Effect of Pharmacological Intervention(s) in Skeletal Muscle of Diabetic rat

SUMMARY

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Anand Kumar

Enrollment No. 416/16

CO-SUPERVISOR

Dr. Sapana Kushwaha

Assistant Professor

NATIONAL INSTITUTE OF PHARMACEUTICAL EDUCATION AND RESEARCH (NIPER), RAEBARELI, LUCKNOW, UTTAR PRADESH -226002, INDIA

SUPERVISOR

Dr. Vikas Mishra

Assistant Professor

DEPARTMENT OF PHARMACEUTICAL SCIENCES
SCHOOL OF BIOMEDICAL & PHARMACEUTICAL SCIENCE
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
(A CENTRAL UNIVERSITY)
LUCKNOW, UTTAR PRADESH -226025, INDIA

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1. Introduction

Diabetes mellitus (DM) is a prevalent chronic condition that can be classified into two types based on insufficient insulin or insulin resistance According to the International Diabetes Federation (IDF), India is considered one of the epicenters of diabetes, with approximately 74 million individuals diagnosed with diabetes as of 2021, making it the second-highest number globally after Southeast Asia. As the disease progresses, the likelihood of developing diabetes-related complications increases. While research has primarily focused on complications affecting the cardiovascular, renal, retinal, and neuropathic systems, it is important to highlight that DM can also lead to skeletal muscle atrophy. Diabetic muscular atrophy, often underestimated as a diabetes complication, can result in muscle wasting, reduced grip strength, accelerated aging, and, in severe cases, quadriplegia. These consequences significantly impact the quality of life for individuals managing diabetes.

Research showed that individuals with type 1 diabetes mellitus (T1DM) experience a decline in the repair capacity of their skeletal muscle satellite cells, accompanied by impaired skeletal muscle function. These abnormalities can be attributed to insulin deficiency, disrupting the balance between protein degradation and synthesis. As a result, the rates of protein degradation exceed those of protein synthesis, contributing to skeletal muscle dysfunction. Furthermore, recent research has uncovered intriguing findings concerning the association between obesity, hyperlipidemia, sarcopenia, and low muscle mass in the context of type 2 diabetes mellitus (T2DM). While obesity and hyperlipidemia are generally recognized as risk factors for metabolic diseases and diabetic complications, they paradoxically seem to be linked to a lower risk of developing sarcopenia in T2DM patients. Skeletal muscle fibers are typically classified into fast-twitch and slow-twitch types based on their contractile, oxidative, and metabolic characteristics. The distribution of muscle fiber types can vary across different muscles. For instance, the extensor digitorum longus (EDL) and soleus muscles are predominantly composed of fast-twitch and slow-twitch fibers, respectively. However, in the gastrocnemius (GN) muscle, approximately half of both fiber types are co-expressed. Furthermore, studies clearly indicate that reduced oxidative enzyme activity is observed in the muscles of diabetic patients. The key signaling pathways elucidate the underlying cause of muscle atrophy and regulate muscle protein synthesis through a complex interaction involving myogenic regulatory factors (MRFs), such as Myo D, Myf 5, myogenin, and MRF4. These factors specifically bind to the regulatory DNA elements of

muscle genes. Multiple lines of evidence support the notion that oxidative stress serves as a primary contributor to skeletal muscle impairment in diabetic rats. Also, impaired mitochondrial bioenergetics have been identified as a contributing factor to skeletal muscle dysfunction in young individuals with type 1 diabetes. Mechanisms such as oxidative stress, altered lipid metabolism, insulin resistance, and impaired vascularization have been investigated in relation to diabetes-associated skeletal muscle atrophy. The findings pertaining to the atrophy of glycolytic fibers demonstrate a decreased number of type IIB/X fibers and a reduction in the area of type IIA and IIB/X fibers in the Ins2Akita+/- model of type 1 diabetes. Taken together, these observations suggest that it is crucial to understand the differential impact of muscle fiber types in relation to the development of muscle atrophy.

Physical exercise, protein intake, dietary intervention, acupuncture, electro-stimulation hormonal therapy, and a few repurposed pharmacological interventions are recognized as effective strategies for promoting muscle protein synthesis and managing skeletal muscle atrophy in diabetic myopathy.

Salbutamol, also known as albuterol, is a synthetic medication that targets β2-adrenoceptors and resembles adrenaline. It is commonly used as a bronchodilator for treating chronic obstructive pulmonary disease and bronchial asthma. Previous preclinical and clinical studies have shown that diabetes is associated with skeletal muscle wasting, resulting in a decline in muscle mass, grip strength, and endurance. The β2 adrenergic receptor (β2AR)-mediated signaling pathway in skeletal muscle has been identified as a crucial factor in reducing the breakdown of myofibrillar proteins and facilitating adaptive responses to various physiological and pathological conditions. There is substantial evidence supporting the positive effects of β2 adrenergic receptor (β2AR) agonists in addressing skeletal muscle wasting and its associated complications. Literature reports have provided evidence that salbutamol, a β2AR agonist, can enhance voluntary muscle strength in humans. Additionally, salbutamol has been found to increase muscle weight and protein content in the hindlimb muscles of rats, including those in senescent rats. Recent research has further demonstrated that salbutamol has the ability to enhance protein turnover rates and stimulate muscle protein synthesis in humans. Building on these findings, the present study aims to explore the potential of salbutamol in addressing muscle loss caused by diabetes. The objective is to assess the effectiveness of salbutamol as a therapeutic intervention for mitigating or preventing muscle atrophy in a diabetic rat model. In

order to evaluate the characterization and effectiveness of salbutamol against skeletal muscle atrophy in diabetic rats, an *in-silico* and *in-vivo* study was conducted.

Based on the hypothesis, the study aims to achieve the following objectives:

2. Objectives

1. To investigate the interaction between the β2-adrenergic agonist salbutamol and proteins involved in skeletal muscle atrophy through an *in silico* approach.
2. To characterize the loss of muscle fiber types in both streptozotocin (STZ)-induced diabetic rats and high-fat diet-induced (HFD) diabetic rats in a time-dependent manner.
3. To investigate the effects of salbutamol on skeletal muscle wasting, oxidative stress, and inflammation in both streptozotocin (STZ)-induced diabetic rats and high-fat diet-induced (HFD) diabetic rats.
4. To elucidate the molecular mechanisms of salbutamol through a metabolomics approach in both streptozotocin (STZ)-induced diabetic rats and high-fat diet-induced (HFD) diabetic rats.

3. Experimental Design

For *in-silico* study, the ligand's three-dimensional (3D) structure (CID: 2083) was obtained from PubChem at https://pubchem.ncbi.nlm.nih.gov, while the structures of Akt-1, GDF-8, IGF-1, MuRF-1, MyoD, and TNF-α were obtained from the RCSB PDB online web source at https://www.rcsb.org. Autodock Vina 1.2.0 was utilized for docking simulations. Protein preparation included the addition of non-polar hydrogen atoms, assignment of total Kollman and Gasteiger charges, and removal of unattached atoms. The ligand received partial charges and allowed all torsions to rotate during docking. Grid boxes were created around the active sites of Akt-1, GDF-8, IGF-1, MuRF-1, MyoD, and TNF-α (PDB ID: 6HHG, 5JI1, 1B9G4, 2D8U, 7WZ6, and 3RT4) to cover the binding sites. AutoGrid was employed for grid calculations. One hundred Lamarckian genetic algorithm runs were performed with default parameters. Interactions between ligands (Akt-1, GDF-8, IGF-1, MuRF-1, MyoD, and TNF-α) and their complex conformations were analyzed, including hydrogen bonds and bond lengths. Visualization was carried out using PyMOL and Biovia Discovery Studio.

For *in vivo* study, the following experimental design was used to explore the potential effect of

salbutamol in ameliorating skeletal muscle loss in streptozotocin-induced type 1 and high-fat diet-induced type 2 diabetic rats.

A. Induction of type 1 diabetes

The male *Sprague Dawley* (SD) rats received a single intraperitoneal (*i.p.*) dose of freshly prepared streptozotocin (STZ) at a concentration of 55 mg/kg, dissolved in citrate buffer (pH 4.5). Prior to STZ administration, the rats underwent an overnight fasting period. After 48 hours, blood samples were obtained from the rat tail tip using a glucometer strip and a glucometer (Dr. Morepen GlucoOne, model-BG3) for measuring blood glucose levels. Blood glucose levels equal to or exceeding 250 mg/dL were considered indicative of diabetes. Animals with blood glucose levels ≥250 mg/dL were assigned to the STZ and STZ + Salbutamol groups. Additionally, blood glucose levels were measured at different weeks throughout the entire study (1, 2, 3, and 4 weeks).

B. Induction of type 2 diabetes

In brief, SD male rats were given *ad libitum* access to a high-fat diet (HFD) consisting of 58% fat, 25% protein, and 17% carbohydrate as a percentage of total kcal for a period of two weeks. After the initial two weeks of dietary treatment, a single intraperitoneal injection of a low dose of STZ (35 mg/kg) was administered, and the animals were then continued on the HFD feeding for an additional two weeks. At the end of the four-week period, fasting plasma glucose, and lipid profile (triglyceride, and cholesterol) were measured to confirm the induction of type-2 diabetes. Animals with fasting plasma glucose levels of ≥250 mg/dl or higher were considered diabetic and were used in the present experiments. The STZ was prepared in a vehicle of citrate buffer with a pH of 4.5, while the respective control rats were given a vehicle of citrate buffer with a pH of 4.4 in a dose volume of 1 ml/kg, intraperitoneally. Also, in order to perform muscle fiber typing and characterization, the animals were divided into control and diabetic groups. The diabetic group consisted of rats in which diabetes was induced either through STZ administration or HFD feeding. The animals were sacrificed at specific time points, namely 1, 2, 3, and 4 weeks, for further analysis. Throughout the study, blood glucose levels were measured at different time points (1, 2, 3, and 4 weeks) to monitor the progression of diabetes. Animals with blood glucose levels equal to or exceeding 250 mg/dL were assigned to the STZ and STZ+ Salbutamol groups.

In both diabetic rat models, blood glucose levels were measured using a glucometer (Dr. Morepen GlucoOne, model-BG3) through the tail tip on days 0, 7, 14, 21, and 28. Blood samples were collected from the rats using the retro-orbital plexus procedure under mild anesthesia, and the serum was separated and stored at -20°C for further analysis.

Based on an extensive literature review, it was observed that salbutamol is commonly administered in microgram doses, primarily in studies focusing on asthma. Furthermore, two papers were identified that repurposed salbutamol for different research purposes, specifically the sepsis study and distribution in the central nervous system. These studies employed doses of 4mg/kg and 10mg/kg, respectively, for salbutamol administration in rats. Considering these findings, a dose within the range of these two studies was thoughtfully selected. Therefore, a dose of 6mg/kg was chosen for further investigation in the present study.

After completing both studies, at the end of the 4-week treatment, all groups from both diabetic models were euthanized. Prior to euthanasia, muscle grip strength and muscle coordination parameters were evaluated in the experimental animals. Subsequently, all rats were sacrificed, and serum and gastrocnemius (GN) muscles were collected for endpoint measurements.

4. Endpoints parameters

The effects of salbutamol on diabetic skeletal muscle were evaluated through the assessment of various parameters, including:

a) **Muscle strength and muscle coordination test:** The experimental animals underwent various tests, including forelimb grip strength, hang-wire, actophotometer, rotarod, and footprint tests, to evaluate their muscle strength and coordination.

b) **Inflammatory markers:** Levels of inflammatory markers, including tumor necrosis factor α, interleukin-1β, interleukin-2, and interleukin-6, were measured to investigate the inflammatory response in diabetic skeletal muscle following salbutamol administration.

c) **Muscle markers:** Creatine kinase, myostatin, and testosterone levels were examined to assess the impact of salbutamol on muscle function, growth, and metabolism in diabetic skeletal muscle.

d) **Lipidemic markers:** Parameters related to lipid metabolism, such as lipid profiles and lipid levels, were analyzed to understand the influence of salbutamol on lipid metabolism in diabetic skeletal muscle.

e) **Oxidative stress and antioxidant levels:** The levels of oxidative stress markers (malondialdehyde, protein carbonyl) and antioxidants (glutathione, catalase, and superoxide dismutase) were measured to determine the effect of salbutamol on oxidative stress and antioxidant defense mechanisms in diabetic skeletal muscle.

f) **Histology:** Histological examination was performed to observe structural changes and gastrocnemius (GN) muscle morphology of diabetic rat after salbutamol treatment.

g) **Muscle fiber typing using SDH/COX staining:** Succinate dehydrogenase (SDH) and cytochrome c oxidase (COX) staining techniques were used to classify muscle fiber types based on their oxidative capacity and mitochondrial content. This allowed for the evaluation of the impact of salbutamol on muscle fiber typing in diabetic skeletal muscle.

h) **1H NMR profiling:** Serum and tissue profiling using 1H Nuclear Magnetic Resonance (NMR) was conducted to analyze the metabolites in diabetic skeletal muscle and investigate how salbutamol administration affected the metabolic profile.

***Summary of findings: In-silico* study**

- In the present study, we used Ramachandran plot statistics analysis in PROCHECK to examine the stereochemical quality of six protein models on a residue-by-residue basis. Models demonstrating good stereochemical quality typically exhibit scores close to 100%. The Akt1, GDF-8, IGF-1, MuRF-1, MyoD, and TNF-α models demonstrated acceptable stereochemical quality, characterized by a low proportion of residues with phi/psi angles in the outlier zone. No poor connections or unfavorable main chain or side chain parameter scores were observed. However, the overall G-factor values for Akt1,

GDF-8, IGF-1, MuRF-1, MyoD, and TNF-α fell slightly outside the expected range, as they were below -0.5. In PROCHECK, G-factor values ranging from 0 to -0.5 are considered acceptable, while values below -0.5 are deemed satisfactory. Models with values close to zero indicate the highest quality.

- Furthermore, ERRAT was employed to assess the quality of the revised models. ERRAT, also known as the "overall quality factor" for non-bonded atomic interactions, provides higher scores for higher quality models. In this investigation, MuRF-1 exhibited the highest ERRAT score of 100%, indicating its superior resolution and quality compared to previous protein models. Importantly, none of the residues exceeded the 99% error value limit, further confirming the high quality of MuRF-1.
- Further, PROSA was used to examine three-dimensional protein models for potential flaws. The Z-score serves as an indicator of the overall quality of the models and measures the deviation of the structure's total energy from an energy distribution derived from random conformations. PROSA-web analysis demonstrated that the residue energy of the Akt1, GDF-8, IGF-1, MuRF-1, and TNF-α models was predominantly negative, with some peaks observed in the middle region.
- In this study, we employed molecular docking simulations to predict protein-ligand interactions for anti-atrophy purposes. By combining protein structure predictions with docking, we were able to predict protein-ligand interactions. Our findings revealed that *in silico* approach can successfully predict interactions between essential protein targets, and known β2-adrenergic agonists, salbutamol.

Summary of findings: Characterization of skeletal muscle fiber typing in diabetic rats

- In this study, we focused on the classification of muscle fiber types based on their metabolic characteristics, specifically as fast-twitch (glycolytic) or slow-twitch (oxidative) fibers. Under certain conditions, there can be a shift from slow-twitch to fast-twitch fibers, indicating a change in muscle metabolism. To investigate this, we employed staining techniques using aerobic metabolic enzymes, succinate dehydrogenase (SDH), and cytochrome c oxidase (COX).

- Our findings revealed that in type 1 and type 2 diabetic rats, there was a time-dependent shift in muscle fiber types from fast-twitch to slow-twitch fibers. This change in fiber types occurred over a period of four weeks, with the rats being treated with streptozotocin (STZ) and a high-fat diet (HFD) for one, two, three, and four weeks.
- In this study, we focused on the classification of muscle fiber types based on their metabolic characteristics, specifically as fast-twitch (glycolytic) or slow-twitch (oxidative) fibers. Under certain conditions, there can be a shift from slow-twitch to fast-twitch fibers, indicating a change in muscle metabolism.
- The results indicated that it took a duration of four weeks under diabetic conditions to observe significant changes in muscle fiber types. This time frame was sufficient to induce differential fiber type composition in the rats.
- Overall, our study demonstrates the dynamic nature of muscle fiber types and how they can be influenced by diabetic conditions. The staining techniques using SDH and COX provided valuable insights into the time-dependent shift in fiber types, contributing to our understanding of muscle metabolism in diabetes.

Summary of findings: Effects of salbutamol on muscle biomarkers, oxidative stress, and inflammation in diabetic rats

- The present findings demonstrate that salbutamol significantly increased muscle weight and grip strength in diabetic rats, addressing the negative impact of diabetes on muscular function, strength, and muscle mass. Moreover, diabetes was associated with reduced muscular strength, muscle mass, and decreased lean mass in type 2 diabetic rats, leading to impaired muscle mass and changes in muscle fiber type.

- Salbutamol treatment restored muscle biomarkers, such as testosterone, creatine kinase, and mystatin, in both diabetic rat models. Testosterone, known for its role in muscle growth through androgen receptor stimulation and synthesis of sex steroidogenic enzymes in skeletal muscles, was affected by disrupted androgen receptor homeostasis caused by hyperglycemia in diabetic rats. Lower testosterone levels may contribute to the indirect reduction in diabetic muscle weight. Serum creatine kinase (CK) plays a crucial

role in preserving the phosphocreatine cellular pool and preventing ATP depletion in muscle cells, making it a potential biomarker for muscle atrophy. Additionally, myostatin negatively regulates skeletal muscle growth and atrophy, suggesting a negative correlation between myostatin levels and the diabetic environment.

- The intervention with salbutamol increases the antioxidant status of muscles and decreases the levels of oxidative stress markers in diabetic rats. These changes indicate the crucial role of antioxidants in preserving the integrity of skeletal muscle fibers and suggest that salbutamol may have the ability to remove reactive oxygen species (ROS).
- The findings showed that proinflammatory markers, such as TNF-α, IL-2, IL-6, and IL-1β, which are myokines produced in muscle tissue, were significantly elevated in the diabetic group. However, the results demonstrated that salbutamol effectively reduced the levels of these pro-inflammatory markers in diabetic rats. From these findings, it can be inferred that increased levels of TNF-α can lead to muscle catabolism by promoting ROS generation and activating the ubiquitin/proteasome pathway, ultimately resulting in protein degradation. Additionally, the activation of inflammatory signals under diabetic conditions may contribute to the degradation of myofibrillar proteins. However, it is important to note that the present study did not assess the protein expressions of inflammatory signaling pathways related to muscle atrophy, which is a limitation of the study.
- The histopathological study revealed cellular alterations in the gastrocnemius muscles of diabetic rats. The cross-sectional area (CSA) of muscle fiber cells and Feret's diameter were significantly lower in the diabetic rats compared to the control group. However, administration of salbutamol improved the CSA of muscle fiber cells and Feret's diameter, indicating a significant increase in the CSA and Feret's diameter of muscle fibers. This finding suggests that salbutamol has the potential to improve muscle cellular atrophy in hyperglycemic conditions.

Summary of findings: Effect of salbutamol on molecular mechanisms in diabetic rats using metabolomics approach

- Metabolomics analysis revealed significant metabolic changes in diabetic rats and the effect of salbutamol treatment on these metabolic perturbations.
- In diabetic rats, significant increases were observed in serum and muscle metabolites, including amino acids (histidine, glycine, tyrosine), energy metabolites (lactate, acetate, creatine, choline, succinate), and ketone bodies (betaine, 3-hydroxybutyrate isobutyrate). The phenylalanine-to-tyrosine ratio and glutamine-to-glucose ratio were altered, indicating changes in amino acid metabolism. Elevated succinate levels, an intermediate of the tricarboxylic acid cycle, were associated with inflammation and damage in diabetic skeletal muscle cells. The diabetic group also exhibited a lower pyruvate level, suggesting a shift in the tricarboxylic acid cycle. Also, salbutamol treatment demonstrated positive effects on sarcosine levels, a metabolite associated with type 2 diabetes, as well as on succinate levels, which play a role in skeletal muscle function.
- Amino acids play a vital role in maintaining physiological homeostasis and meeting the energy demands of muscles. Salbutamol treatment increased the levels of gluconeogenic amino acids (glutamine and glutamate) in diabetic rats. Phenylalanine and histidine, which are aromatic amino acids, were significantly elevated in the serum of diabetic rats compared to the control group. This suggests that these amino acids may replenish depleted intermediate levels of the tricarboxylic acid cycle during proteolysis. Oxidative stress can contribute to the deterioration of skeletal muscle and disrupt glutathione (GSH) levels, potentially leading to decreased levels of glutamate and glutamine in diabetic conditions. The present study observed considerably lower levels of glutamate and glutamine in diabetic rats, highlighting their significance as alternative energy sources to glucose in muscle cells.
- Additionally, the higher phenylalanine-to-tyrosine ratio (PTR) in diabetic rat serum indicates an oxidative deficit and catabolic state due to malfunctioning hydroxylase enzymes. The PTR ratio reflects the body's ability to convert phenylalanine to tyrosine, which requires cofactors such as tetrahydrobiopterin (BH4), niacin (B3), and iron. An increased PTR ratio in serum may indicate the presence of inflammatory disease and a person's catabolic stage. However, when diabetic rats were treated with salbutamol, the observed metabolic alterations returned to normal, suggesting that salbutamol possesses

antioxidant and anti-atrophy properties. These findings indicate that salbutamol can counteract oxidative stress and mitigate muscle atrophy in diabetic conditions.

- Oxidative stress may lead to a deterioration of the skeletal muscle and alter the GSH levels, which may be responsible for the lower glutamate and glutamine levels in a diabetic state. Furthermore, a higher PTR ratio in diabetic rat serum indicates an oxidation deficit and catabolic condition owing to hydroxylase enzyme malfunction. The phenylalanine/tyrosine ratio assesses the capacity of the body to convert phenylalanine to tyrosine. The conversion enzyme requires cofactors, such as tetrahydrobiopterin (BH4), niacin (B3), and iron. An increase in the serum PTR ratio may support diagnosing the existence of inflammatory disease and a person's catabolic stage. On the other hand, when diabetic rats were treated with salbutamol, the above-mentioned metabolic alterations reverted to normal, suggesting that salbutamol had both antioxidant and anti-atrophy properties.

- Taken together, these findings highlight the significance of diabetic metabolites as potential early markers for skeletal muscle wasting in diabetes. Additionally, salbutamol was found to ameliorate the altered metabolites in both streptozotocin (STZ)-induced diabetic rats and the high-fat diet-induced diabetic rat model, thus preventing skeletal muscle wasting.

Conclusions

The present study findings suggest that salbutamol has potential as a therapeutic intervention for muscle loss in diabetes. Computational analysis revealed the molecular mechanisms by which salbutamol may counteract muscle atrophy by targeting key genes involved in muscle homeostasis and protein degradation. Salbutamol administration in diabetic rats resulted in improvements in body weight, muscle weight, and antioxidant levels, supporting its therapeutic potential. Salbutamol also enhanced lean muscle mass in type II diabetic rats and reduced serum pro-inflammatory markers in both type 1 and type 2 diabetic rats, indicating its anti-inflammatory properties. The treatment with salbutamol restored the cellular structure of the gastrocnemius muscle and normalized metabolic changes observed in diabetic rats, including

alterations in amino acids, energy metabolites, ketone bodies, and metabolic ratios. Salbutamol treatment also showed positive effects on sarcosine levels, an important metabolite associated with type 2 diabetes, and succinate levels, which are involved in skeletal muscle function. Overall, these findings highlight the potential of salbutamol as a repurposed drug for managing muscle wasting in diabetes, and further research is needed to fully explore its therapeutic benefits and mechanisms of action.