RETINAL AUTOPHAGY AND THE IMPACT OF PHARMACOLOGICAL BLOCKADE OF CELLULAR PROTEIN KINASES IN EXPERIMENTAL DIABETIC RETINOPATHY

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DOI: 10.51587/3068-3580/US-2025-05-1

Usenko, K., Mikhajlovska, V. & Ziablitsev, S. (2025). Retinal autophagy and the impact of pharmacological blockade of cellular protein kinases in experimental diabetic retinopathy / *University Science*, 1(1), 65–80. https://doi.org/DOI: 10.51587/3068-3580/US-2025-05-1-65-80

Received: April 24, 2025
Accepted: May 05, 2025

15 May, 2025,
Volume 1 (1),
ISSN 3068-3580
https://www.us-journal.us

ABSTRACT

Background. Diabetic retinopathy (DR) remains the leading cause of irreversible vision loss among working-age patients, necessitating the search for new therapeutic targets in the early stages of the disease. Studying the effect of pharmacological blockade of cellular protein kinases on Beclin-1 expression in the retina will help uncover the molecular mechanisms of autophagy regulation and develop new treatment methods.

Aim: to determine the content of Beclin-1 protein in retinal tissue and the effect of the pharmacological cellular protein kinase blocker sorafenib on it in experimental DR.

Materials and methods. DR was modeled in male Wistar rats by single administration of streptozotocin (50 mg/kg; Sigma-Aldrich, Co, China). Rats were divided into 4 groups: control, with insulin administration (30 U; NovoNordisk A/S, Bagsvaerd, Denmark), protein kinase inhibitor sorafenib (Cipla, India) at a dose of 50 mg/kg, and with administration of insulin and sorafenib. Immunoblotting was performed using monoclonal antibodies against Beclin-1 (Invitrogen, USA).

Results. During the development of experimental DR, the level of Beclin-1 in the retina increased by 1.8-2.0 times ($p<0.05$) compared to intact animals, indicating activation of autophagy processes. Insulin administration normalized the Beclin-1 level to that of intact animals, while sorafenib monotherapy did not significantly affect its content. Combined use of insulin and sorafenib led to a significant decrease in Beclin-1 levels (1.5 times lower than the level of intact animals; $p<0.05$). The obtained results indicate potentiation of insulin effects with simultaneous blockade of cellular protein kinases, which may have therapeutic significance in DR.

Conclusion. Dysregulation of autophagy involving the Beclin-1 protein plays a key role in the pathogenesis of DR. Investigation of possibilities for pharmacological effects on cellular protein kinases to modulate Beclin-1 levels opens prospects for developing new approaches to retinal neuroprotection.

Key words

Diabetic retinopathy, autophagy, Beclin-1, sorafenib.

15 May, 2025,
Volume 1 (1),
ISSN 3068-3580
https://www.us-journal.us

BACKGROUND

Diabetic retinopathy (DR) is one of the most common and severe complications of diabetes mellitus (DM) and remains the leading cause of irreversible vision loss among working-age adults in developed countries (Simó & Hernández, 2014) . Epidemiological projections estimate that the number of patients with DR in the United States will reach 16 million by 2050, with approximately 3.4 million at risk of vision-threatening complications (Wong & Sabanayagam, 2020). Given the rapidly growing global prevalence of diabetes, the number of people affected is expected to rise to nearly 700 million by 2045, a substantial proportion of whom will be at risk of developing retinopathy (International Diabetes Federation, 2021).

Historically, DR has been considered a microvascular disease characterized by blood-retinal barrier disruption, thickening of the vascular basement membrane, leukocyte adhesion, acellular capillary formation, pericyte loss, and in advanced stages, pathological angiogenesis (Antonetti et al., 2012). According to the classical classification, DR is divided into non-proliferative and proliferative forms. The latter is marked by neovascularization, which may result in vitreous hemorrhage, tractional retinal detachment, and ultimately, complete vision loss (Solomon et al., 2017) .

However, over the past decade, the understanding of DR pathogenesis has significantly evolved. Accumulating experimental and clinical evidence indicates that DR is not solely a vascular disorder, but rather a complex neurovascular pathology of the retina (Simó, Stitt, & Gardner, 2018) Numerous studies have demonstrated that neurodegenerative changes occur early in the disease process, often preceding observable microvascular abnormalities. Functional impairment and apoptosis of retinal neurons, microglial activation, reactive gliosis, and disrupted neurovascular coupling are all evident before classical vascular lesions develop (Dehdashtian et al., 2018).

This paradigm shift is critical for the development of new preventive and therapeutic strategies. Current treatment approaches, primarily involving intravitreal injections of vascular endothelial growth factor (VEGF) inhibitors, target the late stages of DR when structural damage and vision loss are already substantial (Salminen & Kaarniranta, 2013). These therapies are limited by short duration of action, requiring frequent administration, which increases the risk of adverse events such as endophthalmitis, vitreous hemorrhage, retinal detachment, and cataract formation. Moreover, while VEGF blockade effectively suppresses pathological neovascularization, it may interfere with the physiological functions of VEGF, including its roles in neuroprotection and maintenance of normal vasculature (Salminen & Kaarniranta, 2013).

Accordingly, there is a growing interest in identifying new therapeutic targets and interventions aimed at the early stages of DR to prevent or slow disease progression. One promising direction involves the regulation of cellular autophagy–a tightly controlled process responsible for the degradation and recycling of damaged organelles, aggregated proteins, and other cellular components. Under physiological conditions, autophagy is essential for maintaining cellular homeostasis and survival during stress. However, in pathological conditions such as DM, dysregulated autophagy can contribute to cellular dysfunction and death (Dehdashtian et al., 2018).

Beclin-1 is a key regulator of autophagy. It forms part of the class III phosphoinositide 3-kinase (PI3KC3) complex, which initiates

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ISSN 3068-3580
https://www.us-journal.us

autophagosome formation. Beclin-1 interacts with a wide range of cellular proteins, including anti-apoptotic Bcl-2 family members, cell cycle regulators, protein kinases, and transcription factors–linking autophagy to various other cellular processes (Salminen & Kaarniranta, 2013)

Altered expression and activity of Beclin-1 have been implicated in a range of pathologies, including neurodegeneration, cancer, infection, and metabolic disorders. In the context of DR, the role of Beclin-1 and autophagy is not yet fully elucidated, although emerging evidence suggests their critical involvement in the disease's onset and progression.

Particular attention has been given to pharmacological modulation of autophagy via targeting intracellular signaling pathways, especially those involving protein kinases. These enzymes are central to signal transduction and regulate key cellular functions, including metabolism, proliferation, survival, and apoptosis. They are also instrumental in controlling autophagy by modulating Beclin-1 and other components of the autophagic machinery (Simó et al., 2018).

Among the diverse kinase pathways, RAF/MEK/ERK and PI3K/Akt/mTOR are particularly relevant in DR pathophysiology. These pathways influence retinal cell proliferation, survival, metabolism, inflammation, angiogenesis, and autophagy, and their dysregulation is associated with diabetic complications (Solomon, 2017).

Sorafenib, a multikinase inhibitor targeting RAF, VEGFR, and PDGFR, has demonstrated therapeutic potential in conditions involving overactivation of these signaling cascades. In DR, sorafenib may exert dual action: attenuating angiogenesis via VEGFR inhibition and modulating autophagy through intracellular signaling regulation (Antonetti et al., 2012).

Insulin, the cornerstone of DM treatment, exerts multiple effects beyond glycemic control by activating the insulin receptor and downstream signaling pathways. Notably, insulin is a potent activator of the PI3K/Akt/mTOR pathway, a known negative regulator of autophagy. Thus, insulin may also influence Beclin-1 expression and autophagic activity in retinal cells (Simó & Hernández, 2014).

The combined effects of insulin and sorafenib on autophagy in DR warrant particular attention, given their potential interaction at the level of intracellular signaling. However, studies addressing this question are virtually absent in the current literature, underscoring the novelty and importance of this research (International Diabetes Federation (IDF), 2021).

Aim: to determine the content of Beclin-1 protein in retinal tissue and evaluate the effect of the pharmacological cellular protein kinase inhibitor sorafenib in an experimental model of diabetic retinopathy.

MATERIALS AND METHODS

All procedures were conducted in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), Directive 86/609/EEC of the Council of Europe (1986), the Law of Ukraine No. 3447-IV "On the Protection of Animals from Cruel Treatment," the general ethical principles of animal experimentation approved by the First National Congress of Ukraine on Bioethics (2001), and the guidelines of the Bioethics and Scientific Ethics Committee at Bogomolets National Medical University.

The study involved 65 male Wistar rats aged 3 months and weighing 140–160 g. Experimental diabetes mellitus (DM) and diabetic retinopathy (DR) were induced in 60 rats via a

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https://www.us-journal.us

single intraperitoneal injection of streptozotocin (STZ; 50 mg/kg; Sigma-Aldrich, Co., China) dissolved in freshly prepared cold 0.1 M citrate buffer (pH 4.5). Five animals served as the intact control group. Prior to STZ administration, the rats were fasted for 16 hours and provided with a 5% glucose solution for the subsequent 24 hours. Blood glucose levels were measured every three days using a glucometer (ACCU-Chek Instant, Roche Diagnostics, Germany) and test strips from tail vein blood samples collected after fasting. Rats with blood glucose levels ≥15 mmol/L three days post-STZ injection were considered diabetic and were monitored for 3 months.

After 7 days, animals with persistent hyperglycemia (n=60) were divided into 4 groups of 15 individuals by blind random method. In the 1st group (control), hyperglycemia was not treated. In the 2nd group, animals received intraperitoneal injections of short-acting insulin (Actrapid HM Penfill, Novo Nordisk A/S, Bagsvaerd, Denmark) at a dose of 30 U every other day. Animals in the 3rd group were administered per os daily a solution of the protein kinase inhibitor sorafenib (200 mg, Cipla, India) at a dose of 50 mg/kg in the form of sachets. Animals in the 4th group received insulin (according to the 2nd group protocol) as well as sorafenib solution (according to the 3rd group protocol).

Animals were removed from the experiment after 28 days, 2 and 3 months in quantities of 5 individuals in each group by a lethal injection of thiopental (75 mg/kg). Determination of Beclin-1 content in retinal tissue lysates was performed by immunoblotting. Tissue samples were kept in liquid nitrogen, crushed, and homogenized in 50 mmol Tris-HCl buffer (pH 7.4) with the addition of phosphatase and protease inhibitors (Pierce Protease and Phosphatase inhibitor, "ThermoScientific", USA, No. A32961). Electrophoresis was performed in 8% polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) in a vertical gel electrophoresis chamber ("BioRad", USA). Proteins were transferred from the gel to a nitrocellulose membrane using electroblotting. Membranes were incubated with monoclonal antibodies against Beclin-1 (Invitrogen, USA). Antibodies to actin (β-actin (loading control), no. MA5-15739, mouse, 1:3,000, Invitrogen, USA) were used for its detection as a protein loading control.

After washing, membranes were incubated with species-specific horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit or anti-mouse IgG, Invitrogen, USA; cat. nos. G-21234 and 31430, 1:8000 dilution). Bands were visualized and analyzed densitometrically using TotalLab software (version TL120, Nonlinear Inc., USA). Protein levels were normalized to β-actin and expressed as arbitrary units relative to the control group.

Statistical analysis was conducted using Statistica 10.0 software (StatSoft Inc., USA). Data were expressed as mean ± standard error of the mean (SEM). Differences between groups were assessed using one-way ANOVA, with $p < 0.05$ considered statistically significant.

RESULTS

Immunoblotting analysis (Fig. 1) demonstrated a significant and sustained increase in Beclin-1 protein levels in the retinas of rats with experimental DR compared to intact animals. Specifically, Beclin-1 expression was elevated by 1.8-fold on day 28, by 2.0-fold after 2 months, and by 1.9-fold after 3 months ($p<0.05$ in all cases). This persistent elevation indicated continuous activation of early-stage autophagy in the diabetic retina, which, according to previous studies, is associated with reactive gliosis and apoptosis of Müller cells during this period (Usenko, 2025).

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ISSN 3068-3580
https://www.us-journal.us

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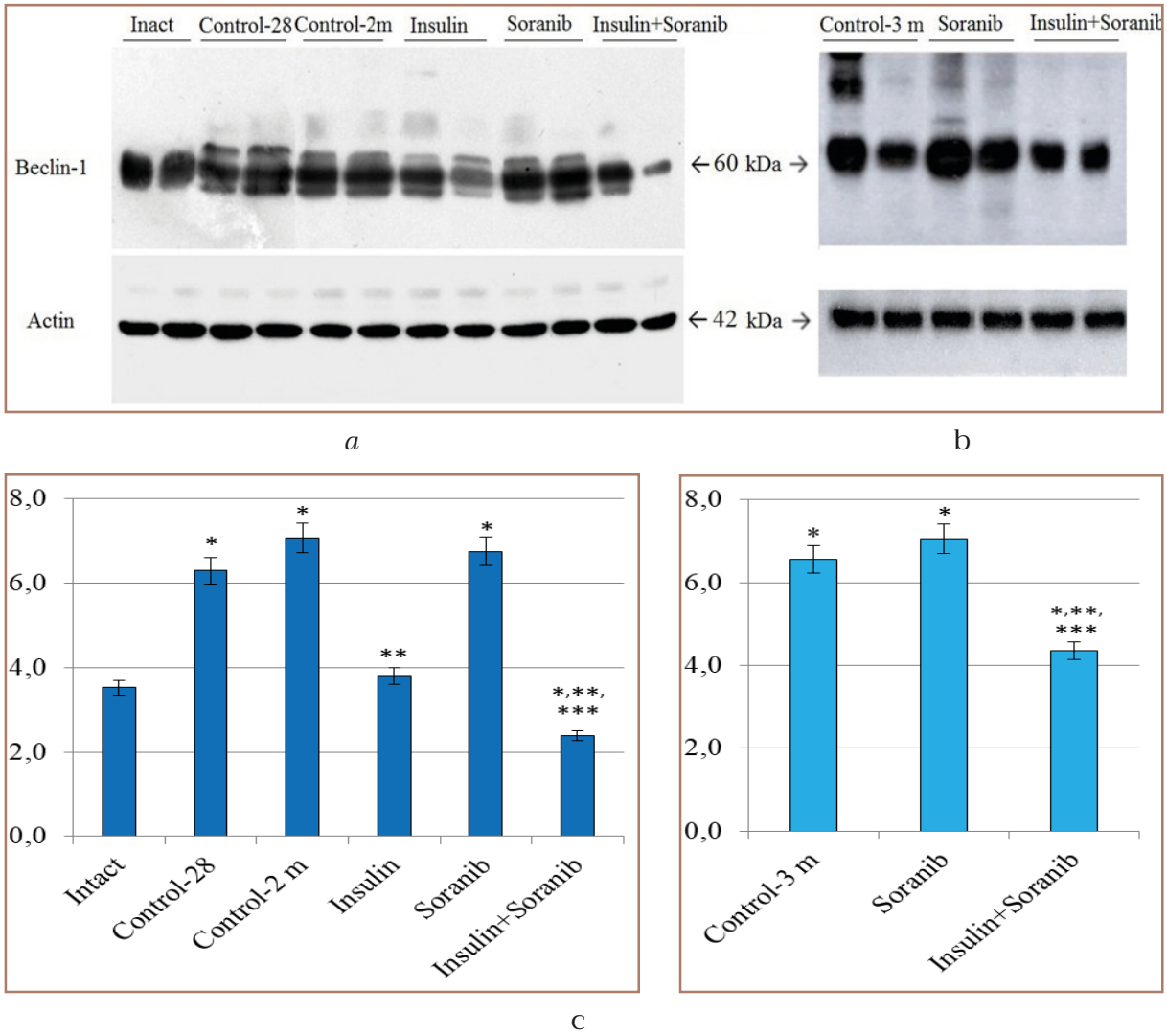

Figure 1. Beclin-1 protein levels in retinal tissue of intact rats (Intact), and in the control group at 28 days (Control-28), 2 months (Control-2 m), and 3 months (Control-3 m); groups treated with insulin (Insulin), sorafenib (Sorafenib), and the combination (Insulin + Sorafenib).

a - representative blotograms of Beclin-1 protein and actin;
b - results of densitometric analysis of Beclin-1 protein blotograms (ratio to actin level);
* - $p<0.05$ compared to the level of intact rats;
** - $p<0.05$ compared to the control level (in a - with control after 2 months, in b - with control after 3 months);
*** - $p<0.05$ compared to the level with sorafenib administration.

Administration of insulin after 2 months of disease progression restored Beclin-1 expression to normal levels (Fig. 1a), suggesting an inhibitory effect of insulin on autophagic activity in DR. In contrast, monotherapy with sorafenib had no significant effect on diabetes-induced Beclin-1 upregulation. However, combined administration of insulin and

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sorafenib led to a further reduction in Beclin-1 levels, falling 1.5-fold below those observed in intact animals ($p<0.05$).

Comparable results were observed at the 3-month time point, where insulin + sorafenib co-treatment significantly reduced Beclin-1 levels compared to the control group (by 1.5-fold; $p<0.05$).

In summary, diabetic retinal tissue exhibited a consistent, nearly twofold increase in Beclin-1, indicating pronounced autophagy activation. Insulin treatment alone suppressed this response, while sorafenib alone had no effect. Their combined administration, however, led to a marked suppression of Beclin-1 expression.

DISCUSSION

Autophagy is an evolutionarily conserved membrane trafficking process responsible for the degradation of cytoplasmic proteins and dysfunctional organelles via lysosomal targeting. This intrinsic "self-cleaning" mechanism plays a fundamental role in maintaining intracellular homeostasis and facilitating cellular adaptation to stress conditions (Cao et al., 2014).

The Beclin-1 protein serves as a central regulator of autophagy initiation by promoting autophagosome formation through the activation of the class III phosphatidylinositol 3-kinase (PI3KC3) complex. Beclin-1 is considered a key driver of the early stages of the autophagic cascade. Experimental models have demonstrated that reduced expression of Beclin-1 is associated with impaired autophagic flux and the accumulation of damaged organelles, contributing to retinal cellular injury in DR (Di Rosa et al., 2016).

At the molecular level, autophagy is regulated by complex signaling pathways. Beclin-1 interacts with anti-apoptotic proteins of the Bcl-2 family, which inhibit autophagy by sequestering Beclin-1 and preventing autophagosome formation. Under stress conditions, disruption of the Beclin-1/Bcl-2 complex leads to the release of Beclin-1, thereby promoting autophagic vesicle formation.

In the retina, autophagy is activated at the early stages of DR, particularly in the dendrites of retinal ganglion cells (RGCs), where it serves a neuroprotective function. However, excessive or sustained autophagy activation may contribute to neuronal loss, particularly through autophagy-mediated cell death mechanisms (Kunchithapautham & Rohrer, 2007). Beclin-1 is essential for autophagosome formation within the ganglion cell layer, highlighting its role in retinal neurodegeneration and homeostasis.

One of the primary negative regulators of autophagy is the PI3K/Akt/mTOR signaling axis. The mammalian target of rapamycin (mTOR), a 289 kDa serine/threonine kinase, governs cell growth, metabolism, proliferation, and autophagy in response to a wide range of extracellular stimuli, including growth factors (IGF-I, VEGF), hormones (e.g., insulin), nutrients, and cytokines (Zoncu et al., 2011). Activation of mTORC1 inhibits autophagy by suppressing the activity of the Beclin-1-containing initiation complex (Di Rosa et al., 2016). Hence, chronic activation of mTOR in the diabetic retina may suppress autophagy, disrupt cellular equilibrium, and exacerbate DR progression.

In DR, hyperactivation of the mTORC1 complex is considered a pivotal pathophysiological event, leading not only to autophagy suppression but also to dysregulated lipogenesis and metabolic dysfunction in retinal cells (Laplante & Sabatini, 2013; Blommaart et al., 1995). Inhibition of autophagy via mTORC1 occurs through the phosphorylation and inactivation of autophagy-initiating proteins, in-

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cluding Beclin-1. In retinal cells, this leads to the accumulation of damaged organelles and proteins, increased oxidative stress, inflammation, and apoptosis (Di Rosa et al., 2016).

A reduction in Beclin-1 expression or functional activity observed in DR may serve as a molecular marker of impaired autophagy and diminished adaptive capacity. These findings highlight the therapeutic potential of targeting autophagy via modulation of protein kinase signaling. Pharmacological inhibition of kinases such as mTOR or MAPKs may help restore autophagic activity by reactivating Beclin-1-dependent pathways, thus promoting retinal cell survival and maintaining structural integrity in DR.

Changes in Beclin-1 Levels in Retinal Tissue in Experimental Diabetic Retinopathy and Their Pharmacological Modulation

Significant alterations in autophagic activity occur in retinal cells under conditions of diabetic retinopathy (DR), directly associated with dysregulation of Beclin-1 expression and its functional activity. Müller glial cells, which represent the principal glial elements of the retina and provide trophic and metabolic support to neurons and blood vessels, exhibit complex dynamics of autophagy under hyperglycemic conditions (Shen et al., 2012).

At the early stages of DR, increased levels of Beclin-1 and the autophagy marker LC3-II have been observed in Müller cells; however, due to lysosomal dysfunction, the degradation of autophagic cargo remains incomplete. This leads to the accumulation of p62, the release of VEGF, and the activation of apoptotic cascades (Lopes de Faria et al., 2016; Adornetto et al., 2021).

Experimental studies in rat Müller glial cell cultures have shown that chronic hyperglycemia progressively decreases the expression of Beclin-1 and LC3-II, while simultaneously increasing p62 accumulation. These findings reflect a deterioration of autophagic flux during the prolonged course of the disease. Pharmacological activation of autophagy with rapamycin—an mTOR inhibitor—promotes an increase in Beclin-1 levels, a decrease in p62, and reduced cell death, thereby supporting the therapeutic potential of autophagy modulation in DR (Adornetto et al., 2021).

Our experimental results revealed a significant and sustained elevation of Beclin-1 levels in retinal tissue under conditions of experimental DR. A progressive increase in the concentration of this protein was observed throughout the entire study period: by day 28, Beclin-1 levels had increased 1.8-fold; by 2 months, 2.0-fold; and by 3 months, they remained elevated at 1.9-fold compared to the control group ($p < 0.05$ in all cases). This dynamic reflects persistent activation of autophagic processes in response to hyperglycemia and oxidative stress, which may be considered a compensatory mechanism aimed at eliminating damaged cellular components (Lopes de Faria et al., 2016). At the molecular level, the regulation of Beclin-1 is largely dependent on its interaction with anti-apoptotic proteins of the Bcl-2 family. Under physiological conditions, Beclin-1 forms a complex with Bcl-2, preventing the initiation of autophagy. In response to cellular stress or external stimuli—such as phosphorylation activity by protein kinases including JNK1, DAPK, and ERK—either Bcl-2 or Beclin-1 becomes phosphorylated, resulting in the dissociation of the complex and activation of autophagy (Pattingre et al., 2005).

Transcription factors also play a crucial role in regulating Beclin-1 expression, particularly NF-κB and E2F. The p65 subunit of the classical NF-κB pathway binds to the Beclin-1 promoter, potentially enhancing its transcription. However, depending on the cellular context, NF-κB may act as either an activator or inhibitor of autophagy. In parallel, E2F direct-

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ly binds to the promoters of various autophagy-related genes, including Beclin-1, stimulating their expression.

Immunohistochemical studies have shown that the spatial distribution of Beclin-1 across retinal layers is altered in DR (Lopes de Faria et al., 2016). Normally, Beclin-1 is predominantly localized to the ganglion cell layer, inner nuclear layer, and photoreceptor segments (Chai et al., 2016). In DR, immunoreactivity for Beclin-1 is reduced in the ganglion and inner plexiform layers, correlating with neuronal injury in these regions (Xu & Chen, 2016).

Pharmacological blockade of cellular protein kinases, including JNK and ERK, can modulate Beclin-1 phosphorylation status and its interaction with Bcl-2, thereby influencing autophagic activity (Pattingre et al., 2005). Notably, inhibition of JNK1 prevents Bcl-2 phosphorylation, stabilizing the Bcl-2/Beclin-1 complex and reducing the pool of free Beclin-1 (Kim & Guan, 2015). This strategy may have therapeutic value in conditions of excessive autophagy associated with neuronal death. Conversely, in states of autophagy insufficiency, characterized by the accumulation of dysfunctional organelles and protein aggregates, activation of Beclin-1 via mTOR inhibition could restore cellular homeostasis.

Thus, pharmacological modulation of Beclin-1 expression and activity via targeted regulation of protein kinase pathways holds promise as a therapeutic strategy in DR, aimed at restoring autophagic balance and preserving retinal structural and functional integrity.

Caspases—cysteine-aspartyl proteases responsible for executing apoptosis—have been shown to cleave Beclin-1, thereby abolishing its pro-autophagic function (Shi, 2002; Wirawan et al., 2010). Caspases-3, -7, and -8 mediate specific proteolytic cleavage of Beclin-1, generating fragments incapable of initiating autophagy. The C-terminal cleavage products localize to mitochondria and enhance cellular susceptibility to apoptosis, establishing a positive feedback loop for cell death progression. Bax, a pro-apoptotic protein, also contributes to autophagy inhibition by promoting caspase-dependent Beclin-1 cleavage at residue D149 (Wang et al., 2006). Interestingly, expression of a cleavage-resistant mutant of Beclin-1 or co-expression with Bcl-XL may mitigate this effect (Pattingre et al., 2005). In certain models, including HeLa cells, TRAIL (TNF-related apoptosis-inducing ligand) has been shown to induce caspase-mediated Beclin-1 cleavage (Thorburn et al., 2014).

However, according to recent data, active caspase-8, an effector molecule in death receptor signaling pathways, can itself be degraded via autophagy, indicating a bidirectional relationship between these processes (Hou et al., 2010). Autophagy and apoptosis share multiple signaling components, and the final cellular fate often depends on the balance between these opposing programs. Notably, while proteolytic cleavage of Beclin-1 and Atg5 during apoptosis leads to autophagy inhibition, caspase-3-mediated cleavage of Atg4D, on the contrary, generates a fragment with enhanced autophagic activity (Betin & Lane, 2009). These findings highlight the complex and dynamic interplay between mechanisms of cellular adaptation and cell death.

The Effect of Insulin on Beclin-1 Levels and Autophagy in DR

Experimental data revealed that administration of insulin two months after the onset of DR led to a significant decrease in Beclin-1 levels to baseline values ($p < 0.05$), indicating an inhibitory effect of insulin on autophagic activity in the retina. The molecular mechanisms and physiological implications of this phenomenon warrant further discussion.

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Insulin is a potent regulator of metabolic processes, including autophagy. The Beclin-1 protein, encoded by the Becn1 gene, is known to facilitate adiponectin secretion through its interaction with the exocyst complex. While Beclin-1 is associated with improved insulin sensitivity, insulin can paradoxically suppress Beclin-1 expression by activating the PI3K/Akt/mTOR pathway—one of the key negative regulators of autophagy (Kuramoto et al., 2021). Therefore, insulin likely attenuates excessive autophagic activity in DR by downregulating Beclin-1 through PI3K/Akt/mTOR-dependent mechanisms, suggesting a compensatory role in restoring disrupted retinal homeostasis.

The Dual Effect of Insulin-Induced Autophagy Inhibition in the DR

One potential benefit of insulin therapy is the suppression of pathological autophagy overactivation. In DR, sustained hyperactivation of autophagy may result in the depletion of cellular resources and impaired function of photoreceptors and other retinal cells (Fu et al., 2016). Insulin-mediated normalization of Beclin-1 expression could mitigate this pathological overload and support cell survival.

Support for Retinal Neuron Survival

Excessive autophagy can progress to autophagy-dependent cell death. Studies have demonstrated that fine-tuned modulation of autophagy protects retinal neurons from degeneration (Rodriguez-Muela et al., 2012). Accordingly, insulin-induced suppression of Beclin-1 expression may help prevent overactivation of autophagy and protect retinal neurons from apoptosis.

Potential Adverse Effects of Insulin-Induced Autophagy Inhibition

However, a possible drawback of insulin therapy is the restriction of autophagy-dependent clearance of damaged organelles and protein aggregates. Autophagy serves as a protective mechanism during light-induced retinal degeneration, and its inhibition may compromise this defense, leading to accumulation of toxic components—especially under conditions of oxidative stress in DR (Chen et al., 2013).

Disruption of Cellular Homeostasis

Research shows that calpain-mediated cleavage of Beclin-1 following ischemic injury in the retina impairs autophagic regulation and increases cell death (Russo et al., 2011). These findings underscore the critical importance of maintaining optimal Beclin-1 levels for preserving retinal structure and function under diabetic conditions.

Impact on Retinal Cellular Homeostasis

The retina is a highly metabolically active tissue with significant energy demands. Disruption in autophagy homeostasis can have critical consequences for retinal function. In the context of diabetic retinopathy (DR), hyperglycemia leads to oxidative stress and inflammation, resulting in damage to cellular structures. Autophagy is a key protective mechanism that facilitates the clearance of such damaged components (Piano et al., 2016).

Insulin, by reducing Beclin-1 levels, may help restore autophagy to physiological levels and protect retinal cells from excessive self-digestion. However, this modulation may also limit the ability of cells to eliminate dysfunctional organelles and protein aggregates, particularly under chronic oxidative stress characteristic of DR.

Effect of Sorafenib on Beclin-1 Levels in Experimental DR

Our experimental data demonstrated that sorafenib monotherapy did not significantly alter the diabetes-induced elevation of Beclin-1 levels in retinal tissue ($p > 0.05$). According to previous studies, sorafenib can activate autophagy without affecting total Beclin-1 ex-

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pression (Tai et al., 2013). The proposed mechanism involves dissociation of the Beclin-1 complex from the anti-apoptotic protein Mcl-1 due to inhibition of the STAT3 signaling pathway. Under physiological conditions, Mcl-1 binds and sequesters Beclin-1, preventing its participation in autophagosome initiation. By inhibiting STAT3, sorafenib reduces Mcl-1 expression, which facilitates the release and activation of Beclin-1, even without increasing its total protein levels (Tai et al., 2013).

This mechanism may explain our finding that sorafenib treatment did not lead to changes in Beclin-1 content in experimental DR. It is likely that sorafenib enhanced Beclin-1 activity indirectly by modulating its interaction with Mcl-1, rather than by altering its expression level.

Effect of Sorafenib on the mTOR Signaling Pathway

An additional important mechanism by which sorafenib induces autophagy is via inhibition of the mTOR signaling pathway. Shimizu et al. (2012) demonstrated that sorafenib promotes autophagy in hepatocellular carcinoma cells by suppressing mTOR signaling (Shimizu et al., 2012). Notably, this induction was not associated with significant changes in Beclin-1 expression, but rather with increased LC3-I to LC3-II conversion—a hallmark of autophagosome membrane formation.

Since mTOR is a well-established negative regulator of autophagy, its inhibition triggers autophagic flux independent of Beclin-1 protein levels. Normally, mTOR phosphorylates and inhibits the ULK1/Atg13/FIP200 complex, thereby blocking autophagy initiation. Sorafenib-mediated inhibition of mTOR results in dephosphorylation and activation of ULK1, which initiates autophagosome biogenesis (Zhu et al., 2011).

Anti-Angiogenic Effects of Sorafenib in DR

Pathological angiogenesis is a central feature of DR progression, particularly in the proliferative stage. Sorafenib, as a multikinase inhibitor that targets vascular endothelial growth factor receptors (VEGFRs), has demonstrated efficacy in suppressing retinal neovascularization and vascular permeability independently of its autophagy-modulating effects.

In a murine model of oxygen-induced retinopathy, sorafenib significantly reduced VEGF expression and inhibited neovascularization (Yang et al., 2018). This was accompanied by improvement in blood-retinal barrier integrity and a reduction in retinal edema. Importantly, these anti-angiogenic effects occurred without concomitant changes in Beclin-1 or other autophagy markers, suggesting that the vascular and autophagic mechanisms of sorafenib are distinct and independently regulated.

Anti-inflammatory and Antioxidant Effects

Chronic inflammation and oxidative stress are key pathogenic factors in the development of DR (Li et al., 2009). Sorafenib has demonstrated anti-inflammatory and antioxidant properties through the inhibition of various kinases and signaling pathways. For instance, it reduces the levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and chemokines (MCP-1) in the retina of diabetic rats by suppressing NF-κB activation (Li et al., 2009). This effect is accompanied by reduced activation of microglia and macrophages, key mediators of retinal inflammation in diabetes.

Our experimental findings showed that sorafenib does not alter Beclin-1 levels in the retina in experimental DR. However, literature analysis indicates that sorafenib may activate autophagy through mechanisms independent of Beclin-1 expression. These include: disruption of the Beclin-1/Mcl-1 complex via STAT3 inhibi-

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tion, allowing Beclin-1 to participate in autophagy initiation; inhibition of the mTOR signaling pathway, leading to ULK1 activation and autophagy induction; and modulation of other autophagy-related proteins such as LC3, p62, ATG5, and ATG12 (Tai et al., 2013; Shimizu et al., 2012).

Furthermore, sorafenib may exert therapeutic effects in DR through autophagy-independent mechanisms, particularly its anti-angiogenic activity via inhibition of VEGFR and PDGFR, as well as its anti-inflammatory and antioxidant effects (Adornetto et al., 2021).

Taken together, these findings highlight the pleiotropic effects of sorafenib, which may offer therapeutic benefits in the integrated management of DR. The lack of effect on Beclin-1 levels does not rule out modulation of autophagy via other mechanisms, nor does it diminish the therapeutic potential of sorafenib through alternative signaling pathways.

Synergistic Effect of Insulin and Sorafenib on Beclin-1 Levels in Experimental DR

Our results revealed a marked increase in Beclin-1 levels in the retina during experimental DR, indicating activation of autophagic processes. Insulin monotherapy restored Beclin-1 to physiological levels, while the combined administration of insulin and sorafenib led to a further reduction in Beclin-1 expression—falling even below that of intact animals (by 1.5-fold; $p<0.05$). Notably, sorafenib alone did not produce a similar effect.

Comparable results were observed after three months of follow-up: combined treatment with insulin and sorafenib significantly reduced Beclin-1 levels in comparison to the control group (by 1.5-fold; $p<0.05$).

Molecular Mechanisms of the Synergistic Effect

The mechanisms underlying the synergistic effect of insulin and sorafenib are complex and require thorough molecular elucidation. Autophagy is regulated by several intracellular signaling pathways, with the PI3K/Akt/mTOR axis playing a central role. Insulin activates this pathway, resulting in suppression of autophagy (Fu et al., 2016). Specifically, activated mTOR phosphorylates and inactivates the ULK1/2–ATG13–FIP200 complex, which is essential for autophagy initiation. It also phosphorylates AMBRA1, leading to dissociation of the Beclin-1–AMBRA1–VPS34 complex and subsequent inhibition of autophagosome formation.

Sorafenib, a multi-target protein kinase inhibitor, exerts more complex and context-dependent effects. Previous studies have demonstrated that sorafenib can exhibit a biphasic influence on autophagy, depending on tissue type, dosage, and exposure duration (Zhang et al., 2019). It inhibits RAF kinase and consequently downregulates the RAF/MEK/ERK pathway, potentially suppressing Beclin-1 expression. Concurrently, sorafenib may induce autophagy under certain conditions through inhibition of the mTOR pathway, either by activating AMPK or suppressing PI3K/Akt signaling.

The seemingly paradoxical finding in our study, that sorafenib, in combination with insulin, enhances autophagy inhibition, can be explained by several mechanisms.

Firstly, interaction at the level of the PI3K/Akt/mTOR cascade: although sorafenib has the capacity to inhibit mTOR, the concurrent presence of insulin may redirect its signaling influence toward alternative pathways such as ERK. This may result in additional suppression of Beclin-1 expression via ERK-dependent transcriptional regulators (Chen et al., 2018).

Secondly, effects on Beclin-1 interactions with anti-apoptotic Bcl-2 family proteins: insulin-induced Akt activation promotes Bcl-2

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phosphorylation, which enhances its binding affinity to Beclin-1, thereby further suppressing autophagy. Meanwhile, sorafenib may downregulate Mcl-1, another Bcl-2 family member, thereby indirectly influencing Beclin-1 availability. In the context of insulin-activated Akt signaling, this could potentiate the sequestration of Beclin-1 by Bcl-2 proteins, leading to enhanced autophagy inhibition.

Clinical Significance of the Synergistic Effect

The observed reduction in Beclin-1 levels below those seen in intact controls following combined insulin and sorafenib treatment is of particular interest for its clinical implications.

Excessive autophagy activation in DR has been linked to pericyte loss, a key event in the breakdown of the blood-retinal barrier (Fu et al., 2016). Hyperglycemic conditions upregulate Beclin-1 expression and autophagic markers, resulting in autophagic cell death of pericytes. Inhibition of autophagy in this context improves cell survival and stabilizes vascular permeability.

Overactivated autophagy also exacerbates inflammatory responses and oxidative stress in the diabetic retina (Zhang et al., 2019). Moderate suppression of this process leads to decreased production of pro-inflammatory cytokines and reactive oxygen species (ROS), and attenuates activation of the NLRP3 inflammasome – one of the primary components of the inflammatory cascade in DR.

Furthermore, Beclin-1 contains a BH3 domain, enabling it to interact with Bcl-2 family proteins. Reduced Beclin-1 levels may increase the availability of anti-apoptotic proteins such as Bcl-2 and Bcl-xL, which can inhibit pro-apoptotic factors like Bax and Bak, thus contributing to neuronal survival in the retina.

However, profound suppression of Beclin-1 and autophagic activity could have adverse consequences. Basal autophagy is critical for maintaining mitochondrial quality control and homeostasis in retinal cells (Kaarniranta et al., 2013). Complete inhibition of this pathway can result in the accumulation of dysfunctional mitochondria, excessive ROS production, and activation of mitochondrial apoptosis.

Moreover, autophagy is essential for the clearance of protein aggregates and adaptation to cellular stressors such as hypoxia and oxidative stress. In the chronic progression of DR, impaired autophagic flux may aggravate tissue damage and worsen visual prognosis.

CONCLUSION:

1. In experimental diabetic retinopathy, the content of Beclin-1 protein in retinal tissue was significantly elevated throughout 3 months (by 1.8-2.0 times compared to the intact group; $p<0.05$), indicating persistent activation of autophagic processes in response to hyperglycemia. Insulin monotherapy contributed to the normalization of Beclin-1 levels by the 2nd month of observation, indicating its inhibitory effect on autophagy under DR conditions.
2. The use of sorafenib as monotherapy did not affect Beclin-1 levels; however, its combination with insulin reduced the protein content below the indices of the intact group (by 1.5 times; $p<0.05$), suggesting a potential synergistic effect in inhibiting autophagy.
3. The obtained data demonstrate the promising nature of combined use of insulin with cellular protein kinase inhibitors (specifically, sorafenib) for modulation of autophagy and prevention of structural and functional retinal damage in diabetic retinopathy.

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ISSN 3068-3580
https://www.us-journal.us

SOURCE OF FUNDING

The work was performed at the Department of Ophthalmology and Optometry of Postgraduate Education at Bogomolets National Medical University, Ministry of Health of Ukraine, and is a fragment of the department's research project "Development of new methods for diagnosis, treatment, and prevention of refractive, inflammatory, dystrophic, and traumatic diseases of the visual organ and their clinical and experimental substantiation" (state registration number 0120U105324).

ACKNOWLEDGMENTS

The authors express their immense gratitude to the Head of the Department of Chemistry and Biochemistry of Enzymes at the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine (Kyiv), A.O. Tykhomyrov, for his support and conducting the immunoblotting studies.

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Volume 1 (1),
ISSN 3068-3580
https://www.us-journal.us