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ORIGINAL ARTICLE

The combination of insulin-like growth factor 1 and erythropoietin protects against ischemic spinal cord injury in rabbits

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Abstract
Introduction Insulin-like growth factor 1 (IGF-1) and erythropoietin (EPO) have been reported to independently protect against ischemic spinal cord injury in rabbits. In the present study, we investigated whether the combination of IGF-1 and EPO protects against ischemic spinal cord injury in rabbits.
Methods Animals were assigned to 1 of 4 groups ($n = 6$ in each): a control group (saline), an IGF-1 group (IGF-1 0.3 mg/kg), an EPO group (EPO 800 U/kg), or an IGF-1 + EPO group (IGF-1 0.3 mg/kg + EPO 800 U/kg). Spinal cord ischemia was produced by occluding the abdominal aorta for 15 min. Saline, IGF-1, and EPO were administered intravenously just after the start of reperfusion. Hindlimb motor function was assessed daily for 7 days, after which histopathological evaluation was performed. To analyze phosphorylation of signal transduction molecules, animals were assigned to 1 of the 4 groups ($n = 8$ in each). Spinal cord ischemia and the treatment were the same as those described above. The spinal cords were removed at 15 or 30 min after reperfusion and used to analyze phosphorylation of signal transduction molecules. Four animals served as the preischemic control, and the spinal cord was removed just before the start of ischemia.
Results In the IGF-1 + EPO group, both neurological and histopathological outcomes were significantly improved as compared to the control group, which was consistent with the increase of Janus kinase-2 (JAK2) phosphorylation.
Conclusions The combination of IGF-1 and EPO protects against ischemic spinal cord injury in rabbits. JAK2 might contribute to the protective effect.

Keywords Spinal cord ischemia · Insulin-like growth factor 1 · Erythropoietin · Rabbit

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Introduction

A devastating complication of thoracoabdominal aortic surgery is paraplegia, which is thought to result from spinal cord ischemia during aortic occlusion. To protect against ischemic spinal cord injury, many drugs have been tested in animal experiments. However, no drug has been approved for use in humans.

Recently, after the failure of clinical trials for pharmacological therapies that inhibit a specific ischemia response pathway for cerebral ischemia, endogenous neuroprotection has emerged as a more promising strategy. Among several possible drugs that are thought to activate endogenous

neuroprotection, insulin-like growth factor 1 (IGF-1) [1] and erythropoietin (EPO) [2, 3] may have substantial potential. In a rabbit model of spinal cord ischemia, IGF-1 administered before ischemia [1] and EPO administered after ischemia [2] have independently shown protective effects. In cultured rat cortical neurons, IGF-1 and EPO have been reported to have a synergistic neuroprotective effect on *N*-methyl-D-aspartate (NMDA)-induced apoptosis [4]. Both IGF-1 and EPO appear to have common bioactivities that lead to the activation of phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (Akt) [4]. However, it has been unknown whether the combination of IGF-1 and EPO protects against ischemic spinal cord injury through Akt activation in an in vivo model.

Both IGF-1 and EPO are endogenously produced and also available as recombinant drugs clinically. If the combination of IGF-1 and EPO administered after ischemia is proven protective, it would provide an important strategy for spinal cord protection during thoracoabdominal aortic aneurysmal surgery because drugs can be administered only when spinal cord ischemia is diagnosed during the surgery. In the present study, we sought to investigate whether the combination of IGF-1 and EPO administered after the start of reperfusion could protect against ischemic spinal cord injury in rabbits, comparing the protective effects of IGF-1 and EPO to those of IGF-1 or EPO alone. The previous studies that demonstrated the protective effects of IGF-1 or EPO assessed neurological function for only 48 h [1, 2]. As the rabbit spinal cord ischemia model is known to show delayed spinal cord dysfunction, we observed the neurological function for 7 days after reperfusion in the present study. In addition, we comprehensively investigated the activation of signal transduction molecules involved in spinal cord protection, including Akt, extracellular signal-regulated kinase (ERK), and Janus kinase-2 (JAK2) under the experimental conditions.

Methods

The study protocol was approved by the Animal Care and Use Committee at Yamaguchi University Graduate School of Medicine. Sixty male New Zealand white rabbits weighing 2.7 ± 0.1 (mean ± SD) kg were used in this study.

Experiment 1

After an overnight fast with unrestricted access to water, rabbits were anesthetized in a plastic box with 4 % isoflurane in oxygen. A catheter was inserted in an ear vein for administration of fluid (lactated Ringer's solution 10 ml/kg/h) and drugs, and another catheter was inserted in an ear artery to measure blood pressure. Pentobarbital (30 mg) was administered to facilitate tracheal intubation. After placing a 3-mm cuffed endotracheal tube, the rabbit's lungs were mechanically ventilated with 2–3 % isoflurane in 40 % oxygen/60 % nitrogen. Fentanyl was administered as needed during surgical preparation. Temperatures were monitored with a calibrated esophageal thermistor (Model MG-Type 209; Nihon Kohden, Tokyo, Japan) and a needle-type thermistor (Model PTC-201; Unique Medical, Tokyo, Japan) inserted into the paravertebral muscle at the level of L5. The paravertebral muscle temperature was controlled throughout the study at approximately 38.0 °C with a heating lamp and warming pad. After infiltration with 1 % lidocaine, a PE-50 catheter was inserted into the right femoral artery and advanced 5 cm to measure blood pressure below the aortic occlusion performed subsequently.

Spinal cord ischemia was produced as previously reported [5]. Briefly, in the right lateral decubitus position, the abdominal aorta was exposed retroperitoneally at the level of the left renal artery. A PE-50 catheter was placed around the aorta immediately distal to the left renal artery for later occlusion of the aorta. Then, an occluder tube (16F rubber tube) was tunneled through the skin. After completion of surgery, the end-tidal isoflurane concentration was maintained at 2 % (1 minimum alveolar concentration in rabbits).

Rabbits were randomly assigned to 1 of 4 groups ($n = 6$ in each): a control group, an IGF-1 group, an EPO group, or an IGF-1 + EPO group. The control group received saline, the IGF-1 group received 0.3 mg/kg IGF-1 (Astellas Pharma Inc., Tokyo Japan), the EPO group received 800 U/kg EPO (Kyowa Hakko Kirin Co., Ltd., Tokyo Japan), and the IGF-1 + EPO group received 0.3 mg/kg IGF-1 + 800 U/kg EPO intravenously just after the start of reperfusion. If proximal mean arterial blood pressure dropped below 55 mmHg, phenylephrine was infused to maintain blood pressure.

Immediately before aortic occlusion, 400 U heparin was administered. Ischemia was induced by pulling the PE catheter and clamping an occluder tube for 15 min. Both a loss of pulsatile distal aortic pressure and the decrease in distal blood pressure less than 10 mmHg were observed immediately after the occlusion of the abdominal aorta. At the end of the 15-min period of ischemia, reperfusion of the spinal cord was established by removal of the occluder tube and PE catheter. Then, all catheters were removed and incisions were sutured. An antibiotic (cephazolin 30 mg/kg) was administered intramuscularly. Isoflurane was discontinued and lungs were mechanically ventilated with 100 % oxygen. Extubation of the trachea was performed when vigorous spontaneous ventilation and movement occurred. The animals were allowed to recover in a warmed plastic box that contained supplemental oxygen until they appeared conscious and alert. Bladder contents were expressed manually as required.

The animals were neurologically assessed daily for 7 days after reperfusion by an observer unaware of the treatment groups, according to the 5-point score system proposed by Drummond and Moore [6]: 4 = normal motor function; 3 = ability to draw legs under body and hop but not normally; 2 = some lower-extremity function with good antigravity strength but inability to draw legs under body and/or hop; 1 = poor lower-extremity function and weak antigravity movement only; 0 = paraplegic with no lower-extremity function.

After the final neurological assessment (7 days after reperfusion), the rabbits were re-anesthetized with 3 % isoflurane in oxygen. Transcardiac perfusion and fixation were performed with 10 % phosphate-buffered formalin. Coronal sections of the spinal cord at the level of L5 were cut at a thickness of 8 μm, and stained with hematoxylin and eosin. Normal neurons in the anterior spinal cord (anterior to a line drawn through the central canal perpendicular to the vertical axis) were counted in 2 sections for each rabbit and averaged using a light microscope (Optiphot, Nikon, Tokyo, Japan) by an observer unaware of the treatment groups. Damaged neurons were identified by cytoplasmic eosinophilia with loss of Nissl substances and the presence of pyknotic homogenous nuclei as reported previously [5].

Experiment 2

Thirty-two animals were randomly assigned to 1 of the 4 groups ($n = 8$ in each), and spinal cord ischemia was produced in the same manner as in Experiment 1. All the groups were further divided into 2 subgroups, depending on whether the spinal cord was removed at 15 or 30 min after the start of reperfusion ($n = 4$ in each). Four animals served as the preischemic control, and the spinal cords were removed just before ischemia.

For the analysis of phosphorylation proteins in signal transduction, immediately after the animals were killed, the spinal cord was quickly removed with the plunger of a 1-ml syringe, frozen in liquid nitrogen, and then stored at −80 °C.

The frozen tissues were minced, placed into a 2-ml micro tube containing a bead, and homogenized in 9 volumes of lysis buffer [1 % Triton X-100, 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride] with a Retsch MM300 mixer mill. After removal of the bead, the samples were centrifuged at 12,000 × *g* for 20 min at 4 °C; and the supernatants were immediately used for western blotting after the protein concentration was measured. Electrophoresis and western blotting were carried out as described previously [7]. Briefly, the extracts and molecular mass standards were electrophoresed in 10 % (w/v) polyacrylamide gels in the presence of sodium dodecyl sulfate and transferred to polyvinylidene fluoride membranes (0.45 μm; Millipore Co., Bedford, MA) in the case of phosphorylation proteins, or nitrocellulose membranes in the case of Akt and ERK. The blots were blocked with 10 % bovine serum albumin for anti-p-Akt antibody (Ser473) (Cell Signaling Technology, Beverly, MA), anti-p-ERK antibody (Tyr204) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-phosphotyrosine antibody (PY99) (Santa Cruz Biotechnology, Inc.), anti-phosphoserine antibody (PSR45) (Novus, Littleton, CO), anti-phosphothreonine antibody (18F6) (Novus), anti-phospho-JAK2 antibody (Tyr1007/Tyr1008) (Santa Cruz Biotechnology, Inc.), or 5 % non-fat dry milk in Tris-buffered saline containing 0.05 % (w/v) Tween-20 and incubated with anti-Akt antibody (Santa Cruz Biotechnology, Inc.), anti-ERK antibody (Santa Cruz Biotechnology, Inc.), anti-JAK2 antibody (Santa Cruz Biotechnology, Inc.), or anti-α-actin antibody (Sigma-Aldrich, St. Louis, MO). The blots were then washed, and the antigens were visualized by enhanced chemiluminescent detection reagents.

Physiological variables are presented as mean ± SD and were analyzed by a repeated-measures analysis of variance. Hindlimb motor function and the number of normal neurons in the anterior spinal cord were statistically analyzed by a nonparametric method (the Kruskal–Wallis test followed by the Mann–Whitney *U* test with Bonferroni corrections). Data on band densities measured from western blots are presented as mean ± SE and were analyzed by one-way analysis of variance followed by the Bonferroni's multiple comparison test. A value of $p < 0.05$ was considered statistically significant.

Results

Physiological variables for Experiment 1 are shown in Table 1. There were no significant differences among the 4 groups.

In Experiment 1, all the rabbits survived until the final neurological assessment (7 days after reperfusion). Figure 1 shows the time course of changes in motor function score in each group. The control group showed a score of 0–1 at 7 days after reperfusion. The motor function scores were significantly better in the IGF-1 and IGF-1 + EPO groups than in the control group from 2 to 7 days after reperfusion ($p < 0.008$). However, there was no significant difference in the motor function scores between the IGF-1 group and the IGF-1 + EPO group or between the EPO group and the IGF-1 + EPO group.

Figure 2 shows the representative microphotographs of the lumbar spinal cord (L5) of a paraplegic animal (score = 0) in the control group and a normal animal in

Table 1 Physiological variables

	MAP(mmHg)		HR (bpm)	Temperature (°C)		pH	PaO_2 (mmHg)	$PaCO_2$ (mmHg)	Glucose (mg/dl)	Hematocrit (%)
	Proximal	Distal		Esophageal	Paravertebral					
Control ($n = 6$)										
Preischemia	64 ± 5	65 ± 4	312 ± 19	38.5 ± 0.3	38.0 ± 0.1	7.39 ± 0.05	205 ± 5	40 ± 1	127 ± 11	35 ± 1
Ischemia 7.5 min	65 ± 3	7 ± 1	317 ± 19	38.4 ± 0.4	37.5 ± 0.2					
Reperfusion 15 min	62 ± 4	61 ± 3	299 ± 24	38.2 ± 0.2	38.1 ± 0.1	7.36 ± 0.04	204 ± 23	39 ± 2	131 ± 16	34 ± 2
IGF-1 ($n = 6$)										
Preischemia	64 ± 5	68 ± 7	305 ± 20	38.1 ± 0.2	38.0 ± 0.1	7.38 ± 0.06	201 ± 25	39 ± 2	128 ± 7	36 ± 2
Ischemia 7.5 min	67 ± 5	8 ± 1	298 ± 25	38.1 ± 0.2	37.6 ± 0.2					
Reperfusion 15 min	63 ± 4	65 ± 4	298 ± 28	38.2 ± 0.3	38.1 ± 0.2	7.36 ± 0.06	202 ± 31	39 ± 1	106 ± 12	37 ± 2
EPO ($n = 6$)										
Preischemia	64 ± 5	68 ± 4	315 ± 13	38.5 ± 0.3	38.0 ± 0.1	7.40 ± 0.08	199 ± 24	39 ± 2	128 ± 9	37 ± 1
Ischemia 7.5 min	67 ± 2	7 ± 1	308 ± 8	38.4 ± 0.2	37.5 ± 0.4					
Reperfusion 15 min	63 ± 5	66 ± 4	298 ± 20	38.2 ± 0.2	37.9 ± 0.2	7.39 ± 0.07	220 ± 12	39 ± 3	133 ± 13	37 ± 2
IGF-1 + EPO ($n = 6$)										
Preischemia	64 ± 3	65 ± 3	318 ± 18	38.3 ± 0.2	38.0 ± 0.1	7.36 ± 0.04	221 ± 9	39 ± 2	123 ± 12	36 ± 3
Ischemia 7.5 min	65 ± 4	7 ± 1	315 ± 15	38.2 ± 0.2	37.3 ± 0.2					
Reperfusion 15 min	64 ± 4	65 ± 4	303 ± 24	38.0 ± 0.1	37.9 ± 0.2	7.34 ± 0.04	211 ± 23	38 ± 1	111 ± 16	36 ± 2

Data are mean ± SD

MAP mean arterial blood pressure, *HR* heart rate

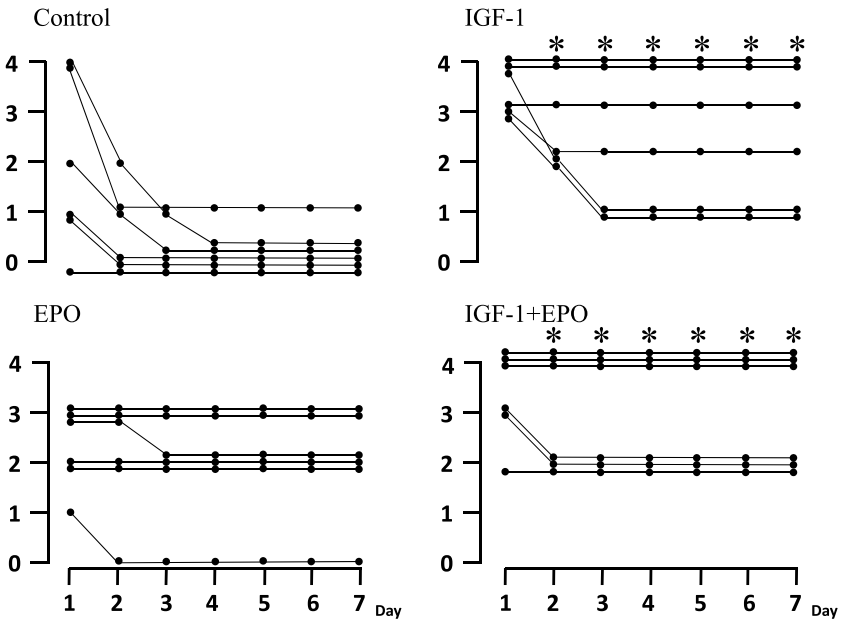

Fig. 1 Individual motor function score change from 1 to 7 days after reperfusion. Motor function scores range from 0 (paraplegia) to 4 (normal). Each symbol represents data for one animal. The motor function scores were significantly better in the IGF-1 and IGF-1 + EPO groups than in the control group from 2 to 7 days after reperfusion (*$p < 0.008$)

the IGF-1 + EPO group. In the animals with severe motor dysfunction (score ≤ 1) in the control, IGF-1, and EPO groups, the structure of the spinal cord gray matter was destroyed, most motor neurons had disappeared, and prominent inflammatory cell infiltration was observed. Vacuolation in the white matter was also observed in these animals. In contrast, the structure of the gray and white matter of the spinal cord was well maintained and motor neurons retained a normal appearance in the animals with normal motor function in the IGF-1 + EPO group (Fig. 2).

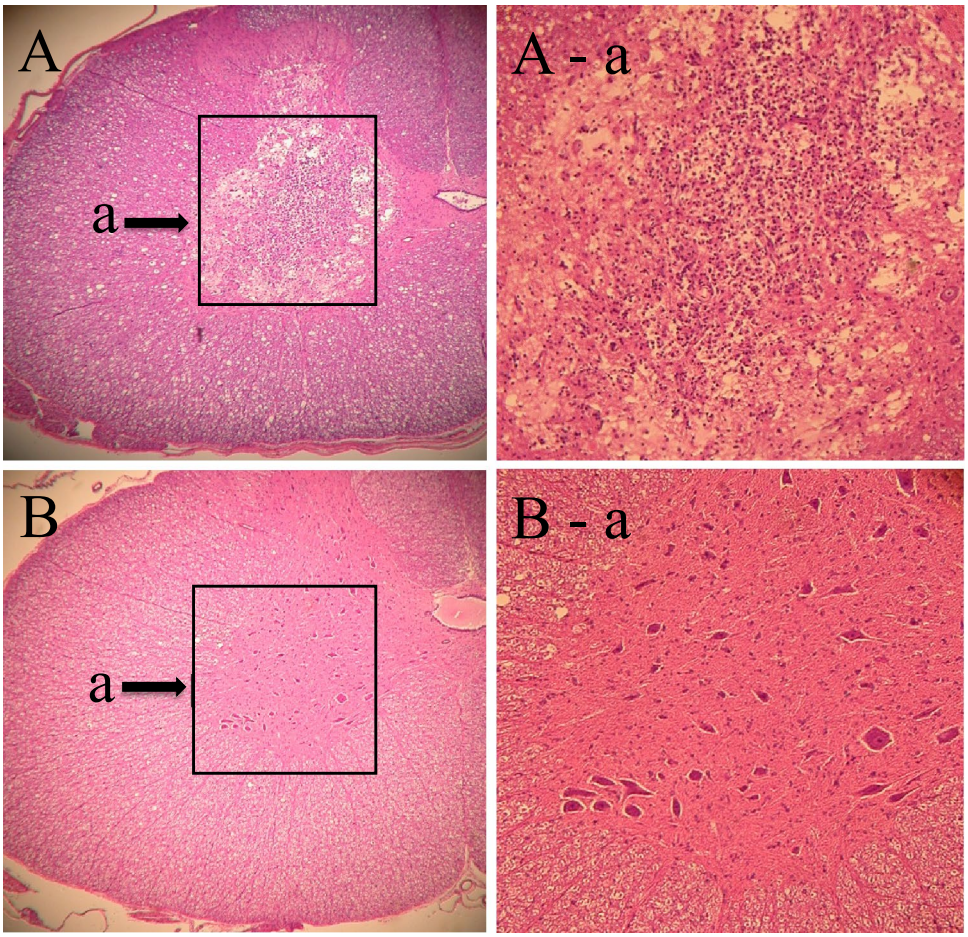

Fig. 2 The representative light microphotographs of the lumbar spinal cord of the paraplegia animal (score = 0) in the control group (**A, A-a**) and the normal animal in IGF-1 + EPO group (**B, B-a**). (L5 level, hematoxylin-eosin stain). Few normal neurons and the destruction of the entire gray matter with inflammatory changes were seen in the control group (**A, A-a**). In contrast, the structure of the gray matter of the spinal cord was well maintained and motor neurons preserved almost normal appearance in the IGF-1 + EPO group (**B, B-a**). **A** and **B**: original magnification × 40, **A-a** and **B-a**: original magnification × 100

Figure 3 shows the number of morphologically normal neurons (L5 level) at 7 days after reperfusion. There were significantly more normal neurons in the IGF-1 + EPO group than in the control group at the level of L5 ($p = 0.0039$). However, there was no significant difference in the number of normal neurons at the level of L5 between the IGF-1 group and the IGF-1 + EPO group or between the EPO group and the IGF-1 + EPO group.

To examine whether any intracellular signaling molecules were involved in the neuronal protection in the presence of IGF-1 and EPO after reperfusion, the phosphorylation levels of proteins in the spinal cord were examined by western blotting. Phosphorylation of Akt significantly increased at 30 min after the start of the reperfusion in the EPO group ($p = 0.0004$). In the IGF-1 + EPO group, however, the phosphorylation of Akt did not increase at either 15 or 30 min (Fig. 4a, d). Phosphorylation of ERK significantly increased at 15 min after the start of reperfusion in the control group and at 30 min in all groups ($p < 0.005$), but the treatment with IGF-1, EPO, or both had little effect on the phosphorylation (Fig. 4b, e). We comprehensively analyzed phosphorylation of intracellular molecules involved in neuronal protection by using antibodies against

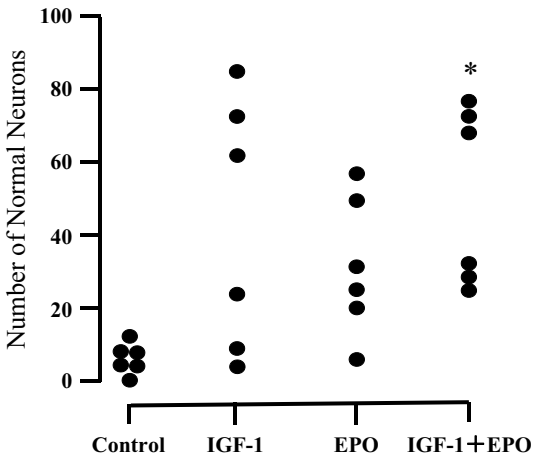

Fig. 3 The number of normal neurons in the anterior spinal cord (L5 level) 7 days after reperfusion. Each symbol represents data for one animal. There were significantly more normal-appearing neurons in the IGF-1 + EPO group than in the control group at the level of L5 (*$p = 0.0039$)

phosphotyrosine, phosphoserine, and phosphothreonine. The simultaneous administration of IGF-1 and EPO tended to increase tyrosine phosphorylation of a protein with a

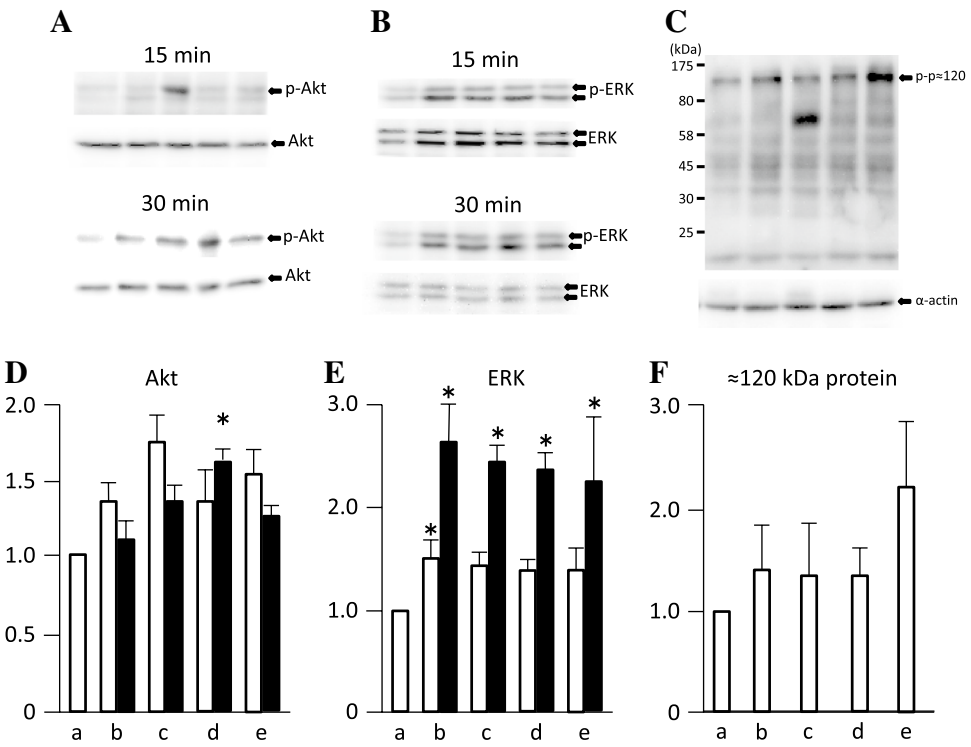

Fig. 4 Phosphorylation of Akt, ERK, and tyrosine in the lumbar spinal cord during reperfusion in the presence of IGF-1, EPO, or both. At the indicated time after reperfusion, the reactions were stopped, and the tissue extracts were subjected to western blotting with anti-phospho-Akt or Akt, anti-phospho-ERK or ERK, or phospho-tyrosine antibodies. The *Upper panels* show representative immunoblots in phospho-Akt and Akt (**A**) phospho-ERK and ERK (**B**) and phospho-tyrosine (**C**) *Lower panels* show the ratio of signals for phospho-Akt to Akt (**D**) phsopho-ERK to ERK (**E**) and phospho-tyrosine to α-actin (**F**) densitometrically determined from the immunoblots. (*a* preischemia, *b* the control group, *c* the IGF-1 group, *d* the EPO group, *e* the IGF-1 + EPO group, the *open bar* 15 min after reperfusion, the *solid bar* 30 min after reperfusion, mean ± SE, $n = 4$ in each, $^*p < 0.005$ versus preischemia)

molecular mass of approximately 120 kDa at 15 min after the start of reperfusion, but the difference did not reach statistical significance (Fig. 4c, f). The phosphorylation of serine and threonine in the proteins was not enhanced under the experimental conditions (data not shown).

To identify the tyrosine-phosphorylated protein with a molecular mass of 120 kDa, phosphorylation of JAK2 was examined by immunoblotting, because the molecular mass of JAK2 is approximately 125 kDa. As expected, phosphorylation of JAK2 was significantly increased in the IGF-1 + EPO group ($p = 0.0045$) (Fig. 5), which was consistent with the data using anti-tyrosine phosphorylation antibody.

Discussion

The combination of IGF-1 and EPO significantly improved both neurological and histopathological outcomes compared to the control group in a transient spinal cord ischemia model in rabbits. JAK2 activation might be involved in the protective mechanism because phosphorylation of JAK2 in the lumbar spinal cord was increased after reperfusion in the IGF + EPO group in agreement with the protective effects. As the neurological function score did not change from 4 days after reperfusion in all groups, a 7-day observational period in the present study was thought to be enough to assess neurological outcome.

IGF-1 was originally found to have protective effects in hypoxic-ischemic brain injury in rats [8]. Later, IGF-1 was demonstrated to cross the blood–brain barrier [9]. In a spinal cord ischemia model in rabbits, Nakao et al. [1] reported strong protective effects of IGF-1. However, we did not observe the strong protective effects of IGF-1 in the present study, although IGF-1 improved neurological outcome. The dose of IGF-1 was the same as that in Nakao's study [1]. Differences between Nakao's study [1] and ours include the timing of the administration of IGF-1 and the blood glucose concentrations in the peri-ischemic period. In Nakao's study, IGF-1 was administered 30 min before ischemia and the mean blood glucose concentration at 15 min after aortic occlusion was about 60 mg/dl (no data were shown before ischemia); by contrast, in the present

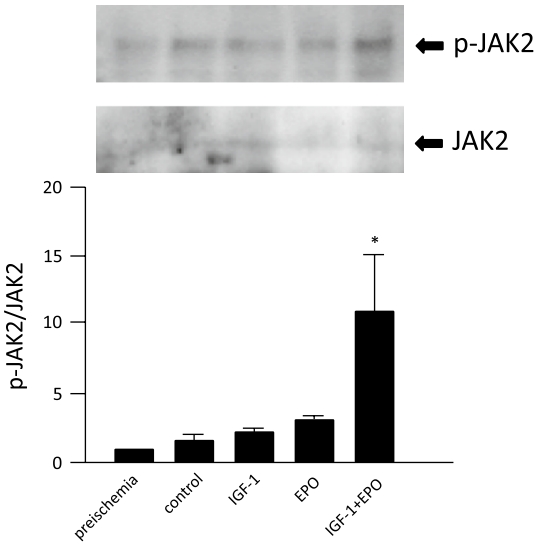

Fig. 5 Phosphorylation of JAK2 in the lumbar spinal cord during reperfusion in the presence of IGF-1, EPO, or both. At 15 min after reperfusion, the reactions were stopped, and the tissue extracts were subjected to western blotting with anti-phospho-JAK2 (Tyr1007/Tyr1008) or JAK2 antibodies. *Upper panels* show representative immunoblots in phospho-JAK2 and JAK2. *Lower panels* show the ratio of signals for phospho-JAK2 to JAK2 densitometrically determined from the immunoblots. (mean ± SE, $n = 4$ in each, $^*p = 0.0045$ versus preischemia)

study, IGF-1 was administered just after the start of reperfusion and the mean blood glucose concentration before aortic occlusion was about 125 mg/dl. Preischemic administration of IGF-1 and mild hypoglycemia might augment the protective effects of IGF-1.

EPO was originally shown to have protective effects in forebrain ischemia in gerbils [10]. Later, EPO was shown to cross the blood–brain barrier [11]. It was also demonstrated that EPO receptors are localized on motor neurons in the spinal cord [2]. Celik et al. [2] found that EPO administered intravenously immediately after the onset of reperfusion had protective effects on neurological and histopathological outcomes after transient spinal cord ischemia in rabbits. They performed a dose–response study to examine the protective effects of EPO and found that the protective effects basically showed a bell-shaped profile [2]. According to their results, we chose the maximal protective dose (800 U/kg) in the present study. However, the protective effects of EPO alone did not reach the level of statistical significance in the present study. In Celik et al.'s study, the average neurological function score in the 800 U/kg group was lower than 3 (3 = can hop, but not normally) 48 h after reperfusion, but no damage was observed in histopathological examination [2]. In our previous studies using almost the same model as that in Celik et al.'s study, animals with neurological function score 3 or lower always showed histopathological damage [12, 13]. In fact, two animals that showed neurological function score 3, 7 days after reperfusion in the EPO group, exhibited mild histopathological damage in the present study. It appears that the protective effects of EPO against ischemic spinal cord damage are not as strong as previously reported.

There was no difference in the neurological and histopathological outcomes between the IGF-1 group and the IGF-1 + EPO group or between the EPO group and the IGF-1 + EPO group. However, when compared with the control group, both neurological and histopathological outcomes improved only in the IGF-1 + EPO group. In fact, no animal in the IGF-1 + EPO group showed a poor neurological outcome (score < 2) (Fig. 1). Also, the neurological score and the number of normal neurons were greater in each animal in the IGF-1 + EPO group than in any animal in the control group (Figs. 1 and 3). Therefore, it might be reasonable to conclude that the combination of IGF-1 and EPO protects against ischemic spinal cord injury in this model more than IGF-1 or EPO alone.

The combination of IGF-1 and EPO has been reported to exhibit neuroprotective effects during NMDA-induced apoptosis in cultured rat cerebrocortical neurons [4]. In cultured cerebrocortical neurons, simultaneous exposure to IGF-1 and EPO has been reported to promote increased PI3-K activity, leading to Akt activation [4]. Activation of Akt promotes neuronal survival in the cultured cells. In the present study, Akt was not activated at either 15 or 30 min after the start of reperfusion in the IGF-1 + EPO group, although the phosphorylation of Akt was enhanced during reperfusion in the EPO group. We examined phosphorylation of intracellular molecules to investigate the protective mechanism of the combination of IGF-1 and EPO. As a result, we demonstrated that the combination of IGF-1 and EPO tended to increase tyrosine phosphorylation of a protein with molecular mass of approximately 120 kDa. We have identified the tyrosine-phosphorylated protein as JAK2 for the following reasons. First, the calculated molecular mass of JAK2 is approximately 125 kDa, which corresponds to the molecular mass of the protein detected in this study [14]. Second, it is well known that the EPO receptor binds directly to JAK2 [15]. IGF-1 also activates the JAK/signal transducer and activator of transcription (STAT) pathway, leading to neuronal survival [16, 17]. Finally, the anti-phospho-JAK2 antibody (Tyr1007/Tyr1008) recognized the protein with molecular mass of 120 kDa, and the phosphorylation was significantly increased under the experimental conditions that exerted the protective effects. The downstream pathway activated by JAK2 may contribute to the protective effect of the combination of IGF-1 and EPO. There is a possibility that STAT3, a downstream molecule of JAK2, may be involved in the production of neuroprotective factor(s) as a transcription factor, since tyrosine

phosphorylation of a protein of approximately 80 kDa was weakly detected with the administration of both IGF-1 and EPO (Fig. 4c). Further studies detecting which cellular phenotype expresses phosphorylated JAK2 and determining causal relationships between activation of JAK2 and neuroprotective effects with the combination of IGF-1 and EPO by using an inhibitor of JAK2 should be performed to elucidate the role of JAK2.

We chose the IGF-1 and EPO doses that have been reported to have strong protective effects [1, 2]. More appropriate combinations of the doses may exist, such as a larger dose of IGF-1 combined with a smaller dose of EPO and vice versa. From a clinical standpoint, side effects including hypoglycemia with IGF-1 should be avoided. As the dose of IGF-1 is increased, control of blood glucose concentrations becomes more difficult. Intravenous administration of 33,000 U EPO once daily for 3 days has been demonstrated to be safe in humans [18]. However, the approved maximum daily dose of EPO is 24,000 U (about 400 U/kg). Also, a more appropriate timing of the drug administration may exist. Further studies are needed to explore the therapeutic time window.

In summary, we demonstrated the neuroprotective effects of the combination of IGF-1 and EPO with the activation of JAK2 in a transient spinal cord ischemia model in rabbits. Because IGF-1 and EPO are clinically available recombinant drugs, the combination of IGF-1 and EPO might be a promising strategy for spinal cord protection during thoracoabdominal aortic surgery.

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Conflict of interest The authors declare no conflicts of interest.

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