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Angiogenic Effects of Dimeric Dipeptide Mimetic of Loop 4 of Nerve Growth Factor

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Angiogenic action of compound GK-2, a dimeric dipeptide mimetic of loop 4 of nerve growth factor (NGF), was studied in *in vitro* and *in vivo* experiments. Experiments on human endothelial cell culture HUVEC showed that compound GK-2 significantly ($p<0.05$) stimulated the initial stage of angiogenesis, and its angiogenic activity was not inferior to the reference neurotrophin NGF. In experiments with hindlimb ischemia modeled in rats, GK-2 (1 mg/kg intraperitoneally for 14 days) significantly increased the total length of capillary vessels ($p<0.003$) and the number of vessels per 1 mm^2 ischemic tissue ($p<0.001$) in comparison with the control. Our findings indicate that under experimental conditions compound GK-2 exhibits not only angiogenic, but also anti-ischemic activity.

Key Words: *angiogenesis; dimeric dipeptide mimetic; nerve growth factor; human endothelial cell culture (HUVEC); limb ischemia*

Despite significant progress in their prevention and treatment of chronic ischemia, in particular, coronary heart disease and chronic lower limb ischemia, these pathologies remain the leading cause of death in developed countries, especially in situations when the use of highly effective surgical and/or endovascular technologies is impossible or is associated with high risk of serious complications. An alternative approach is the use of pharmacological stimulators of angiogenesis providing adequate blood flow in the ischemic tissues. This line of therapy, "therapeutic angiogenesis" or "biological bypass" implies the use of drugs, predominantly biological agents that improve perfusion of ischemic tissues by enhancing natural, but insufficient in critical situations, tissue neovascularization processes, *i.e*. stimulating the growth and branching (arborization) of blood vessels and/or modulating endothelium functions [10]. The development and introduction into clinical practice of exogenous analogs of endogenous growth factors and/or chemical activators of growth factors and the use of genetically modified progenitor cells, are most promising in this field [4]. There is quite convincing evidence that nerve growth factor (NGF) is synthesized and excreted not only in the CNS, but also by endothelial cells and vascular smooth muscle cells, and these cells carry NGF-specific membrane TrkA-receptors [5] mediating the angiogenic effects of NGF. NGF-mediated activation of TrkA-receptors located on the membrane of vascular smooth muscles and endothelium leads to activation of PI3K-Akt, Ras-MAPK, and PLCγ1-IP3 signaling that initiates angiogenesis [3]. This phenomenon is described for both normal and pathological tissues. After many-year basic research in V. V. Zakusov Research Institute of Pharmacology we synthesized compound GK-2, a dimeric dipeptide mimetic of NGF loop 4 exhibiting properties of TrkA-receptor agonist [2]. According to pharmacological data, compound GK-2 possesses marked neuroprotective activity *in vitro* and *in vivo* [7] due to its ability to activate TrkA-conjugated intracellular PI3K-Akt signaling pathway [1].

Here we studied angiogenic activity of compound GK-2 in *in vivo* and *in vitro* experiments.

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MATERIALS AND METHODS

***In vitro* experiments.** Angiogenic activity of compound GK-2 was tested on cultured HUVEC cells (isolated human endothelial cells). NGF (BD Bioscience) was used as the positive control. Endothelial cells were seeded in DMEM medium (HyClone) containing 20 mM HEPES (ICN), 2 mM L-glutamine (ICN), heparin (5 U/ml; Panfarma), ECGF (20 μg/ml; Sigma), 10% fetal calf serum (Invitrogen) at a density of 4000 cells on 96-well polylysine-precoated plates. GK-2 (10^{-6} M) and NGF (10^{-9} M) were added 30 min after cell seeding on the plates and then every 48 h (a total of 3 applications). On the next day after application of the studied compounds, the cells were photographed (Nikon Eclipse TS100-F microscope) in phase contrast at ×100. Microtubule length was measured in 5 fields of view in each well using WCIF-ImageJ software and recorded in micrometers.

Normality of sample distribution was tested using Shapiro–Wilk test, homogeneity of variance was assessed by Levene test. Since the data fitted normal distribution, and sample dispersions were non-homogenous, statistical significance of differences was determined using Student's *t* test in the approximation for samples with unequal variances, for multiple comparisons Bonferroni correction was used. The results were expressed as the arithmetic means and standard errors. The differences were considered statistically significant at $p \leq 0.05$.

***In vivo* experiments.** Experiments were carried out on mongrel male rats weighing 180-200 g kept in a vivarium in accordance with the Directive No. 267 of the Ministry of Health of Russian Federation "On Establishment of the Rules of Good Laboratory Practice" (June 06, 2003). The animals were randomized into two groups: control (n=18) and experimental (n=17). Hindlimb ischemia was produced in anesthetized rats (thiopental sodium, 50 mg/kg, intraperitoneally) by single-stage resection of the femoral artery segment, after which the wound was sutured layer-by-layers. GK-2 (1 mg/kg) was administered intraperitoneally for 14 days starting from the moment of femoral artery resection. The first injection was made 1 h after the end of surgery. Control animals received 0.3 ml saline intraperitoneally by the same scheme. One day after the last injection, the animals were sacrificed and the gastrocnemius muscle of the ischemic limb was isolated. The intensity of necrobiotic processes in the ischemic gastrocnemius muscle was assessed by light microscopy. The muscle was fixed in 10% neutral formalin, 5-μ sections were prepared using freezing microtome, and stained routinely with hematoxylin and eosin. Evans blue was used for visualization of capillaries. For each field of view total vessel length and their number in 1 mm^2 were calculated. Vascularization was expressed in vascularization index, calculated as a product of capillary length and their number in 1 mm^2 of ischemic tissue.

Statistical significance of the changes, produced by compound GK-2, was assessed by Mann–Whitney *U* test. Results are expressed as medians and upper and lower quartiles. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

***In vitro* experiments.** Compound GK-2 in concentration of 10^{-6} M (n=12) stimulated the initial stage of angiogenesis – tubulogenesis, as evidenced by significantly ($p<0.05$) increased, in comparison with control (n=12), total length of microtubules: 312±18 and 162±6 μ, respectively (Fig. 1). When angiogenic activity of GK-2 was in comparison with that of NGF in concentration of 100 ng/ml (≈10^{-9} M) (n=12), they almost equally stimulated tubulogenesis: the total length of microtubules increased up to 312±18 and 330±9 μ respectively (Fig. 1). Preliminary addition of GK-2 (10^{-6} M) (n=12) 30 min prior to NGF did not affect the effects of the neurotrophin: total length of microtubules was 321±6 μ, when NGF was administered alone it was 330±9 μ (Fig. 1). Hence, GK-2 under experimental conditions compound exhibited pronounced angiogenic activity, comparable to that of NGF.

***In vivo* experiments.** In animals, treated with GK-2, total length of the capillary bed was significantly greater (p=0.003), than in control animals: 19,531 (16,085÷24,511) and 14,456 (10,901÷17,404) μ/mm^2, respectively. The number of blood vessels in 1 mm^2 of ischemic tissue in animals of experimental group was also significantly higher ($p<0.001$) than in the control: 70 (70÷78) and 53 (50÷57), respectively. Mathematical analysis of the data indicates that the intensity of ischemic tissue vascularisation in animals of experimental group was almost 2 times higher than in control: vascularisation index was 27,794 (25,218÷35,941) and 14,725 (9030÷19,630) respectively ($p<0.001$).

According to light microscopy data, in control animals the core of the tissue was formed by necrobiotically altered muscle with areas of wax-like necrosis. The sarcoplasm was brightly colored, homogeneous, cross-striation was absent (Fig. 2). A large number of inflammatory infiltrates was noted. Nuclei of the striated muscles were small, hyperchromatic, or absent. Blood vessels were full-blooded, perivascular edema was well expressed, capillaries were convoluted, small, thin, poorly distinguishable. Thus, removal of a segment of femoral artery in the gastrocnemius muscle resulted in pronounced necrotic and necrobiotic changes, accompanied by inflammatory response

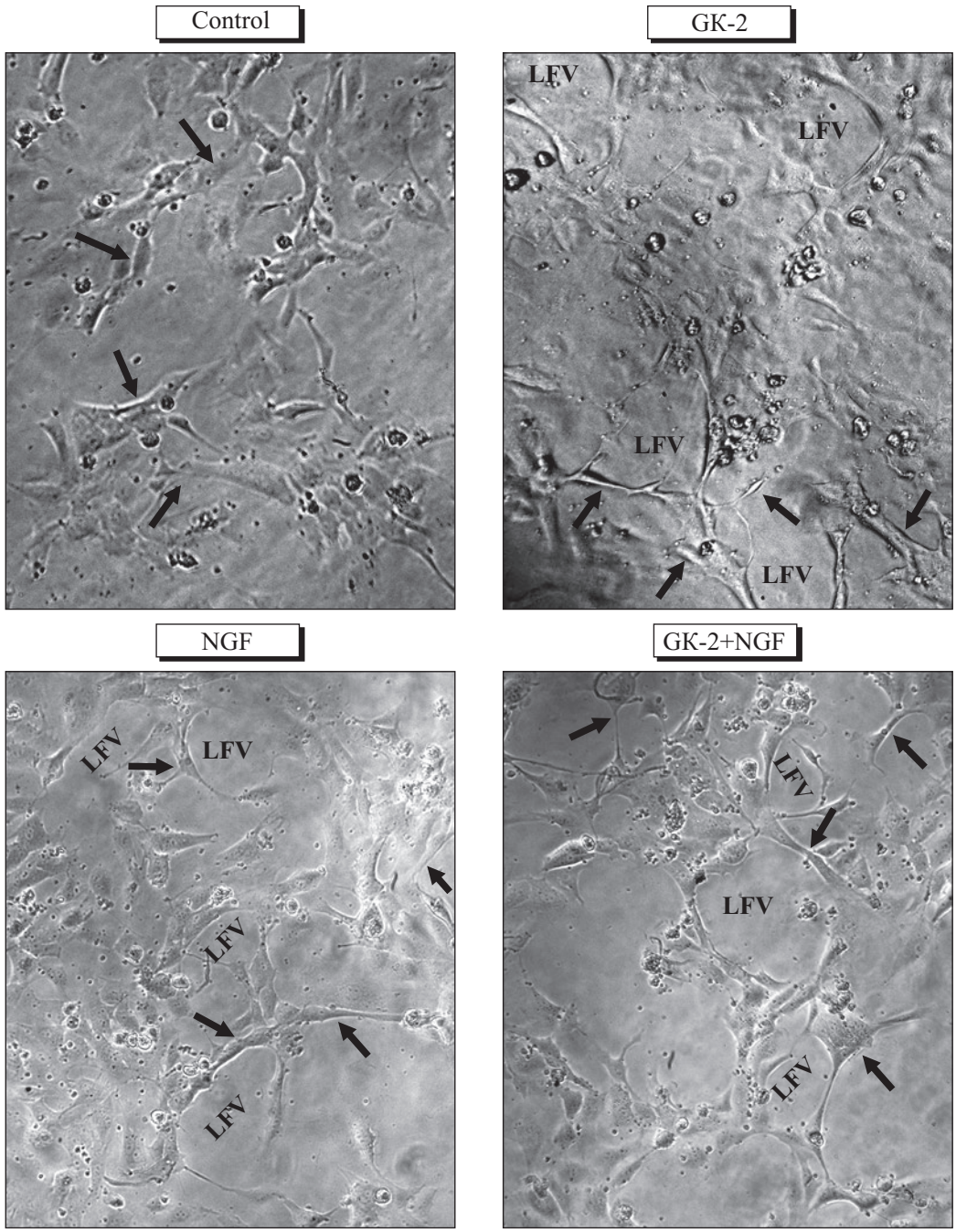

Fig. 1. Effect of GK-2 (10^{-6} M) and NGF (10^{-9} M) on formation of microtubules in the culture of human vascular endothelial cells HUVEC. Light microscopy, ×100. Arrows indicate endothelial cells with microtubules. LFV, lumen-forming vessel.

and blood circulation disorder. In animals treated with compound GK-2, the microscopic picture of gastrocnemius muscle was significantly different: the intensity of alterative processes in the muscle of these rats was less pronounced, the number and size of wax-like necrosis areas was significantly smaller than in control animals. Area of inflammatory infiltrates was insignificant. Vascular endothelium was well-defined, cross striations of the skeletal muscle was preserved. The capillary network was well distinguishable, capillaries were straight and located along muscle fibers (Fig. 2).

Thus, results of *in vivo* experiments not only confirmed *in vitro* data, but also showed that compound GK-2 along with angiogenic activity possesses expressed anti-ischemic action that appears to be related to the ability of the compound to stimulate angiogenesis in the injured gastrocnemius muscle.

Numerous experimental studies in the late twentieth century demonstrated high effectiveness of various

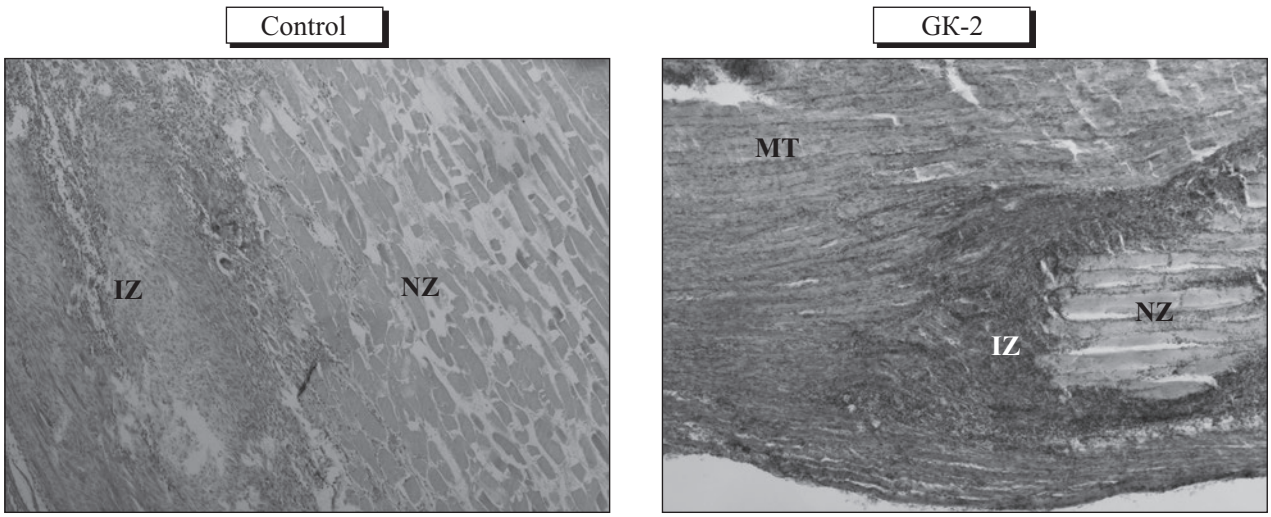

Fig. 2. Morphology of rat gastrocnemius muscle. Light microscopy, ×40. IZ, inflammation zone; NZ, necrosis zone; MT, muscle tissue.

viral vector systems, containing such factors as VEGF, FGF, placental growth factor (PLGF), and stem cell factor (FSC), in promoting angiogenesis in ischemic tissue of cardiac and/or limb muscle [6.12]. However, results of the first clinical randomized trials (VIVA, FIRST, TRAFFIC, and others), which assessed angiogenic activity of growth factors, did not live up to expectations [8,11,15]. For example, in a randomized placebo-controlled FIRST study, CHD patients with were administered intracoronary with FGF-2, and there were no difference from the placebo group [15]. Similar results were obtained when stem cells were used as angiogenic agents – in ASTAMI and REPAIR-AMI studies [14].

Over the years passed since the time of these studies, in spite of the great theoretical interest to therapeutic angiogenesis and numerous clinical trials of various biosimilar drugs, no definitive positive results have been achieved [13]. On the basis of the available data it can be suggested that low clinical efficacy of biosimilar drugs is largely due to lack of angiogenic "power" as far as they stimulate only angiogenic intracellular cascades, directly coupled with them. For instance, VEGF-A implements its angiogenic effects through dimerization of its specific tyrosine kinase receptors – VEGFR-1 and VEGFR-2. According to available data, in vascular endothelial cells, the NGF-activated specific TrkA-receptors not only activate the angiogenic intracellular cascades, coupled with them, but also VEGF-A [9], which in turn activates VEGFR-2 receptors, resulting in activation of the signaling cascade, mediated by NO synthase, and/or initiates expression of urokinase and its receptor. The latter appears to be extremely important, because urokinase expression is the initial stage of the program, necessary for translocation of cells from one tissue compartment to another, which is achieved by lateral migration of activated urokinase receptors (uRAR) in the phospholipid layer of the cell membrane to its "leading" pole. Having reached the "leading" pole, activated uRAR perform strictly directed local degradation of extracellular matrix, which is necessary for migration and invasion of cells. In this context, it seems quite promising to investigate further the biosimilar dimeric dipeptide mimetic of loop 4 of NGF – compound GK-2 with properties of the TrkA-receptor agonist as an agent, stimulating therapeutic angiogenesis.

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