

Pleiotropic neuroprotective and metabolic effects of Actovegin's mode of action

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ABSTRACT

This article reviews the mechanisms of action of Actovegin in the context of its preclinical effects and new concepts in the pharmacological treatment of neurological disorders. Actovegin is an ultrafiltrate of calf blood, composed of more than 200 biological substances. The drug is used for a broad spectrum of diseases, including disturbances of peripheral and cerebral blood circulation, burns, impaired wound healing, radiation-induced damage and diabetic polyneuropathy. Actovegin is composed of small molecules present under normal physiological conditions, therefore pharmacokinetic and pharmacodynamic studies to determine its active substance are not feasible. Preclinical data have revealed that it improves metabolic balance by increasing glucose uptake and improving oxygen uptake under conditions of ischemia. Actovegin also resists the effects of gamma-irradiation and stimulates wound healing. More recent preclinical studies have suggested that anti-oxidative and anti-apoptotic mechanisms of action specifically underlie the neuroprotective properties of Actovegin. The drug has been found to exert these beneficial effects experimentally, in primary rat hippocampal neurons and in an STZ-rat model of diabetic polyneuropathy, while also providing evidence that it positively affects the functional recovery of neurons. Latest data suggest that Actovegin also has a positive influence on the NF- κ B pathway, but many molecular and cellular pathways remain unexplored. In particular, Actovegin's influence on neuroplasticity, neurogenesis and neurotrophic activity are questions that ideally should be answered by future research. Nevertheless, it is clear that the multifactorial and complex nature of Actovegin underlies its pleiotropic neuroprotective mechanisms of action and positive effect on clinical outcomes.

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1. Introduction

Currently, it is difficult to find the optimum therapeutic approach for brain protection and recovery, especially as we do not fully understand all of the endogenous neurobiological processes and complete nature of the pathophysiological mechanisms involved, and the link between the two. Moreover, we continue to use a simplistic approach in this respect.

The reductionistic perception of the effects of brain lesions is that they are the linear sum of independent pathology causing mechanisms, such as excitotoxicity, inflammation, apoptosis and oxidative stress [1].

The classic concept of neuroprotection has developed the subtractive suppressive strategy for neuroprotective therapies. The backbone of this therapeutic strategy is the presumption that if a certain pathophysiological mechanism is pharmacologically suppressed by a chemical drug, the amount of damage caused by the specific mechanism will simply subtract from the total amount created by all pathophysiological mechanisms. However, what is clear from clinical trials in neuroprotection is that the suppression subtractive strategy with chemical drugs does not work [2,3].

The current tendency to exclusively frame drug activity in terms of single mechanisms and single focus effect may distract from other paradigms with greater explanatory power and hinder the development of more effective treatment strategies. A change of concept is required in pharmacological brain protection and recovery. The current understanding is that neurotrophic activity, neuroprotection, neuroplasticity and neurogenesis are the fundamental neurobiological processes that contribute to endogenous defense activity (EDA) and attempt to counteract pathophysiological damage mechanisms (DMs), and stimulate endogenous recovery (Fig. 1) [4].

The key issue in the new theory of brain protection and recovery is that the neurobiological processes of EDA share a common biological background with the pathophysiological mechanisms of DM. For example, excitotoxicity (a pathophysiological mechanism) and neuroplasticity (a neuroreparatory process) share N-methyl-D-aspartate receptors (NMDAR) activity as their common important driver. Therefore, when one acute pathophysiological process is pharmacologically suppressed (e.g. excitotoxicity or inflammation) by a single mechanism chemical drug, the long-term endogenous drivers of recovery (e.g. neuroplasticity and neurotrophic activity) are also disturbed [1].

Consequently, it is not appropriate to approach pharmacological neuroprotection and neurorecovery separately, because this dichotomy

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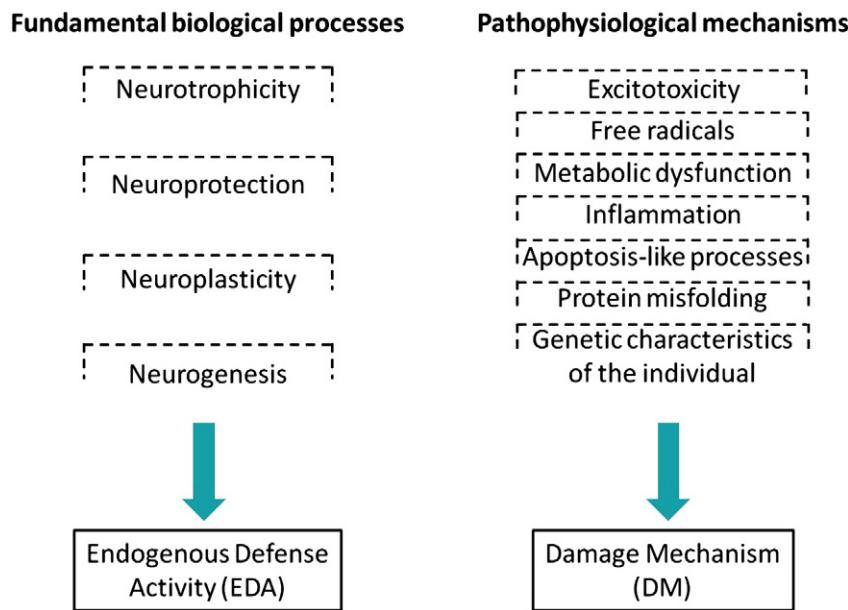


Fig. 1. Endogenous defense activity (EDA) and damage mechanisms (DMs).

might mislead basic and clinical research in the field. In fact, in clinical practice, a complex recovery outcome is evaluated, which is generated by all of the protection and recovery processes of EDA in an inseparable way [1].

Prospective considerations include an integrated pharmacological approach, focusing on agents with multimodal activity and pleiotropic neuroprotective effects which are biological drugs, rather than single mechanism drugs — which are usually chemically designed small molecules [5].

Actovegin is a deproteinized, endotoxin and antigen-free, ultrafiltrate from calf blood, which contains more than 200 bioactive constituents. The manufacturing process of Actovegin is both lengthy and complex and involves two ultrafiltration steps, which make use of different molecular mass cut-off sieves. During the production process, a cut-off sieve of 5000 Da is first used, followed by vacuum distillation removal of the remaining precipitate by filtration (0.45 µm) and titration to pH 6.4. One further ultrafiltration step followed by sterile filtration with prefilters of 0.2 µm and 0.45 µm, complete deproteinization of the product, which is finally tested negative for the presence of protein by SDS-polyacrylamide gel electrophoresis. The molecular weight of components in the final ultrafiltrate is less than 5000 Da.

The composition of Actovegin has been re-analyzed using state-of-the-art analytical techniques, including gas chromatography and validated high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). These metabolite-targeted quantification techniques (Biocrates Life Sciences AG, Innsbruck, Austria) demonstrated that Actovegin contains a combination of more than 200 bioactive-molecules. Actovegin's main constituents are low-molecular weight substances, including amino acids, biogenic amines and polyamines, sphingolipids, hexoses, eicosanoids, lactate, succinate, choline, vitamins, adenosine monophosphate (AMP) and inositol phospho-oligosaccharides (IPOs). Only small amounts of acylcarnitines, phospholipids, free fatty acids, and oxysterols have been detected; prostaglandins, oxidized polyunsaturated fatty acids, and bile acids are present in even smaller amounts (data on file).

Owing to the fact that Actovegin is a biological drug consisting of molecules present under normal physiological conditions, pharmacokinetic and pharmacodynamic studies to determine its active molecule(s) are not feasible. Nevertheless, it is obvious that the drug has many active components that influence numerous intracellular processes, and experimental studies have confirmed that Actovegin

influences specific cellular biochemical pathways. However, associations between the multiple molecular mechanisms and beneficial effects *in vitro* and *in vivo* remain only partly understood.

2. *In vitro* and *in vivo* effects of Actovegin

In early preclinical studies, Actovegin has been shown to influence the wound healing process by stimulation of cell growth including collagen synthesis and matrix contraction [6,7]. Actovegin also has beneficial, regenerative effects in the treatment of radiation-induced damage [8], as well as in disturbances of blood circulation [9,10]. However, most recently, its neuroprotective effects on related distinct pathophysiological cellular pathways have been examined closely.

Actovegin was recently demonstrated to have neuroprotective effects on neurons by increasing neuron and synaptic numbers. In an *in vitro* study [11], hippocampal neurons were isolated from rat brains and cultured under optimized conditions in the presence of different concentrations of Actovegin (0.3–1000 µg/mL). Cultured hippocampal neurons normally undergo a rapid decline due to apoptosis. However, after ten days in culture, a dose-dependent increase in the number of viable neurons of up to 2.4-fold was measured at concentrations above 10 µg/mL of Actovegin, compared to untreated neurons. In parallel, the number of excitatory synaptic connections between the neurons was measured by expression of a marker protein of pre-synaptic terminals, VGlut1. Analysis of VGlut1 using immunohistochemistry also revealed a dose-dependent increase of up to 3.6-fold per cell, induced by Actovegin at doses greater than 300 µg/mL.

Neuroprotective effects of Actovegin have also been observed in the peripheral nervous system in streptozotocin (STZ)-rats with severe symptoms of diabetic neuropathy [12]. By treating diabetic rats with intraperitoneal Actovegin with a dose equivalent to the human dose used in the clinical treatment of patients with distal peripheral neuropathy, the diabetes-induced degeneration of peripheral neurons was significantly decelerated. Beneficial effects of Actovegin were reflected by histomorphological improvements, i.e. an increase of 32% in intraepidermal nerve fiber density (IENFD) in rat paws by Actovegin treatment, at study end. Furthermore, a significant improvement in peripheral nerve functionality, i.e. a reversal in the decline of sensory nerve conduction velocity (SNCV), of up to 91% was detected in rats treated with Actovegin. N-acetyl-cysteine was

used as a positive control and interestingly, was not effective in this respect.

3. Actovegin modifies glucose uptake and aerobic metabolism in the brain

Preclinical studies have shown that at the molecular level, Actovegin improves oxygen utilization and uptake, as well as energy metabolism and glucose uptake [13–15]. Glucose is the primary energy source for the brain and in the CNS, glucose transporters play an essential role in neuronal homeostasis and glucose utilization [16–18]. Disruption of glucose homeostasis in the CNS leads to impairment of neuronal activity and cognitive function, which is particularly threatening to the hippocampus, the brain area important for learning and memory. Insulin receptors and insulin-sensitive glucose transporters, e.g. GLUT4, are crucial for glucose uptake and utilization in the CNS. They are expressed in brain areas such as the hypothalamus and cerebellum [19], as well as on the endothelium of the blood-brain barrier [20]. Basic and clinical research findings have demonstrated a clear relationship between brain insulin levels and cognitive functions, including learning and memory [21]. For instance, impairment of neuronal insulin receptor transduction has been demonstrated in Alzheimer's disease. Hoyer and colleagues have induced severe abnormalities in oxidative energy metabolism by intracerebroventricular administration of a diabetogenic agent into the brain, and found that it was associated with behavioral disturbances [22–24]. These findings are in agreement with the hypothesis of Salkovic-Petrisic and Hoyer [25] that an insulin-resistant state in the brain may have an impact on the cellular and molecular abnormalities seen in Alzheimer's disease.

Actovegin has positive effects on the utilization of glucose and oxygen, thereby enhancing energy metabolism in the brain. It was described that inositol phospho-oligosaccharides may play an important role in the regulation of insulin-dependent enzymes [26,27]. Experimentally, Actovegin has demonstrated increased oxygen uptake and utilization [28], which can lead to the stabilization of CNS cells during ischemia, and reduce the formation of lactate. It also up-regulates glucose transporters GLUT1 and GLUT4, which in cerebrovascular disorders may translate into an improvement of glucose transport across the blood-brain barrier.

The parietal cortex is responsible for processing visual information and spatial-directed attention, while shrinkage of this area leads to dementia. It has appeared from clinical studies, that Actovegin improves cognitive processing in the parietal cortex in age-associated memory impairment, which may be associated with its positive influence on hypoxic states [29]. In line with these clinical findings, Hoyer and Betz [30] showed that Actovegin provides cellular protection in a hypoxia model of cerebral ischemia.

4. Amelioration of A β _{25–35} peptide-induced apoptosis

Amyloid β -peptides (A β s) are the main component of amyloid plaques, which are the pathological hallmarks of Alzheimer's disease. The neurotoxic peptides increase oxidative stress and activate inflammatory pathways, ultimately causing neuronal cell death [31]. However, A β fragments vary significantly in terms of their pathological properties. For example, the A β _{25–35} fragment is highly toxic [32,33]

and has more rapid aggregation properties than other commonly studied amyloid peptides [34]. These properties have been reflected in studies using animal models where A β _{25–35} impaired short-term memory, and induced neuropathological processes, resulting in neuronal cell death [35,36]. There is a suggestion that the cytotoxic and abnormal aggregative properties of A β _{25–35} underlie its neurodegenerative properties in Alzheimer's disease and other disorders, but further work is needed in order to fully elucidate this.

In a recent study investigating the potential mode of action of Actovegin, A β _{25–35} peptides were used to test whether or not it has an anti-apoptotic effect on neuronal cells *in vitro* [11]. Rat primary hippocampal neurons were cultivated with A β _{25–35}, in the presence of Actovegin, to measure the levels of activated caspase-3 on a per-cell basis, reflecting the degree of neuronal apoptosis. In the absence of A β _{25–35} peptides, levels of apoptosis were similar in both Actovegin-treated and untreated cells. In hippocampal neurons exposed to A β _{25–35} peptides and treated with increasing doses of Actovegin, the level of activated caspase-3 decreased significantly ($p < 0.001$ at Actovegin concentrations $> 300 \mu\text{g/mL}$) and followed a trend towards dose-dependency [11].

Since there is evidence to suggest that the A β _{25–35} peptide plays a key role in the pathogenesis of Alzheimer's disease and it has been characterized in the brains of patients with Alzheimer's disease [37], the amelioration of apoptosis induced by this peptide extends our understanding of Actovegin-mediated mechanisms of neuroprotection.

5. NF- κ B pathway activation by Actovegin

The transcription factor NF- κ B has wide-ranging and important roles within the mammalian central and peripheral nervous systems. Specifically, NF- κ B regulates inflammatory processes that exacerbate diseases such as ischemia and Alzheimer's disease, and plays a role in pain, learning and memory, and importantly, neuroprotection [38–41]. Regulation of NF- κ B activity is complicated by the fact that numerous pathways can either upregulate or downregulate its activity, resulting in varying effects, in different tissues.

The involvement of NF- κ B in neuroprotective pathways is mediated by pro-inflammatory cytokines [42]. It has been shown that treatment of primary hippocampal neurons with tumor necrosis factor- α (TNF- α) prevents hypoxia and nitric oxide-induced neuronal cell death, through the elevation of NF- κ B-dependent expression of the anti-apoptotic factors Bcl-2 and Bcl-x [43]. However, it has been found that TNF- α may also induce neuronal apoptosis and trigger degenerative processes [44], while soluble TNF receptor-1 has been demonstrated to induce apoptosis in monocytes [45]. *In vivo*, it was demonstrated that TNF- α pretreatment in rats with focal ischemia reduced brain injury [46], while transgenic TNF receptor knock-out mice presented larger infarctions [47]. This phenomenon could be explained by the existence of two TNF receptor types: R1 – inducing demyelination and increasing cell death, and R2 – inducing remyelination and mediating neuroprotection by activation of oligodendrocytes through BDNF and CNTF [5,48].

Other work supporting a role for the neuroprotective properties of NF- κ B suggests it protects against neuronal cell death in the presence of A β , and may therefore influence apoptotic pathways [40,49]. Rodent models have also shown that downregulation of inhibitory kappa beta (IkB), a central component of the NF- κ B signaling pathway,

Table 1

Response of the beta-lactamase reporter gene (% of baseline of non-stimulated cells) in the reporter gene of NF- κ B-*bla* HEK 293T cells to Actovegin, TNF- α stimulation and placebo control. Data presented are mean values from five wells of a microplate; experiments were repeated three times (Hundsberger and Pflüger).

Test substance	Placebo 1*	Placebo 2*	Actovegin 4 mg/mL	Actovegin 0.4 mg/mL	Actovegin 0.04 mg/mL	TNF- α 10 ng/mL	TNF- α 0.4 ng/mL
Promoter activation \pm SD	295 \pm 31	32 \pm 8	1089 \pm 29	767 \pm 21	614 \pm 19	1253 \pm 32	1115 \pm 15

* Osmolarity of placebo 1 corresponding to Actovegin 4 mg/mL and of placebo 2 to Actovegin 0.4 mg/mL.

Table 2

Summary of preclinical findings on the neuroprotective and metabolic effects of Actovegin.

Actovegin effects	Study
Accelerated wound healing	Mochida et al. [6]; Schonwald et al. [7]
Improved blood circulation	Hegner [9]; Giarola [10]
Protection from radiation-induced damage	Basu et al. [8]
Improved oxygen utilization	Reichel et al. [28]; de Groot et al. [29]; Hoyer and Betz. [30]
Improved glucose metabolism	Machicao et al. [26]
Increased peripheral nerve fiber density	Dieckmann et al. [12]
Increased neuron survival	Elmlinger et al. [11]
Reduced oxidative stress	Elmlinger et al. [11]; Dieckmann et al. [12]
Reduced apoptosis	Elmlinger et al. [11]
Improved nerve conduction velocity	Dieckmann et al. [12]

results in the loss of neuroprotection and can lead to defects in learning and memory [50].

Hundsberger and Pflüger (FH Krems, Austria) have conducted a novel study *in vitro* to investigate the potential of Actovegin to modulate the NF-κB pathway. For their experimental system, the investigators chose the well-established CellSensor® human embryonic kidney cell line, NF-κB-blA HEK 293T, which contains a stably transfected β-lactamase reporter gene under control of the NF-κB response element. In order to exclude osmotic side effects of Actovegin in the cells, a placebo solution with a salt concentration equimolar to Actovegin was used. Measurement of fluorescence emission revealed that Actovegin activates the reporter gene of NF-κB expression in a dose-dependent manner (Table 1; unpublished observations). The effective concentration corresponded to the stimulatory effectiveness of a TNF-α concentration of approximately 400 pg/mL. Coupled with the results of Elmlinger et al. [11] these data suggest that the neuroprotective and anti-apoptotic properties of Actovegin can at least in part be attributed to transient activation of NF-κB.

6. Anti-oxidative stress and poly(ADP-ribose) polymerase pathway

Oxidative stress-induced single-strand DNA breakage can lead to the activation of the nuclear enzyme, PARP. PARP plays a key role in the detection and repair of single-strand DNA breaks, but it has been shown that excessive activation of the enzyme is detrimental; it can trigger a sequence of cellular processes, which ultimately halt glycolysis and mitochondrial respiration, resulting in cell death arising due to energy depletion [51,52].

Experimental studies have shown that the disruption of genes coding for PARP protects rodents from a range of pathophysiological processes [52–54]. PARP gene disruption has been found to offer significant protection to neurons following focal ischemia [52] and prevents the progression or appearance of diabetes following STZ induction [53,54]. Further

work has identified PARP as an important pathway of endothelial dysfunction in diabetes [55] and more recently it has been shown that PARP may underlie the development of diabetic polyneuropathy; PARP inhibition in rats protects against a reduction in nerve conduction velocities [56]. Taken together, these data suggest an important and fundamental role of PARP in chronic disorders such as cerebrovascular disease and diabetic polyneuropathy.

Work by Elmlinger and colleagues found that oxidative stress in primary hippocampal neurons is positively affected by Actovegin, as tested using a fluorescence-based assay system (proportional to total reactive oxygen species (ROS) content). Neurons treated with increasing concentrations of tertiary-butyl hydroperoxide (TBHP) (>0.2 mM) exhibited increased intracellular ROS levels ($p<0.001$), but treatment with Actovegin dose-dependently reduced oxidative stress in neurons after 10 days of culture ($p<0.001$ at concentrations >0.3 µg/mL) [11].

An investigation into the effects of Actovegin on parameters of experimental diabetic polyneuropathy has corroborated the findings of this study. STZ diabetic rats, which were administered Actovegin intraperitoneally from day 11 to day 40 post-STZ exposure, had significantly reduced PARP activity, at an Actovegin dose of 600 mg/kg, as measured by poly(ADP-ribose) content [12]. The reduction in PARP activity may underlie the functional and morphological improvements observed with Actovegin in the peripheral and central nervous systems, as mentioned earlier. The data obtained from these two studies also suggest that the anti-oxidative properties of Actovegin underpin its effects on PARP activation, but further research is needed in order to verify this.

7. Discussion

The data reviewed above describe how Actovegin improves cellular metabolic balance and influences a number of mechanisms implicated in different disorders. Since neurological disorders are underpinned by a complex array of pathophysiological events, an integrated pharmacological approach, rather than a simplistic one, may be required to effectively treat them. As a biological agent with pleiotropic neuroprotective and metabolic effects (Table 2), Actovegin's mode of action fits this future vision of an integrated treatment paradigm.

The pleiotropic neuroprotective effect describes different simultaneous ways to modulate pathological mechanisms of DM (excitotoxicity, inflammation, apoptosis, oxidative stress, and more). In contrast, a single-mechanism molecule is only able to suppress one pathophysiological mechanism. The pleiotropic neuroprotective and metabolic effects of Actovegin's mode of action have been briefly described in this article. Actovegin has been demonstrated to have neuroprotective effects on the neurovascular unit due to its anti-apoptotic and anti-oxidative effects. Positive effects have also been demonstrated on the utilization of glucose and oxygen, thereby enhancing energy metabolism in the brain. A simple diagram describing the pleiotropic neuroprotective and metabolic effects of Actovegin is depicted in Fig. 2. Further effects on other endogenous

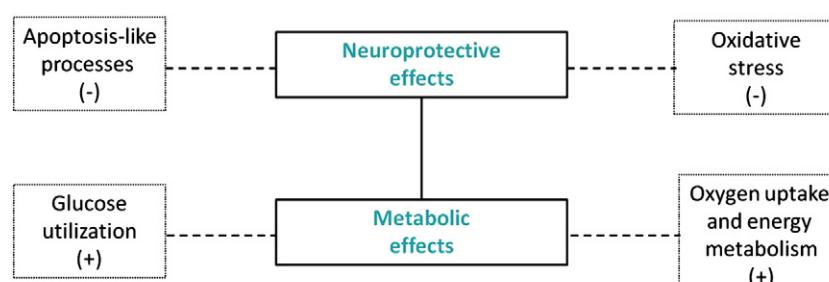


Fig. 2. Pleiotropic neuroprotective and metabolic effects of Actovegin.

neurobiological processes such as neurotrophicity, neuroplasticity and neurogenesis are yet to be fully explored in future studies.

8. Conclusions

Actovegin is composed of more than 200 biological constituents, which influence many intracellular processes and pathways. A range of findings have revealed possible mechanisms of action underlying the neuroprotective and metabolic effects of Actovegin; it has been shown to modulate the activation of NF- κ B, reduce apoptosis, inhibit PARP and positively affect both glucose and oxygen utilization. However, the effects of Actovegin on other potential pathways involving neurotrophicity, neuroplasticity and neurogenesis still remain to be investigated. Nevertheless, the complex composition of the drug, which has biological origin, underlies its pleiotropic neuroprotective and metabolic mode of action.

Conflicts of interest

All authors have received honoraria from Nycomed and Takeda for scientific clinical trial advice, lectures and consultancy work.

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