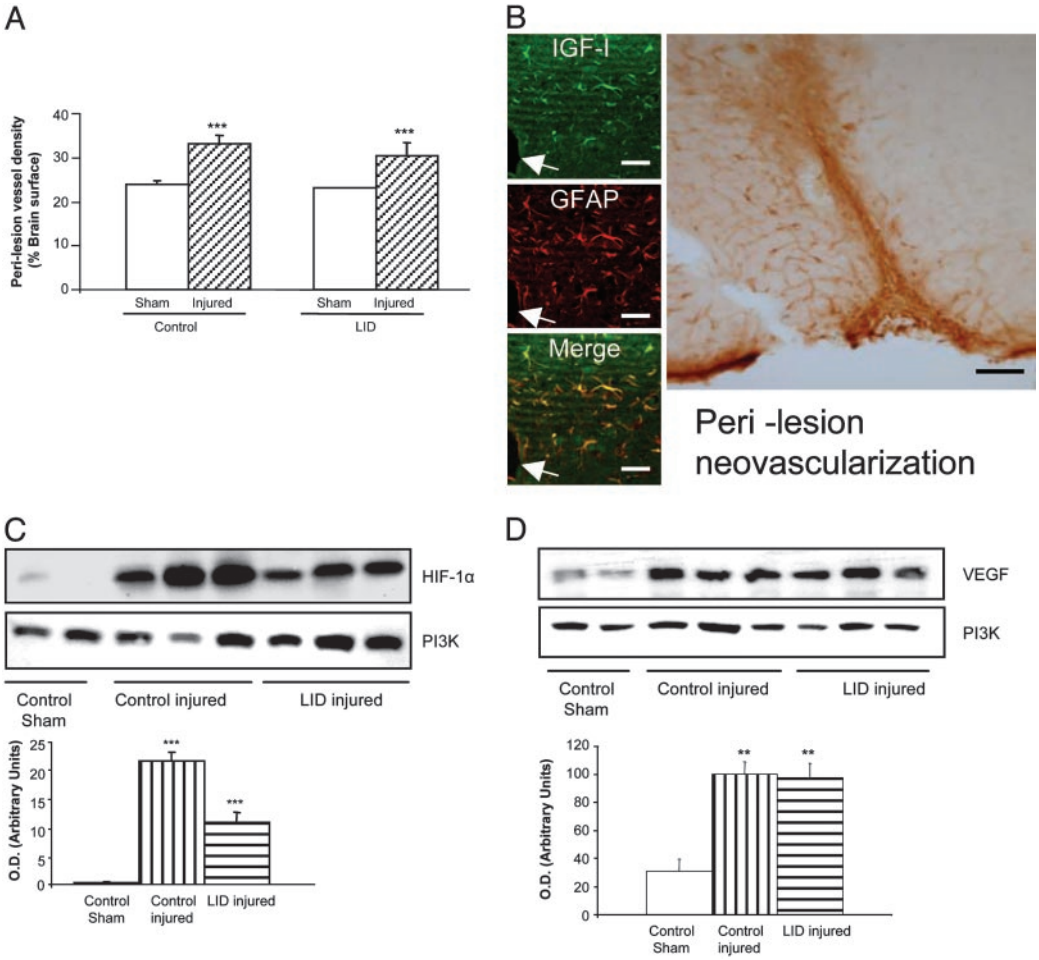

Fig. 3. Vessel remodeling after brain trauma. (*A*) Density of vessels in the area surrounding the lesion significantly increases in control as well as in LID mice (***, $P < 0.001$; $n = 4$ animals per group). (*B Left*) Fluorescence photomicrographs of the perilesion site (arrow) in LID mice showing IGF-I immunoreactive cells. The majority of the IGF-I$^+$ cells are astrocytes (GFAP$^+$) as determined by confocal analysis (Merge). Control littermate mice show an identical local IGF-I response. (Bars = 50 μm.) (*Right*) Neovascularization is restricted to the perilesioned area as determined by lectin staining of brain vessels. The arrow indicates the cannula tract (14 days after injury). Note the heavy lectin staining concentrated around the lesion area. (Scale bar = 100 μm.) (*C*) Levels of HIF-1α increase 3 days after injury in the perilesion area in both groups, although in LID mice, the increase is smaller. The upper gel shows a representative HIF-1α blot, and the lower gel shows the control of protein load with anti-PI3K. Histograms show densitometric quantitation of HIF-1α (***, $P < 0.001$ vs. sham-injured mice; $n = 4$). (*D*) Levels of VEGF around the lesion area increase 3 days after injury in both experimental groups. The upper gel shows a representative VEGF blot, and the lower gel shows the control of protein load. Histograms show quantitation of VEGF (**, $P < 0.01$ vs. sham mice; $n = 4$).

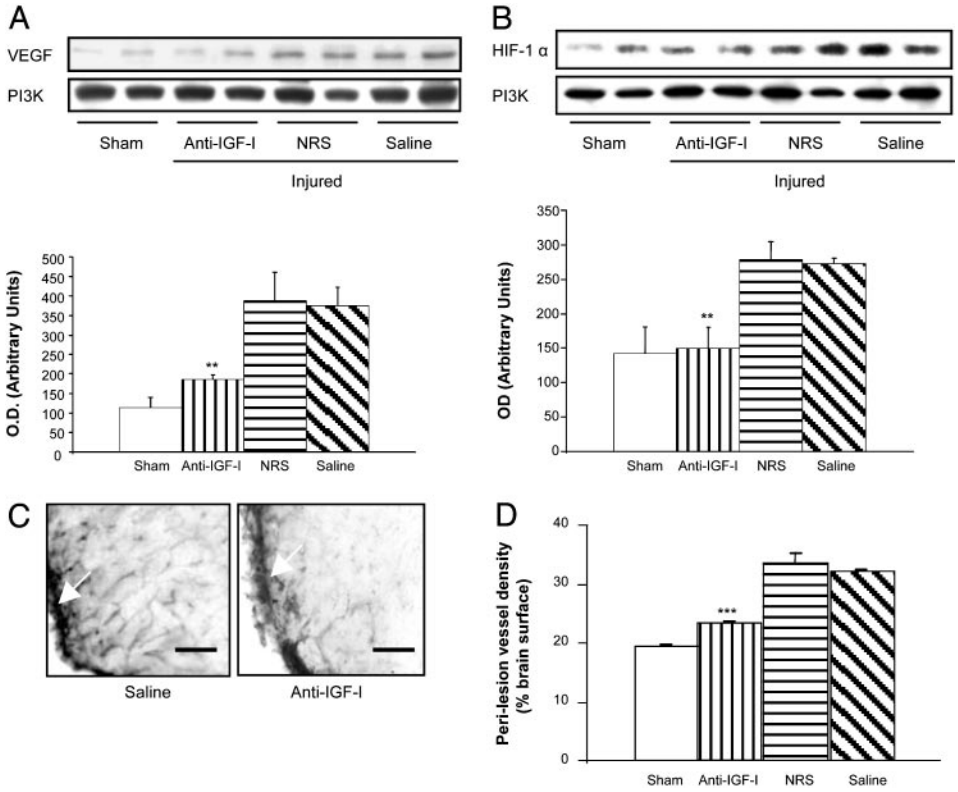

Fig. 4. Local IGF-I is required for lesion-induced angiogenesis. (*A*) LID mice receiving a s.c. anti-IGF-I infusion simultaneously to the lesion show a significantly diminished increase in VEGF levels 3 days after the lesion, compared with control injured animals receiving either NRS infusion or saline. The upper gel shows a representative VEGF blot. The lower gel shows the PI3K blot for control of protein load. Histograms indicate quantitation of VEGF levels in the perilesion area (**, $P < 0.01$ vs. NRS and saline-treated brain injured mice; $n = 4$). (*B*) Similar results are found in peak HIF-1α levels 3 days after injury in anti-IGF-I-treated LID mice. The upper gel shows the HIF-1α blot, and the lower gel shows the protein load control with PI3K. Histograms show that HIF-1α is significantly diminished in the anti-IGF-I group (**, $P < 0.01$ vs. NRS and saline treated; $n = 5$). (*C*) Lectin immunocytochemistry of the perilesioned site indicates prominent vessel growth after 1 month of stab injury in saline-treated LID mice, whereas after infusion of anti-IGF-I, angiogenesis is absent. The white arrow indicates the position of the cannula tract. (Scale bar = 100 μm.) (*D*) Vessel density in the perilesion area increases in saline-treated and NRS-treated LID mice, compared with sham-operated animals, but not in injured anti-IGF-I-treated LID mice (***, $P < 0.001$ vs. NRS and saline treated; $n = 4$ per group).

receptor α_6 subunit; data not shown). In LID mice, this response was absent. No increases in the number of endothelial cells, vessel density, or in the levels of the endothelial cell marker CD31 were seen (Fig. 2 *A–C*). This lack of vessel growth after exercise in LID mice was corrected by simultaneous infusion of IGF-I during training (Fig. 2*B*). Accordingly, a threefold increase in CD31 levels in IGF-I-treated vs. saline-treated exercising LID mice was found (from 177.5 ± 11 densitometric units in saline-treated to 542.5 ± 8.5 in IGF-I-treated mice; $n = 4$; $P < 0.001$). Endothelial cell growth was determined also in the hippocampus, where similar responses to exercise were found. As previously documented, a higher total number of $BrdUrd^+$ cells and a greater increase in the number of $BrdUrd^+$ cells stained with neuronal markers such as NeuN or β_3 tubulin was seen in this brain area (data not shown).

Levels of the angiogenic markers VEGF and HIF-1α were also analyzed. VEGF was not affected by exercise either after 3 (data not shown) or 30 days (Fig. 2*D*), as determined by using Western blot analysis and confirmed by using ELISA (data not shown). The levels of HIF-1α were significantly increased in control mice but not in LID mice, at both early (3 days; data not shown) and late (30 days; Fig. 2*E*) times after exercise.

IGF-I in Injury-Induced Brain Angiogenesis. Increased local synthesis and accumulation of IGF-I is a typical response to brain injury (27). The cells surrounding the lesion site, mostly astroglial cells, contain higher IGF-I and IGF-binding protein immunoreactivity after injury (28, 29). Fig. 3*B* (*Left*) shows the up-regulation of IGF-I levels in astrocytes surrounding the lesion in LID mice. Control littermates show an identical up-regulation. Therefore, brain insult with damage to the blood–brain barrier results in accumulation at the lesion site of IGF-I that probably originated from both local increased synthesis and from the circulation. We examined the contribution of serum IGF-I to this type of reactive angiogenesis by determining the angiogenic response to a stab wound in LID mice. After 2 weeks, increased vessel density in the perilesion area (Fig. 3*B Right*) is similar in both LID and control mice (Fig. 3*A*). This increase in vessel density is preceded by enhanced levels not only of HIF-1α, but also of VEGF, peaking at 3 days after injury (Fig. 3 *C* and *D*) and returning to basal values after 30 days (data not shown). LID mice have a similar response, although with a smaller increase in the levels of HIF-1α.

To determine whether IGF-I is required after injury-induced angiogenesis, we administered to LID mice a chronic s.c. infusion of a blocking anti-IGF-I antibody. As shown in detail before, this treatment effectively blocks IGF-I input to the target cells within the brain (9). Under these conditions, early activation of HIF-1α and VEGF in the perilesion area was significantly attenuated (Fig. 4 *A* and *B*). Moreover, 1 month after injury, vessel density in the perilesional area remained within control values in anti-IGF-I-infused animals, whereas mice that were injected with either saline or normal serum showed prominent vessel growth (Fig. 4 *C* and *D*).

Discussion

IGF-I is a ubiquitous growth factor present at high levels in the circulation and synthesized in many organs, including the brain (30).

Based on previous observations, we recently suggested that not only brain but also systemic IGF-I is a trophic factor for the adult brain (7). Our present observations reinforce this notion indicating that both local and serum IGF-I play a role in brain angiogenesis.

Systemic injection of IGF-I promotes brain vessel growth. This agrees with angiogenic actions of IGF-I in other organs (3) and the presence of IGF-I receptors in brain vessels (25). This action of IGF-I appears to be physiologically relevant, because in mice with low systemic levels of IGF-I, the angiogenic response to physical exercise, a physiological stimulus for brain vessel growth (2), was abrogated. The impaired angiogenic response was restored to normal when serum-IGF-I-deficient mice received IGF-I. In turn, neovascularization after injury proceeded normally even with low serum IGF-I. In this case, injury-induced brain accumulation of IGF-I was required. When IGF-I input to the injured area was blocked by anti-IGF-I infusion (9), perilesional angiogenesis was inhibited. Local increases of IGF-I at the injury site are achieved in part through up-regulated synthesis of IGF-I in the damaged parenchyma (29), and through local increased levels of IGF-binding proteins that will probably retain both brain and bloodborne IGF-I at the lesion site (31). Therefore, due to the disruption of the vascular bed and surrounding tissue, damaged brain vessels in the lesioned area will have access to both brain and serum IGF-I. In the case of serum-IGF-I-deficient mice, angiogenesis after brain trauma was normal probably because local increased synthesis of IGF-I together with the remaining available circulating IGF-I appeared to be sufficient to trigger endothelial growth. Indeed, LID mice showed increased IGF-I immunoreactivity around the lesioned site indistinguishable from that seen in control mice (Fig. 3*B*). Altogether, these data indicate that both local and blood-derived IGF-I is essential for reactive brain vessel remodeling.

As our *in vitro* studies show, the molecular pathway underlying the effects of IGF-I on brain endothelium includes HIF-1α and its downstream effector, VEGF. Both HIF-1α (32) and VEGF (33) were stimulated by IGF-I. However, whereas HIF-1α was increased either after injury, IGF-I administration, or physical exercise, VEGF increased after the first two types of stimuli but not after exercise. A possible explanation is that HIF-1α under normoxic conditions is usually at very low levels. Therefore, any increase over basal levels will easily be detected. On the contrary, VEGF is constitutively expressed by endothelial cells because it is essential for vessel maintenance. This may mean that VEGF increases specifically in regions of vessel sprouting induced by exercise will remain undetected because of already high overall basal levels. In the case of increases in VEGF levels detected *in vitro* and in perilesion tissue blocks, small changes in VEGF levels could be more easily measured. At any rate, the role of VEGF in exercise-induced angiogenesis requires further clarification.

The extent of the angiogenic response may dictate the amount of IGF-I input that is required. When angiogenesis is a widespread phenomenon, such as after exercise, high IGF-I input may be required (i.e., both local and circulating IGF-I may be recruited). When angiogenesis is a localized process, locally available IGF-I input suffices possibly because additional injury-associated processes such as hypoxia and pro-inflammatory cytokines also recruit the HIF-1α/VEGF angiogenic pathway. Consequently, in neurodegenerative conditions where widespread pathology would trigger a wide angiogenic response, it is likely that serum IGF-I input would be required. Notably, in stroke patients and in Alzheimer's disease patients where pathology affects ample brain regions, serum IGF-I levels are increased (34, 35), maybe to cope with the increased brain demand of circulating IGF-I. We hypothesize that in these cases, neovascularization of damaged brain areas will require a higher IGF-I input.

In summary, the adult brain requires IGF-I for reactive vessel remodeling. This proangiogenic activity of IGF-I likely contributes to its neuroprotective actions because increased vascularization assures proper nutrient and oxygen supply to neurons. Low IGF-I input to the brain may compromise angiogenic responses both under physiological and pathological circumstances, which may be of clinical consequence.

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