**UNIVERSIDADE FEDERAL DE UBERLÂNDIA**
**INSTITUTO DE GENÉTICA E BIOQUÍMICA**
**PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA**

# Análise do papel da metformina na via insulínica, não-insulínica e inflamatória

**Aluno:** Leonardo Gomes Peixoto

**Orientador:** Foued Salmen Espindola

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**Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica - Área Bioquímica**

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(Foued Salmen Espindola)

Dedicatória

Dedico a todas as pessoas que conseguiram compartilhar comigo além daquilo que tem, aquilo que é.

Despedidas e saudades

Encontros e desencontros

Começo e recomeço

Tristezas e alegrias

Sonhos e agora realidade

Tudo isso, misturado com um sincero sorriso demistifica a apessoa que sou!

E tenho que admitir: - Sou grato. Por cada palabra amiga, cada abraço inesperado, cada sonho partilhado e por cada realidade vivida.

Palavras estas vindas de abraços que se tornaram sonhos nesta realidade que sou.

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# Contents

## Apresentação

Os estudos apresentados nesta tese foram delineados com o intuito de (1) avaliar o papel da metformina sobre os marcadores inflamatórios séricos de indivíduos diabéticos; de (2) descrever o papel da metformina e da insulina na via de sinalização relacionada à diminuição da sensibilidade insulínica no músculo esquelético de ratos diabéticos e de (3) avaliar o papel da metformina e da insulina na via de sinalização inflamatória e apoptótica em músculo esquelético de ratos diabéticos.

A metformina é uma droga amplamente usada no tratamento do diabetes tipo 2. Esta droga é considerada um sensibilizador de insulina, uma vez que diminui os níveis glicêmicos sem aumentar a secreção de insulina. Além isso, há estudos que comprovam que a metformina diminui a produção hepática de glicose, além de aumentar a disponibilidade de glicose periférica e reduzir a absorção intestinal deste carboidrato.

O mecanismo de ação molecular da metformina ainda não é claro. Até o momento, evidências sugerem quatro vias: 1ª inibir o complexo 1 mitocondrial (NADH-coenzima Q oxidorredutase, também conhecida como NADH desidrogenase), 2ª ativar a proteína quinase dependente de AMP (AMPK), 3º inibir a adenosina 3',5'-monofosfato cíclico induzida por glucagon e 4º inibir a enzima mitocondrial glicerol-3-fostato desidrogenase (GPDH).

Dentre estas 4 vias de sinalização, a melhor estudada é a inibição do complexo 1 mitocondrial, que leva a um desbalanço na relação da adenosita tri-fosfato/ adenosina di-fosfato (ATP/ADP). Este desbalanço desencadeia uma complexa rede de reações de ativação e inibição das vias de sinalização inflamatória e apoptótica. Assim, no capitulo 1 são resumidos os estudos sobre a captação da metformina e seu mecanismo de ação, bem como seu mecanismo de ação no músculo esquelético, destacando a via de sinalização infamatória desencadeada por “toll like receptor” (TLRs).

Além disso, e devido a seu complexo mecanismo de ação, a metformina tem sido avaliada em diferentes patologias. Vários estudos demonstraram que a metformina além de atenuar a resitência insulínica e melhorar a homeostase da glicose, tem sido relacionada ao tratamento de  várias doenças, como o câncer e a sindrome do ovário policístico. Sabe-se que o diabetes associado ou não à outras patologias leva a um aumento do quadro de inflamação do paciente. Assim, o capítulo 2 é um manuscrito de meta-analise sobre o papel da metformina sobre os marcadores inflamatórios séricos e parâmetros metabólicos em pacientes diabéticos. Aqui, excluímos todos os manuscritos que relacionavam o diabetes a outras doenças que aumentariam os marcadores inflamatórios. Assim, neste estudo, mostramos que a inflamação causada pelo diabetes aumenta marcadores inflamatórios como a proteína C reativa (CRP), o fator de necrose tumoral (TNFα) e o hormônio adiponectina. Como esperado, a metformina contribui diminuindo os valores dos marcadores inflamatórios séricos regulando a glicemia e hemoglobina glicada (HbA1c).

Embora existam vários estudos mostrando as diferentes vias de sinalização intracelular desencadeadas pela metformina, ainda há poucas evidências sobre o papel desta droga sobre a via de sinalização inflamatória/apoptótica no músculo esquelético de ratos diabéticos induzidos por estreptozotocina. Por isso, neste estudo avaliamos a via de sinalização relacionada à resistência insulínica no músculo esquelético de ratos diabéticos tratados com metformina. Assim demonstramos no capítulo 3 que a metformina melhora a sensibilidade insulínica a partir da via de ativação da AMPK, Proteína Kinase dependente de Cálcio (CAMKKβ) e Miosina-V, além de diminuir a via inflamatória intracelular relacionada à resistência insulínica. No capítulo 4, avaliamos a via de sinalização inflamatória e apoptótica desencadeada pelo sinal TLRs em ratos hiperglicêmicos. A metformina, além de diminuir os marcadores inflamatórios desencadeados por TLRs, inibe a via de sinalização apoptótica.

Em resumo, demonstramos que a metformina desempenha um importante papel tanto nos controle dos marcadores inflamatórios séricos quanto nos intracelulares, melhorando a sensibilidade à insulina e aumentando a captação de glicose.

### *1.1 Metformin uptake and its action mechanism: systematic review*

Metformin is an oral antidiabetic drug of the biguanide class. According of American Diabetes Association 2015 [1] metformin is one of the first-line drug of choice for the treatment of type 2 diabetes. This drug decreases hyperglycemia primarily by suppressing hepatic gluconeogenesis [2], and increases the peripheral glucose uptake as well as reduces hepatic glucose output [3].

It is well documented that the leg muscles have different morphological characteristics. Muscle fibers can be classified by their contractile property and its color: slow fibers – oxidative, red fibers, or Type I, and fast twitch fibers – anaerobic, white fibers, or Type II. The gastrocnemius muscle contains approximately 50% of anaerobic fibers while the soleus muscle has 75% to 90% of oxidative fibers. In this study we evaluated the effect of metformin action on gastrocnemius and soleus muscles of diabetic rats.

Metformin is actively transported into cells by organic cation transporters (OCT) 1, 2, and 3 (encoded by *SLC22A1*, *SLC22A2* and, *SLC22A3*), which are tissue specifically expressed at significant levels in various organs such as liver, muscle, and kidney [4, 5]. Dujic et al., showed that reduced OCT1 transport leads to metformin intolerance [6]. The liver is a major site of drug action, as it is exposed to high levels of oral metformin and expresses the metformin transporter OCT1, facilitating uptake and allowing for drug accumulation in this tissue. However, another tissues has been identify as sites to metformin action [7].

Evidence suggest that mechanisms of metformin action are: inhibition of the mitochondrial respiratory chain (complex I), activation of AMP-activated protein kinase (AMPK), inhibition of glucagon-induced elevation of cyclic adenosine monophosphate (cAMP) [8] as well as inhibition of mitochondrial glycerophosphate dehydrogenase [9].

To date, the best-known role for metformin is on the inhibition of mitochondrial complex. Complex I inhibition by metformin interrupts mitochondrial respiration and decreases proton-driven synthesis of adenosine triphosphate (ATP) [10], causing cellular energetic stress and elevation of the AMP/ATP ratio [11]. However, Hawley

et al., showed that effects of metformin on mitochondrial respiration vary among cells [12] indicating that it has not yet been possible to confirm whether or not complex I is the only mitochondrial target of metformin. Thus, more work is required to understand the underlying reason(s) for these variations.

The imbalance of ATP/ADP ratio from metformin action leads to activation of 5' AMP-activated protein kinase (AMPK). However, there are evidences that metformin activates AMPK independent of imbalance of APT/ADP [13]. Other studies showed that metformin activates AMPK through inhibition of AMP deaminase [14] or by upstream kinases serine/threonine kinase 1 (LKB1) in liver [15]. Recent finding show that metformin promote the formation of the AMPK αβγ heterotrimeric complex, resulting in an increase of AMPKα phosphorylation by LKB1 [16]. Taken together, it is suggested that AMPK activation by metformin may occur by different pathway. In addition, it is know that this activation of the AMPK by metformin can inhibit gluconeogenesis and activates glycolysis in liver [17] and increase glucose consumption in muscle [18].

In condition as fasting or starvation the hormone glucagon is released and acts via cyclic AMP (cAMP) and cAMP-dependent protein kinase, promoting hepatic glucose production by inhibiting glycolysis and activating gluconeogenesis, both acutely and by long term effects on gene expression. Study shows that metformin rapidly reduces cAMP levels induced by glucagon in primary hepatocytes, and reduces phosphorylation of protein kinase A (PKA) substrates, including the 6-phosphofructo-2-kinase isoform (PFKFB1) [19]. Lowering of cAMP could inhibit the switch from glycolysis to gluconeogenesis triggered by glucagon [20]. These finding also suggest that metformin action is involved in inhibition of glucagon action by decrease of cAMP, and represents one of the AMPK-independent mechanisms by which biguanides acutely inhibit glucose production.

Finally, metformin treatment inhibits mitochondrial glycerophosphate dehydrogenase non-competitively and modulates cytosolic and mitochondrial redox state, inducing an effective reduction in endogenous glucose production [9]. These results identify mitochondrial glycerophosphate dehydrogenase as one of the primary molecular targets by which guanides/biguanides inhibit hepatic gluconeogenesis

Thus, metformin suppresses gluconeogenesis independently of AMPK, instead altering hepatic energy charge and inducing allosteric inhibition of glycolytic enzymes or adenylate cyclase and glucagon-activated gluconeogenic transcription.

### *1.2 Intracellular mechanism of metformin in muscle cells*

Insulin is a glucose regulating hormone. When blood glucose is high, insulin facilitates the uptake of glucose into cells via translocation of glucose transporter 4 (GLUT4) independently AMPK activation. Insulin binds to its receptor (IR) which phosphorylates the substrate (IRS1) following an activation cascade: phosphatidylinositol 4,5 biphosphate - inositol 1,4,5 triphosphate - serine threonine kinase (AKT) - TBC1 domin family members; Akt substrate 160 (AS160) triggering the translocation of GLUT4.

On the other hand, metformin activates GLUT4 translocation by alternative pathway to uptake glucose from AMPK activation [14-16]. Several evidence indicated that in peripheral tissue the activation of AMPK increase GLUT4 translocation [21]. These facts indicate the existence of a metformin–AMPK–GLUT4 axis.

Lee et al., show that metformin induces GLUT4 translocation via increase of Rab4 expression by AMPK pathway [22]; and that the activities of Akt substrate 160 (AS160) and PKC-zeta are involved in metformin-induced Rab4 regulation [22]. The Rab protein is a member of the Ras G proteins. Rab GTPases regulate many steps in membrane traffic, including vesicle formation, movement, and membrane fusion [23]. Furthermore, study showed that myosin Va and RAB8a were co-localized in exocytose of GLUT4 vesicle of muscle cells [24].

Another mechanism for action of metformin is related to the decreased of insulin resistance by the inactivation of pro-inflammatory mediators produced by diabetes [25-27]. Increase of tumor necrosis factor-α (TNFα) inhibits AMPK [28] which also may activates the insulin-mediated glucose uptake pathway [29]. Thus, metformin inhibits TNFα – induced IKKα/β phosphorylation [28]. Thereby, there is a decrease of the intracellular signal for dissociation of nuclear factor (NF-κB) from its inhibitor (IκB) [30] allowing the action of NF-κB. Inflammatory mediator stimulated by NF-κB leads to phosphorylation of c-Jun-N-terminal kinase (JNK) [29]. p-JNK, in

turn, induces serine phosphorylation in insulin receptor substrate (IRS1), which inhibits insulin signal transduction [31] and decreased GLUT4 translocation. In addition, the metformin increases the tyrosine kinase activity of the IR in liver [32]. See below the scheme of molecular mechanism of insulin and metformin action (Figure 1).

The scheme – (figure 1. studied in chapter 3) shows different signaling pathways of the metformin action: 1$^{th}$ inhibition of complex I mitochondrial, 2$^{th}$ activation AMPK, 3$^{th}$ maintenance of NF-κB/IκB complex and 4$^{th}$ inhibition of TNFα. Once AMPK activates, this mechanism can regulate the insulin and non-insulin pathway by phosphorylation both IR and PI3K (insulin pathway) as AS160 (non-insulin pathway). On the other hand, metformin may improve insulin sensitivity by decrease of NF-κB and increase of IκB concentration and consequently inhibition of TNFα. This inhibition decrease the p-JNK and the phosphorylation of IRS1*ser*. Thus, metformin enhance of IR activity and GLUT4 translocation.

### *1.2 Inflammatory pathway on skeletal muscle triggered by toll like receptor and the metformin role.*

Inflammation is pivotal in the development of insulin resistance in part via the activation of the toll-like receptors (TLRs), as TLR4 and TLR2 [33, 34]. TLRs are a class of proteins that play a key role in the innate immune system. TLR4 expression have been shown to be increased in conventional insulin resistance target tissues like skeletal muscle tissue of type 2 diabetic subjects [35]. TLR4 and TLR2 expression are increased in diabetes [36] and the activation of these receptors contributes to insulin resistance in muscle [35]. The signaling pathways triggered by TLRs involve the activation of the transcription factors NF-κB and JNK, which initiates the transcription of proinflammatory cytokine genes [37-39].

NF-κB plays a key role in regulating of genes involved in inflammatory pathways such as TNFα, adhesion molecules [40, 41] and chemokines CXCL1/KC [42]. Boyd et al show that TLR4 activates 20-fold NF-κB pathway than TLR2 in skeletal muscle [43]. NF-κB is inhibited by IκB under basal condition, and remain into cytoplasm. Upon proinflammatory pathway activation, as present in diabetes,

the kinase complex Ikk is activated and catalyzes the phosphorylation of IκB leading to its degradation, which liberates NF-κB to translocate into the cell nucleus and stimulate the transcription of inflammatory mediators. JNK is activated by both mitogen-activated protein kinases (MKK4 and MKK7), which phosphorylate Thr-Pro-Tyr residues induced by several stress stimuli and is inhibited by JNK interacting protein (JIP) [44].

Evidence indicates that both JNK and NF-κB also participate of pro-apoptotic and anti-apoptotic pathways. JNK activation stimulated apoptosis by bcl-2-like protein 4 (BAX) translocation to mitochondria [45] and the phosphorylation of B-cell lymphoma 2 (Bcl2), suppressing its function [46]. NF-κB in turn, stimulates anti-apoptotic signal activates Bcl-2 expression [47]. BAX and Bcl2 are members of Bcl-2 family of proteins, which can be divided into pro-apoptosis (BAX, Bid, Bad, Bak, Bic, Bok, Bcl-XS, and Hrk) or anti-apoptosis (Bcl2, Bcl-XL, Bcl-W, A1 and Mcl-1) proteins [48].

There are few studies about metformin role in inflammatory pathway triggered by TLRs. Two studies show that metformin treatment is able the decrease TLR4 mRNA [49, 50]. On the other hand, several studies show that metformin acts on inflammatory markers triggered by TLRs as describe above.

The scheme – (figure 2. studied in chapter 4) shows the molecular mechanism of metformin action on inflammatory markers triggered by TLRs in different signaling pathways: 1$^{th}$ maintenance of NF-κB/IκB complex and 2$^{th}$ inhibition of both TNFα and CXCL1/KC and consequently inhibition of p-JNK, 3$^{th}$ inhibition of BAX and activation of Bcl2. Taken together, our data point out that metformin may attenuate the activation of the inflammatory pathway TLRs/NF-κB/TNFα/CXCL1/KC and the apoptotic signaling BAX:Bcl2 ratio/p-JNK independent of p-AMPK, which could be accompanied by a reduction of the inflammatory damage caused by hyperglycemia in skeletal muscle of diabetic rats.

## References


1. Standards of medical care in diabetes-2015 abridged for primary care providers. Clin Diabetes. 2015;33(2):97-111. Epub 2015/04/22. doi: 10.2337/diaclin.33.2.97. PubMed PMID: 25897193; PubMed Central PMCID: PMCPmc4398006.
2. Kirpichnikov D, McFarlane SI, Sowers JR. Metformin: an update. Ann Intern Med. 2002;137(1):25-33. Epub 2002/07/03. PubMed PMID: 12093242.
3. Stumvoll M, Nurjhan N, Perriello G, Dailey G, Gerich JE. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. N Engl J Med. 1995;333(9):550-4. Epub 1995/08/31. doi: 10.1056/nejm199508313330903. PubMed PMID: 7623903.
4. Nies AT, Koepsell H, Winter S, Burk O, Klein K, Kerb R, et al. Expression of organic cation transporters OCT1 (SLC22A1) and OCT3 (SLC22A3) is affected by genetic factors and cholestasis in human liver. Hepatology. 2009;50(4):1227-40. Epub 2009/07/11. doi: 10.1002/hep.23103. PubMed PMID: 19591196.
5. Nies AT, Hofmann U, Resch C, Schaeffeler E, Rius M, Schwab M. Proton pump inhibitors inhibit metformin uptake by organic cation transporters (OCTs). PLoS One. 2011;6(7):e22163. Epub 2011/07/23. doi: 10.1371/journal.pone.0022163. PubMed PMID: 21779389; PubMed Central PMCID: PMCPmc3136501.
6. Dujic T, Zhou K, Donnelly LA, Tavendale R, Palmer CN, Pearson ER. Association of Organic Cation Transporter 1 With Intolerance to Metformin in Type 2 Diabetes: A GoDARTS Study. Diabetes. 2015;64(5):1786-93. Epub 2014/12/17. doi: 10.2337/db14-1388. PubMed PMID: 25510240; PubMed Central PMCID: PMCPmc4452716.
7. Strack T. Metformin: a review. Drugs Today (Barc). 2008;44(4):303-14. Epub 2008/06/10. PubMed PMID: 18536788.
8. Rena G, Pearson ER, Sakamoto K. Molecular mechanism of action of metformin: old or new insights? Diabetologia. 2013;56(9):1898-906. Epub 2013/07/10. doi: 10.1007/s00125-013-2991-0. PubMed PMID: 23835523; PubMed Central PMCID: PMCPmc3737434.
9. Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. Nature. 2014;510(7506):542-6. Epub 2014/05/23. doi: 10.1038/nature13270. PubMed PMID: 24847880; PubMed Central PMCID: PMCPmc4074244.
10. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. Biochem J. 2000;348 Pt 3:607-14. Epub 2000/06/07. PubMed PMID: 10839993; PubMed Central PMCID: PMCPmc1221104.
11. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. J Biol Chem. 2000;275(1):223-8. Epub 2000/01/05. PubMed PMID: 10617608.
12. Hawley SA, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, et al. Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. Cell Metab. 2010;11(6):554-65. Epub 2010/06/04. doi: 10.1016/j.cmet.2010.04.001. PubMed PMID: 20519126; PubMed Central PMCID: PMCPmc2935965.
13. Hawley SA, Gadalla AE, Olsen GS, Hardie DG. The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. Diabetes. 2002;51(8):2420-5. Epub 2002/07/30. PubMed PMID: 12145153.
14. Ouyang J, Parakhia RA, Ochs RS. Metformin activates AMP kinase through inhibition of AMP deaminase. J Biol Chem. 286. United States2011. p. 1-11.
15. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science. 310. United States2005. p. 1642-6.
16. Meng S, Cao J, He Q, Xiong L, Chang E, Radovick S, et al. Metformin activates AMP-activated protein kinase by promoting formation of the alphabetagamma heterotrimeric complex.

J Biol Chem. 2015;290(6):3793-802. Epub 2014/12/30. doi: 10.1074/jbc.M114.604421. PubMed PMID: 25538235; PubMed Central PMCID: PMCPmc4319043.
17. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nat Cell Biol. 2011;13(9):1016-23. Epub 2011/09/06. doi: 10.1038/ncb2329. PubMed PMID: 21892142; PubMed Central PMCID: PMCPmc3249400.
18. Galuska D, Zierath J, Thorne A, Sonnenfeld T, Wallberg-Henriksson H. Metformin increases insulin-stimulated glucose transport in insulin-resistant human skeletal muscle. Diabete Metab. 1991;17(1 Pt 2):159-63. Epub 1991/05/01. PubMed PMID: 1936469.
19. Miller RA, Chu Q, Xie J, Foretz M, Viollet B, Birnbaum MJ. Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. Nature. 2013;494(7436):256-60. Epub 2013/01/08. doi: 10.1038/nature11808. PubMed PMID: 23292513; PubMed Central PMCID: PMCPmc3573218.
20. Hardie DG. Metformin-acting through cyclic AMP as well as AMP? Cell Metab. 2013;17(3):313-4. Epub 2013/03/12. doi: 10.1016/j.cmet.2013.02.011. PubMed PMID: 23473026.
21. Magnoni LJ, Vraskou Y, Palstra AP, Planas JV. AMP-activated protein kinase plays an important evolutionary conserved role in the regulation of glucose metabolism in fish skeletal muscle cells. PLoS One. 2012;7(2):e31219. Epub 2012/02/24. doi: 10.1371/journal.pone.0031219. PubMed PMID: 22359576; PubMed Central PMCID: PMCPmc3281052.
22. Lee JO, Lee SK, Jung JH, Kim JH, You GY, Kim SJ, et al. Metformin induces Rab4 through AMPK and modulates GLUT4 translocation in skeletal muscle cells. J Cell Physiol. 2011;226(4):974-81. Epub 2010/09/22. doi: 10.1002/jcp.22410. PubMed PMID: 20857458.
23. Stockli J, Fazakerley DJ, James DE. GLUT4 exocytosis. J Cell Sci. 2011;124(Pt 24):4147-59. Epub 2012/01/17. doi: 10.1242/jcs.097063. PubMed PMID: 22247191; PubMed Central PMCID: PMCPmc3258103.
24. Sun Y, Chiu TT, Foley KP, Bilan PJ, Klip A. Myosin Va mediates Rab8A-regulated GLUT4 vesicle exocytosis in insulin-stimulated muscle cells. Mol Biol Cell. 2014;25(7):1159-70. Epub 2014/01/31. doi: 10.1091/mbc.E13-08-0493. PubMed PMID: 24478457; PubMed Central PMCID: PMCPmc3967978.
25. Vozarova B, Stefan N, Lindsay RS, Saremi A, Pratley RE, Bogardus C, et al. High alanine aminotransferase is associated with decreased hepatic insulin sensitivity and predicts the development of type 2 diabetes. Diabetes. 2002;51(6):1889-95. Epub 2002/05/29. PubMed PMID: 12031978.
26. Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. Diabetes Care. 2004;27(3):813-23. PubMed PMID: 14988310.
27. Pickup JC. Diabetes: Insulin pump therapy for type 2 diabetes mellitus. Nat Rev Endocrinol. 2014. doi: 10.1038/nrendo.2014.142. PubMed PMID: 25112234.
28. Steinberg GR, Michell BJ, van Denderen BJ, Watt MJ, Carey AL, Fam BC, et al. Tumor necrosis factor alpha-induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. Cell Metab. 2006;4(6):465-74. Epub 2006/12/05. doi: 10.1016/j.cmet.2006.11.005. PubMed PMID: 17141630.
29. Plomgaard P, Bouzakri K, Krogh-Madsen R, Mittendorfer B, Zierath JR, Pedersen BK. Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. Diabetes. 2005;54(10):2939-45. Epub 2005/09/28. PubMed PMID: 16186396.
30. Hoffmann A, Levchenko A, Scott ML, Baltimore D. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science. 2002;298(5596):1241-5. Epub 2002/11/09. doi: 10.1126/science.1071914. PubMed PMID: 12424381.
31. Hancer NJ, Qiu W, Cherella C, Li Y, Copps KD, White MF. Insulin and metabolic stress stimulate multisite serine/threonine phosphorylation of insulin receptor substrate 1 and inhibit tyrosine phosphorylation. J Biol Chem. 2014;289(18):12467-84. Epub 2014/03/22. doi: 10.1074/jbc.M114.554162. PubMed PMID: 24652289; PubMed Central PMCID: PMCPmc4007441.

32. Gunton JE, Delhanty PJ, Takahashi S, Baxter RC. Metformin rapidly increases insulin receptor activation in human liver and signals preferentially through insulin-receptor substrate-2. J Clin Endocrinol Metab. 2003;88(3):1323-32. Epub 2003/03/12. doi: 10.1210/jc.2002-021394. PubMed PMID: 12629126.
33. Kim JJ, Sears DD. TLR4 and Insulin Resistance. Gastroenterol Res Pract. 2010;2010. Epub 2010/09/04. doi: 10.1155/2010/212563. PubMed PMID: 20814545; PubMed Central PMCID: PMCPMC2931384.
34. Galbo T, Perry RJ, Jurczak MJ, Camporez JP, Alves TC, Kahn M, et al. Saturated and unsaturated fat induce hepatic insulin resistance independently of TLR-4 signaling and ceramide synthesis in vivo. Proc Natl Acad Sci U S A. 110. United States2013. p. 12780-5.
35. Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, et al. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes. 57. United States2008. p. 2595-602.
36. Devaraj S, Dasu MR, Rockwood J, Winter W, Griffen SC, Jialal I. Increased toll-like receptor (TLR) 2 and TLR4 expression in monocytes from patients with type 1 diabetes: further evidence of a proinflammatory state. J Clin Endocrinol Metab. 2008;93(2):578-83. Epub 2007/11/22. doi: 10.1210/jc.2007-2185. PubMed PMID: 18029454; PubMed Central PMCID: PMCPmc2243229.
37. Takeuchi O, Akira S. Toll-like receptors; their physiological role and signal transduction system. Int Immunopharmacol. 1. Netherlands2001. p. 625-35.
38. Lin HY, Tang CH, Chen JH, Chuang JY, Huang SM, Tan TW, et al. Peptidoglycan induces interleukin-6 expression through the TLR2 receptor, JNK, c-Jun, and AP-1 pathways in microglia. J Cell Physiol. 2011;226(6):1573-82. Epub 2010/10/15. doi: 10.1002/jcp.22489. PubMed PMID: 20945380.
39. Wang H, Wu Y, Ojcius DM, Yang XF, Zhang C, Ding S, et al. Leptospiral hemolysins induce proinflammatory cytokines through Toll-like receptor 2-and 4-mediated JNK and NF-kappaB signaling pathways. PLoS One. 7. United States2012. p. e42266.
40. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med. 1997;336(15):1066-71. Epub 1997/04/10. doi: 10.1056/nejm199704103361506. PubMed PMID: 9091804.
41. Baker RG, Hayden MS, Ghosh S. NF-kappaB, inflammation, and metabolic disease. Cell Metab. 13. United States: A 2011 Elsevier Inc; 2011. p. 11-22.
42. Burke SJ, Lu D, Sparer TE, Masi T, Goff MR, Karlstad MD, et al. NF-kappaB and STAT1 control CXCL1 and CXCL2 gene transcription. Am J Physiol Endocrinol Metab. 2014;306(2):E131-49. Epub 2013/11/28. doi: 10.1152/ajpendo.00347.2013. PubMed PMID: 24280128; PubMed Central PMCID: PMCPmc3920007.
43. Boyd JH, Divangahi M, Yahiaoui L, Gvozdic D, Qureshi S, Petrof BJ. Toll-like receptors differentially regulate CC and CXC chemokines in skeletal muscle via NF-kappaB and calcineurin. Infect Immun. 2006;74(12):6829-38. Epub 2006/09/20. doi: 10.1128/iai.00286-06. PubMed PMID: 16982839; PubMed Central PMCID: PMCPmc1698076.
44. Vlahopoulos S, Zoumpourlis VC. JNK: a key modulator of intracellular signaling. Biochemistry (Mosc). 2004;69(8):844-54. Epub 2004/09/21. PubMed PMID: 15377263.
45. Tsuruta F, Sunayama J, Mori Y, Hattori S, Shimizu S, Tsujimoto Y, et al. JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. EMBO J. 23. England2004. p. 1889-99.
46. Liu J, Lin A. Role of JNK activation in apoptosis: a double-edged sword. Cell Res. 2005;15(1):36-42. Epub 2005/02/03. doi: 10.1038/sj.cr.7290262. PubMed PMID: 15686625.
47. Heckman CA, Mehew JW, Boxer LM. NF-kappaB activates Bcl-2 expression in t(14;18) lymphoma cells. Oncogene. 2002;21(24):3898-908. Epub 2002/05/29. doi: 10.1038/sj.onc.1205483. PubMed PMID: 12032828.
48. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 1999;13(15):1899-911. Epub 1999/08/13. PubMed PMID: 10444588.

49. Soraya H, Clanachan AS, Rameshrad M, Maleki-Dizaji N, Ghazi-Khansari M, Garjani A. Chronic treatment with metformin suppresses toll-like receptor 4 signaling and attenuates left ventricular dysfunction following myocardial infarction. Eur J Pharmacol. 2014;737:77-84. Epub 2014/05/21. doi: 10.1016/j.ejphar.2014.05.003. PubMed PMID: 24842192.
50. Andrews M, Soto N, Arredondo M. [Effect of metformin on the expression of tumor necrosis factor-alpha, Toll like receptors 2/4 and C reactive protein in obese type-2 diabetic patients]. Rev Med Chil. 2012;140(11):1377-82. Epub 2013/05/17. doi: 10.4067/s0034-98872012001100001. PubMed PMID: 23677182.

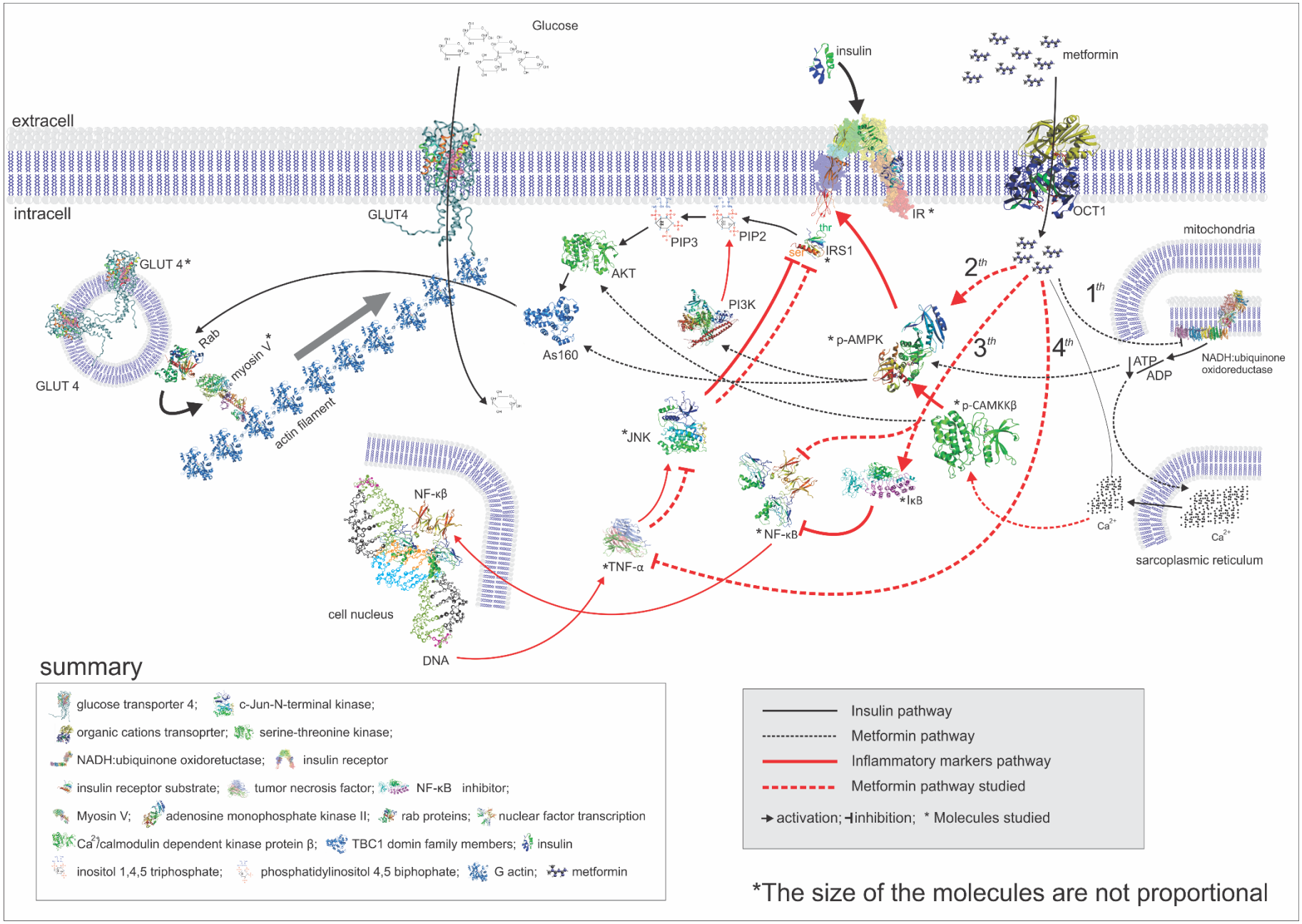


**Figure 1. Scheme of insulin and metformin role on skeletal muscle of diabetic rat**

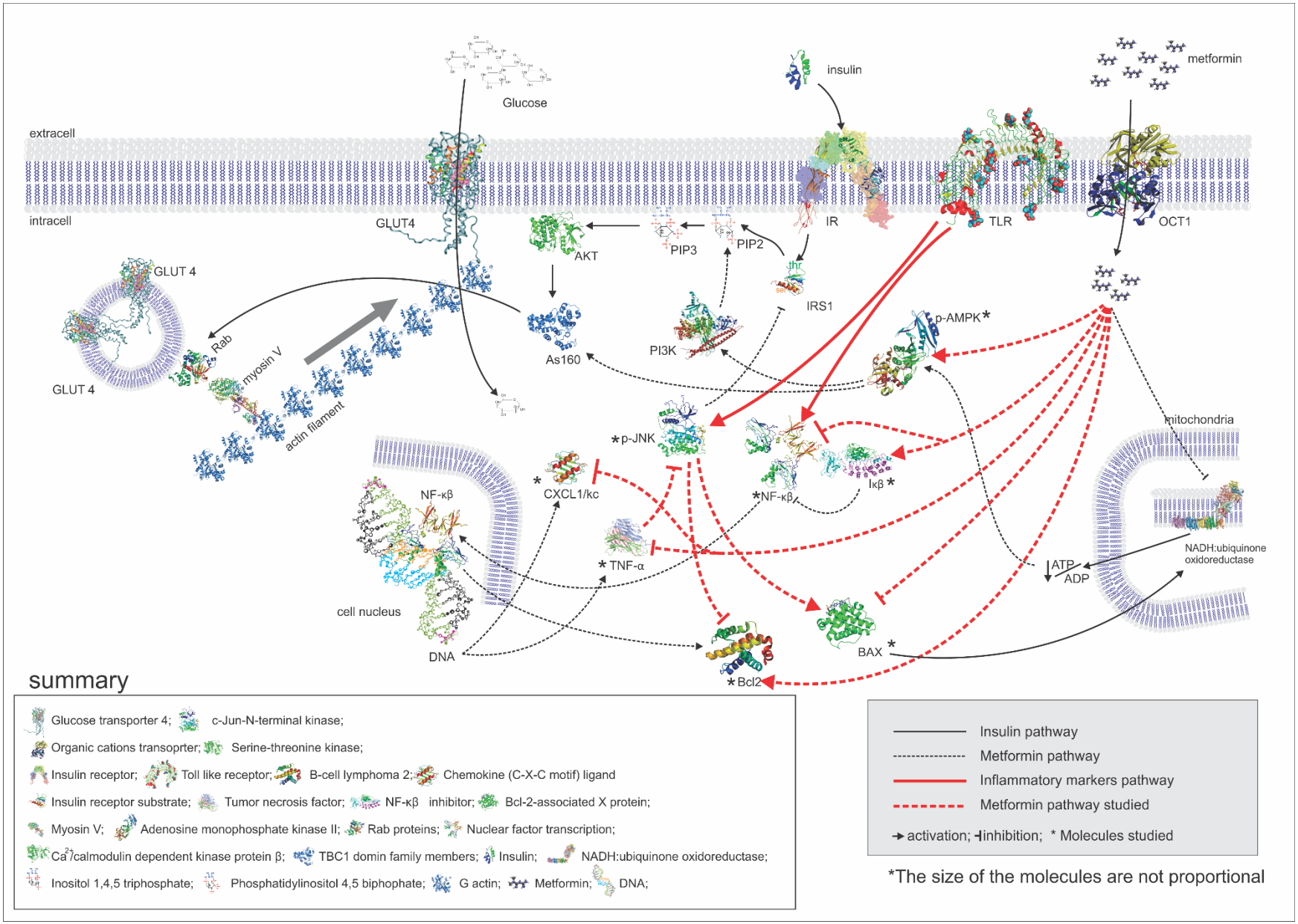


**Figure 2. Scheme of metformin role on inflammatory and apoptotic pathway triggered by TLRs in skeletal muscle of diabetic rats.**

**Chapter 2 – paper 1**

It will be submitted to the Journal of Inflammation

**Metformin on inflammatory markers: Meta-Analysis.**

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**Abstract**

**Purpose:** We performed a meta-analysis of randomized trials to assess the effect of metformin on inflammatory markers and metabolic parameters in subjects with diabetes.
**Methods:** We performed comprehensive searches on NCBI, Cochrane, Science Direct databases from 1966 to Jun of 2015. We included randomized trials of at least 4 weeks duration that compared groups with diabetes before and after the treatment with metformin or metformin plus other drugs, and evaluated body mass index, blood glucose, HbA1c and inflammatory parameters such as C-reactive protein, tumor necrosis factor and adiponectin.
**Results:** Pooled results of the 26 trials, with 1760 participants at the end of treatment reduce BMI in 0.9% p=0,0043, as well as, decrease of blood glucose level [SMD -0,411 mg/dL, 95%CI -0,463 to -0,369, $I^2$= 56.62%], HbA1c [SMD -0.479%, 95%CI -0,568 to -0,390, $I^2$= 55.02%], CRP levels [SMD -0,274mg/dL, 95%CI -0,419 to -0,129, $I^2$= 72.78%], TNFα concentration [SMD -0,103pg/ml, 95%CI -0,514 to 0,309, $I^2$= 87.67%] and increase of adiponectin [SMD 0,171µg/ml, 95%CI 0,098 to 0,440, $I^2$= 81.09%] compared with pretreatment.
**Conclusion:** The long-treatment with metformin monotherapy or metformin plus other drugs improves metabolic parameters and induced changes in inflammatory markers in diabetic subject.

**Key words:**
C-reactive proteins, tumor necrosis factor (TNFα), adiponectin, blood glucose level, glycated hemoglobin (HbA1c)

## Introduction

Metformin is one of the first-line drug of choice for the treatment of diabetes [1]. This drug controls hyperglycemia by suppressing hepatic gluconeogenesis [2], and improve the peripheral glucose uptake [3]. Evidence suggests that metformin has an important role on inflammatory markers linked to diabetes and obesity. Risk factors such as family history of diabetes, physical inactivity, obesity, insulin resistance, hypertension among others can lead to the development of diabetes [1]. Diabetes is a group of metabolic diseases characterized by the high blood sugar levels over a prolonged period. Diabetes leads to the increase of the inflammatory markers such as C-reactive protein (CRP), proinflammatory cytokines such as tumor necrosis factor-α (TNFα) and increasing insulin resistance [4].

Several studies have shown that the use of metformin decrease body mass index (BMI) attenuating obesity [5]. The increase of BMI is associated with the inflammation caused by diabetes [6]. Recent finding suggest that metformin decreases both TNFα and C-reactive protein [7]. Higher TNFα and CRP levels have been linked to the increase of insulin resistance in obese subjects [8, 9] and to the increase of the risk on later development of diabetes [10]. On the other hand, when the inflammatory signal increases there are an overexpression of the adiponectin by adipocytes. This hormone modulates a number of metabolic processes, including glucose regulation and fatty acid oxidation [11]. Adiponectin decreases insulin resistance and has a potent anti-inflammatory and anti-atherogenic effects [12]. Recently, an study showed that metformin is capable to up-regulate adiponectin gene expression [13]. These finding together probably indicate that metformin decreases the systemic inflammation.

This meta-analyses study on data of inflammatory markers and metabolic parameters from randomized trials with diabetics subjects treated with metformin or metformin plus other drugs was conducted to add new evidence to the effect of metformin on these parameters.

## Material and Methods

Selection of Studies and statistical analysis

We performed comprehensive searches on NCBI, Cochrane, Science Direct databases from 1966 to Jun of 2015 using the terms metformin, diabetes, inflammatory cytokines, glucose uptake, TNF and trial and scanned selected journals and references of identified articles. We included randomized trials of at least 4 weeks duration that compared groups with diabetes before and after the treatment with metformin or metformin plus other drugs, and evaluated body mass index, blood glucose, HbA1c and inflammatory parameters such as C-reactive protein, tumor necrosis factor and adiponectin. We excluded trials evaluating inflammation associated with other diseases, because the metabolic derangements may be different from other forms of inflammatory. Interventions included metformin alone or in combination with other drugs.

Inclusion and exclusion criteria are shown in Table I.

The included studies used different outcome measures to quantify CRP. We used the mean values to this analyses and the difference was verified with the test sensitivity. Some studies reported the p-value in the group before and after treatment and, in cases that this value was not available the p-value was calculated by the value of the mean, standard deviation and number of sample by the statistical method of multiple t-test in the GraphPad Prism 6.

We used the Comprehensive Meta-Analysis software, version 3 free trial (www.meta-analysis.com) for statistical calculations. We analyzed the *effect size* data *continuous* for one group – pre and post treatment with metformin or metformin and other drugs – by standardized mean difference (SMD) to *normalize the results*, the *size* and *expression interference effect* in each study regarding the observed variability.

The random effect was decided at $I^2$ statistic value which represent heterogeneity (by the Chi-squared test). A value of 0% to 40% may not be important, from 30% to 60% may represent moderate, 50% to 90% may represent substantial and 75% to 100% considerable heterogeneity. Results were displayed in tables and graphically by Forest plots.

## Results and Discussion

*Search results*

The search identified approximately 3,015 articles, of which 182 were potentially relevant (Figure 1). Of these, 82 were according to the inclusion criteria. Trials were excluded for the following reasons: 32 trials did not compare metformin before and after treatment, 11 trials conducted the study for less than 4 weeks duration, 13 trials evaluated another disease beyond diabetes, and 2 trials provided data of participants included in another trial.

*Trial characteristics and data synthesis*

The analysis included 26 trials, with a total of 2006 participants at the beginning of the treatment with a 13% dropout. The mean duration of the trials was 22 weeks (range, 4-52 weeks), with a mean study size of 95 participants (range 20-473). The mean dose of metformin was 1.2 g/day (range, 500-2250 mg/day). In some studies the treatment with metformin was combined with the following drugs: Arcabose, Rosiglitazone, Pioglitazone, Garlic, Glargine, Exenatide and Atovarstatin (Tables 2).

BMI is express as weight (kilograms) divided by height (meters) squared, this parameter is often used to classify overweight (BMI>25) and obesity (BMI>30) [5]. This meta-analysis showed that metformin treatment reduce BMI (0.9%) compared with pretreatment (table 2). In the studies evaluated was not reported physical exercise for the participants, indicating that this weight loss was associated with the metformin action. Study show that 1% reduction in weight is associated with a reduction in insulin resistance of 3% to 7% and a decrement in new cases of diabetes of 5% to 15% [37, 38]. Considering the 1760 participants, we observed a reduction of approximately 0.9% in BMI, representing a reduction in insulin resistance by approximately 4% and new cases of diabetes by approximately 7%. Thus, these finding suggest that metformin treatment can attenuate weight gain and decrease insulin resistance.

*Glycaemia and HbA1c levels*

We performed the meta-analysis to investigate the metformin role on serum inflammatory and metabolic markers levels in diabetic patients. It is well documented that metformin is an anti-glycemic drug. Corroborating with this

postulation, we show that metformin treatment decrease both blood glucose level [SMD -0,411 mg/dL, 95%CI -0,463 to -0,369, $I^2$= 56.62%] and HbA1c levels [SMD -0.479%, 95%CI -0,568 to -0,390, $I^2$= 55.02%]. Another study showed that metformin alone and combined with vildagliptin decrease glycaemia and HbA1c levels [14]. Metformin decreases hyperglycemia by suppressing hepatic glucose production [2] and as well as reducing hepatic glucose output [3]. In addition, metformin increases insulin sensitivity, enhances peripheral glucose uptake and decreases insulin-induced suppression of fatty acid oxidation [15]. In animal models metformin decreases the absorption of glucose from the gastrointestinal tract [16].

In addition, HbA1c is formed in a non-enzymatic/enzymatic glycation pathway by the hemoglobin's exposure to plasma glucose. It is a form of hemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods of time. Our finding is in accordance with other studies that showed a decrease of HbA1c in metformin monotherapy treatment [17, 18].

*Serum inflammatory markers*

Our findings show that metformin treatment with or without combination with other drug reduced CRP levels [SMD -0,274mg/dL, 95%CI -0,419 to -0,129, $I^2$= 72.78%] (Figure 3). CRP is generated by the liver during acute infection or inflammation, and its concentration in serum may increase as much as 1,000-fold under the condition of injury and infection [29]. Elevated CRP levels have been linked to an increase risk of later development of diabetes [10]. Metformin also reduces serum CRP levels in women with polycystic ovary syndrome [39]. The mechanism through which metformin decreases CRP is still unclear. Evidence indicates that the decrease in CRP is associated with the decrease of waist circumference [40] and serum levels of endothelin-1 [41]. In summary, the main results of our meta-analysis were in accordance to previous findings in which metformin may reduce serum CRP levels in diabetic patients, possibly indicating a decrease of the degree of low-grade inflammation.

TNFα is an inflammatory marker also find in serum of diabetic patients. Overproduction of TNFα by adipose tissue is involved in insulin resistance and obesity, which leads to inflammatory responses triggered by macrophages [8, 9].

Thus, TNFα promote unnecessary systemic inflammation and trigger insulin resistance [42]. In this study, we verified that the treatment with the combination of metformin and another drug decrease TNFα concentration compared to pretreatment [SMD -0,261 pg/ml, 95%CI -0,397 to -126, $I^2$= 87.67%] (Figure 4). Interestingly, our results show that metformin treatment without pioglitazone do not reduced the TNFα levels, this can be related to the dose and duration of the treatment. Kim et al. showed that 12 weeks of metformin treatment without rosiglitazone combination do not decrease CRP, TNFα and IL6 levels [28]. Instead, studies in animal models show that metformin modulates the expression of oxidative stress genes, restoring the mRNA of glutathione S-transferase-α (GSTα), NAD(P)H quinone oxidoreductase (NQO1), and catalase genes and inhibiting TNFα and IL-6 pro-inflammatory genes [43]. In addition, metformin is associated with the decrease of transcriptional factor – κB (NF-κB) [44], which decreases TNFα expression. Thus, it is necessary more studies to understand how metformin acts in the decreased of TNFα in diabetic patients.

On the other hand, adiponectin decreases insulin resistance and has a potent anti-inflammatory and anti-atherogenic effect [12]. Adiponectin is a protein hormone exclusively secreted from adipose tissue. This hormone modulates various metabolic processes, including glucose regulation [11]. Several clinical studies indicate that circulating total adiponectin strongly correlates with markers of insulin resistance. In animal models, adiponectin has an insulin-like function [45, 46]. Here, we show that metformin increase the adiponectin level compared to pretreatment [SMD 0,171µg/ml, 95%CI -0,098 to 0,440, $I^2$= 81.09%] (Figure 5). Arita et al. showed that the mean plasma adiponectin levels were 3.7 mg/ml in a group of obese patients, whereas in non-obese subjects these values reached a mean of 8.9 mg/ml [47]. Furthermore, adiponectin levels in patients with type 2 diabetes mellitus were lower than in non-diabetic patients, and were particularly low in subjects with coronary artery disease [48]. The increase of adiponectin may be associated with anti-inflammatory action and decrease of insulin resistance. However, the molecular mechanism to the increase of adiponectin by metformin is unclear. There is an evidence that point out that metformin is able to up-regulate adiponectin gene expression, both in vivo and in vitro, and to stimulate adiponectin protein secretion from human subcutaneous adipose tissue in vitro [13]. Therefore, the main results

of our study were in concordance to previous findings that metformin may increase serum adiponectin levels of diabetic patients.

In summary, our meta-analysis study provides evidence of metformin-induced changes in inflammatory markers and metabolic parameters in diabetic subjects. Taking into account that in the articles analyzed for this meta-analysis the subjects had no disease to alter inflammatory markers level, our findings indicate that metformin monotherapy for more than 4 weeks is able to reduce BMI and inflammatory markers like CRP and TNFα besides increasing adiponectin levels in diabetics. These indicate that this drug has an important clinical effect on inflammation, controlling glucose homeostasis and HbA1c level.

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**Author contributions**
Conceived and designed the experiments: L.G.P., L.N.B., F.S.E. Collection and tabulation of data: L.G.P., L.N.B., Analyzed the data: L.G.P., L.N.B., Wrote the paper: L.G.P., L.N.B., Supervised the entire project: F.S.E.

**Declaration of interest**
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## References


1. Standards of medical care in diabetes-2015 abridged for primary care providers. Clin Diabetes. 2015;33(2):97-111. Epub 2015/04/22. doi: 10.2337/diaclin.33.2.97. PubMed PMID: 25897193; PubMed Central PMCID: PMCPmc4398006.
2. Kirpichnikov D, McFarlane SI, Sowers JR. Metformin: an update. Ann Intern Med. 2002;137(1):25-33. Epub 2002/07/03. PubMed PMID: 12093242.
3. Stumvoll M, Nurjhan N, Perriello G, Dailey G, Gerich JE. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. N Engl J Med. 1995;333(9):550-4. Epub 1995/08/31. doi: 10.1056/nejm199508313330903. PubMed PMID: 7623903.
4. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest. 2005;115(5):1111-9. Epub 2005/05/03. doi: 10.1172/jci25102. PubMed PMID: 15864338; PubMed Central PMCID: PMCPMC1087185.
5. Hedley AA, Ogden CL, Johnson CL, Carroll MD, Curtin LR, Flegal KM. Prevalence of overweight and obesity among US children, adolescents, and adults, 1999-2002. Jama. 2004;291(23):2847-50. Epub 2004/06/17. doi: 10.1001/jama.291.23.2847. PubMed PMID: 15199035.
6. Jensterle M, Salamun V, Kocjan T, Vrtacnik Bokal E, Janez A. Short term monotherapy with GLP-1 receptor agonist liraglutide or PDE 4 inhibitor roflumilast is superior to metformin in weight loss in obese PCOS women: a pilot randomized study. J Ovarian Res. 2015;8(1):32. Epub 2015/06/03. doi: 10.1186/s13048-015-0161-3. PubMed PMID: 26032655.
7. Akbar DH. Effect of metformin and sulfonylurea on C-reactive protein level in well-controlled type 2 diabetics with metabolic syndrome. Endocrine. 2003;20(3):215-8. Epub 2003/05/02. doi: 10.1385/endo:20:3:215. PubMed PMID: 12721499.
8. McArdle MA, Finucane OM, Connaughton RM, McMorrow AM, Roche HM. Mechanisms of obesity-induced inflammation and insulin resistance: insights into the emerging role of nutritional strategies. Front Endocrinol (Lausanne). 2013;4:52. Epub 2013/05/16. doi: 10.3389/fendo.2013.00052. PubMed PMID: 23675368; PubMed Central PMCID: PMCPmc3650620.
9. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. Gastroenterology. 132. United States2007. p. 2169-80.
10. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. Jama. 2001;286(3):327-34. Epub 2001/07/24. PubMed PMID: 11466099.
11. Diez JJ, Iglesias P. The role of the novel adipocyte-derived hormone adiponectin in human disease. Eur J Endocrinol. 2003;148(3):293-300. Epub 2003/03/04. PubMed PMID: 12611609.
12. Goldstein BJ, Scalia R. Adiponectin: A novel adipokine linking adipocytes and vascular function. J Clin Endocrinol Metab. 2004;89(6):2563-8. Epub 2004/06/08. doi: 10.1210/jc.2004-0518. PubMed PMID: 15181024.
13. Zulian A, Cancello R, Girola A, Gilardini L, Alberti L, Croci M, et al. In vitro and in vivo effects of metformin on human adipose tissue adiponectin. Obes Facts. 2011;4(1):27-33. Epub 2011/03/05. doi: 10.1159/000324582. PubMed PMID: 21372608.
14. Bluher M, Kurz I, Dannenmaier S, Dworak M. Efficacy and safety of vildagliptin in clinical practice-results of the PROVIL-study. World J Diabetes.

2012;3(9):161-9. Epub 2012/11/06. doi: 10.4239/wjd.v3.i9.161. PubMed PMID: 23125906; PubMed Central PMCID: PMCPmc3487174.
15. Collier CA, Bruce CR, Smith AC, Lopaschuk G, Dyck DJ. Metformin counters the insulin-induced suppression of fatty acid oxidation and stimulation of triacylglycerol storage in rodent skeletal muscle. Am J Physiol Endocrinol Metab. 2006;291(1):E182-9. Epub 2006/02/16. doi: 10.1152/ajpendo.00272.2005. PubMed PMID: 16478780.
16. Ikeda T, Iwata K, Murakami H. Inhibitory effect of metformin on intestinal glucose absorption in the perfused rat intestine. Biochem Pharmacol. 2000;59(7):887-90. Epub 2000/03/16. PubMed PMID: 10718348.
17. Bredella MA, McManus S, Misra M. Impact of metformin monotherapy versus metformin with oestrogen-progesterone on lipids in adolescent girls with polycystic ovarian syndrome. Clin Endocrinol (Oxf). 2013;79(2):199-203. Epub 2012/08/30. doi: 10.1111/cen.12028. PubMed PMID: 22928702.
18. Laffel L, Chang N, Grey M, Hale D, Higgins L, Hirst K, et al. Metformin monotherapy in youth with recent onset type 2 diabetes: experience from the prerandomization run-in phase of the TODAY study. Pediatr Diabetes. 2012;13(5):369-75. Epub 2012/03/01. doi: 10.1111/j.1399-5448.2011.00846.x. PubMed PMID: 22369102; PubMed Central PMCID: PMCPmc3371323.
19. Hsieh CH, He CT, Lee CH, Wu LY, Hung YJ. Both slow-release and regular-form metformin improve glycemic control without altering plasma visfatin level in patients with type 2 diabetes mellitus. Metabolism. 2007;56(8):1087-92. Epub 2007/07/10. doi: 10.1016/j.metabol.2007.03.018. PubMed PMID: 17618954.
20. Bulcao C, Ribeiro-Filho FF, Sanudo A, Roberta Ferreira SG. Effects of simvastatin and metformin on inflammation and insulin resistance in individuals with mild metabolic syndrome. Am J Cardiovasc Drugs. 2007;7(3):219-24. Epub 2007/07/06. PubMed PMID: 17610348.
21. Chakraborty A, Chowdhury S, Bhattacharyya M. Effect of metformin on oxidative stress, nitrosative stress and inflammatory biomarkers in type 2 diabetes patients. Diabetes Res Clin Pract. 2011;93(1):56-62. Epub 2010/12/15. doi: 10.1016/j.diabres.2010.11.030. PubMed PMID: 21146883.
22. De Jager J, Kooy A, Lehert P, Bets D, Wulffele MG, Teerlink T, et al. Effects of short-term treatment with metformin on markers of endothelial function and inflammatory activity in type 2 diabetes mellitus: a randomized, placebo-controlled trial. J Intern Med. 2005;257(1):100-9. Epub 2004/12/21. doi: 10.1111/j.1365-2796.2004.01420.x. PubMed PMID: 15606381.
23. de Jager J, Kooy A, Schalkwijk C, van der Kolk J, Lehert P, Bets D, et al. Long-term effects of metformin on endothelial function in type 2 diabetes: a randomized controlled trial. J Intern Med. 2014;275(1):59-70. Epub 2013/08/29. doi: 10.1111/joim.12128. PubMed PMID: 23981104.
24. Derosa G, Franzetti IG, Querci F, Carbone A, Ciccarelli L, Piccinni MN, et al. Variation in inflammatory markers and glycemic parameters after 12 months of exenatide plus metformin treatment compared with metformin alone: a randomized placebo-controlled trial. Pharmacotherapy. 2013;33(8):817-26. Epub 2013/06/08. doi: 10.1002/phar.1301. PubMed PMID: 23744726.
25. Fidan E, Onder Ersoz H, Yilmaz M, Yilmaz H, Kocak M, Karahan C, et al. The effects of rosiglitazone and metformin on inflammation and endothelial dysfunction in patients with type 2 diabetes mellitus. Acta Diabetol.

2011;48(4):297-302. Epub 2011/03/23. doi: 10.1007/s00592-011-0276-y. PubMed PMID: 21424914.
26. Genovese S, De Berardis G, Nicolucci A, Mannucci E, Evangelista V, Totani L, et al. Effect of pioglitazone versus metformin on cardiovascular risk markers in type 2 diabetes. Adv Ther. 2013;30(2):190-202. Epub 2013/01/30. doi: 10.1007/s12325-013-0003-x. PubMed PMID: 23359066.
27. Glintborg D, Mumm H, Altinok ML, Richelsen B, Bruun JM, Andersen M. Adiponectin, interleukin-6, monocyte chemoattractant protein-1, and regional fat mass during 12-month randomized treatment with metformin and/or oral contraceptives in polycystic ovary syndrome. J Endocrinol Invest. 2014;37(8):757-64. Epub 2014/06/08. doi: 10.1007/s40618-014-0103-8. PubMed PMID: 24906976.
28. Kim HJ, Kang ES, Kim DJ, Kim SH, Ahn CW, Cha BS, et al. Effects of rosiglitazone and metformin on inflammatory markers and adipokines: decrease in interleukin-18 is an independent factor for the improvement of homeostasis model assessment-beta in type 2 diabetes mellitus. Clin Endocrinol (Oxf). 2007;66(2):282-9. Epub 2007/01/17. doi: 10.1111/j.1365-2265.2006.02723.x. PubMed PMID: 17224000.
29. Kumar R, Chhatwal S, Arora S, Sharma S, Singh J, Singh N, et al. Antihyperglycemic, antihyperlipidemic, anti-inflammatory and adenosine deaminase- lowering effects of garlic in patients with type 2 diabetes mellitus with obesity. Diabetes Metab Syndr Obes. 2013;6:49-56. Epub 2013/02/05. doi: 10.2147/dmso.s38888. PubMed PMID: 23378779; PubMed Central PMCID: PMCPmc3554227.
30. Mao XM, Liu H, Tao XJ, Yin GP, Li Q, Wang SK. Independent anti-inflammatory effect of insulin in newly diagnosed type 2 diabetes. Diabetes Metab Res Rev. 2009;25(5):435-41. Epub 2009/05/01. doi: 10.1002/dmrr.968. PubMed PMID: 19405039.
31. Pradhan AD, Everett BM, Cook NR, Rifai N, Ridker PM. Effects of initiating insulin and metformin on glycemic control and inflammatory biomarkers among patients with type 2 diabetes: the LANCET randomized trial. Jama. 2009;302(11):1186-94. Epub 2009/09/17. doi: 10.1001/jama.2009.1347. PubMed PMID: 19755697.
32. Stocker DJ, Taylor AJ, Langley RW, Jezior MR, Vigersky RA. A randomized trial of the effects of rosiglitazone and metformin on inflammation and subclinical atherosclerosis in patients with type 2 diabetes. Am Heart J. 2007;153(3):445.e1-6. Epub 2007/02/20. doi: 10.1016/j.ahj.2006.11.005. PubMed PMID: 17307426.
33. Tousoulis D, Koniari K, Antoniades C, Papageorgiou N, Miliou A, Noutsou M, et al. Combined effects of atorvastatin and metformin on glucose-induced variations of inflammatory process in patients with diabetes mellitus. Int J Cardiol. 2011;149(1):46-9. Epub 2009/12/26. doi: 10.1016/j.ijcard.2009.11.038. PubMed PMID: 20034685.
34. Derosa G, Mereu R, D'Angelo A, Salvadeo SA, Ferrari I, Fogari E, et al. Effect of pioglitazone and acarbose on endothelial inflammation biomarkers during oral glucose tolerance test in diabetic patients treated with sulphonylureas and metformin. J Clin Pharm Ther. 2010;35(5):565-79. Epub 2010/09/14. doi: 10.1111/j.1365-2710.2009.01132.x. PubMed PMID: 20831680.
35. Derosa G, Fogari E, Cicero AF, D'Angelo A, Ciccarelli L, Piccinni MN, et al. Blood pressure control and inflammatory markers in type 2 diabetic patients

treated with pioglitazone or rosiglitazone and metformin. Hypertens Res. 2007;30(5):387-94. Epub 2007/06/26. doi: 10.1291/hypres.30.387. PubMed PMID: 17587750.
36. Derosa G, Maffioli P, Salvadeo SA, Ferrari I, Ragonesi PD, Querci F, et al. Effects of sitagliptin or metformin added to pioglitazone monotherapy in poorly controlled type 2 diabetes mellitus patients. Metabolism. 2010;59(6):887-95. Epub 2009/12/18. doi: 10.1016/j.metabol.2009.10.007. PubMed PMID: 20015525.
37. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med. 2002;346(6):393-403. Epub 2002/02/08. doi: 10.1056/NEJMoa012512. PubMed PMID: 11832527; PubMed Central PMCID: PMCPmc1370926.
38. Markovic TP, Jenkins AB, Campbell LV, Furler SM, Kraegen EW, Chisholm DJ. The determinants of glycemic responses to diet restriction and weight loss in obesity and NIDDM. Diabetes Care. 1998;21(5):687-94. Epub 1998/05/20. PubMed PMID: 9589225.
39. Morin-Papunen L, Rautio K, Ruokonen A, Hedberg P, Puukka M, Tapanainen JS. Metformin reduces serum C-reactive protein levels in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2003;88(10):4649-54. Epub 2003/10/15. doi: 10.1210/jc.2002-021688. PubMed PMID: 14557435.
40. McLaughlin T, Abbasi F, Lamendola C, Liang L, Reaven G, Schaaf P, et al. Differentiation between obesity and insulin resistance in the association with C-reactive protein. Circulation. 2002;106(23):2908-12. Epub 2002/12/04. PubMed PMID: 12460870.
41. Diamanti-Kandarakis E, Spina G, Kouli C, Migdalis I. Increased endothelin-1 levels in women with polycystic ovary syndrome and the beneficial effect of metformin therapy. J Clin Endocrinol Metab. 2001;86(10):4666-73. Epub 2001/10/16. doi: 10.1210/jcem.86.10.7904. PubMed PMID: 11600523.
42. Patel PS, Buras ED, Balasubramanyam A. The role of the immune system in obesity and insulin resistance. J Obes. 2013;2013:616193. Epub 2013/04/12. doi: 10.1155/2013/616193. PubMed PMID: 23577240; PubMed Central PMCID: PMCPmc3618935.
43. Alhaider AA, Korashy HM, Sayed-Ahmed MM, Mobark M, Kfoury H, Mansour MA. Metformin attenuates streptozotocin-induced diabetic nephropathy in rats through modulation of oxidative stress genes expression. Chem Biol Interact. 2011;192(3):233-42. Epub 2011/04/05. doi: 10.1016/j.cbi.2011.03.014. PubMed PMID: 21457706.
44. Hattori Y, Suzuki K, Hattori S, Kasai K. Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells. Hypertension. 2006;47(6):1183-8. Epub 2006/04/26. doi: 10.1161/01.hyp.0000221429.94591.72. PubMed PMID: 16636195.
45. Ye R, Scherer PE. Adiponectin, driver or passenger on the road to insulin sensitivity? Mol Metab. 2013;2(3):133-41. Epub 2013/09/21. doi: 10.1016/j.molmet.2013.04.001. PubMed PMID: 24049728; PubMed Central PMCID: PMCPmc3773837.
46. Kadowaki T, Yamauchi T, Okada-Iwabu M, Iwabu M. Adiponectin and its receptors: implications for obesity-associated diseases and longevity. Lancet Diabetes Endocrinol. 2014;2(1):8-9. Epub 2014/03/14. doi: 10.1016/s2213-8587(13)70120-7. PubMed PMID: 24622657.

47. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, et al. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun. 1999;257(1):79-83. Epub 1999/03/27. PubMed PMID: 10092513.
48. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, et al. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler Thromb Vasc Biol. 2000;20(6):1595-9. Epub 2000/06/10. PubMed PMID: 10845877.

## Figures and legends

**Table 1:** Selection criteria for inclusion of studies in the meta-analysis

| Inclusion criteria | Exclusion criteria |
|---|---|
| *Study design:*<br>Retrospective or prospective study | *Study design:*<br>Review, meta-analysis, letters to the editor and animal studies |
| *Population:*<br>both gender of any age, ethnicity, BMI, with diabetes | *Population:*<br>Diabetes associated with others disease |
| *data assessment:*<br>pre and post metformin treatment | *data assessment:*<br>No pre and post outcomes |
| Exposure to monotherapy metformin or metformin and other oral anti-diabetic agents | Healthy control group<br><br>Incomplete information data |
| *Outcomes:*<br>Blood glucose/HbA1c<br>Inflammatory markers<br>CRP/hsCRP, TNF, Adiponectin,<br>Language:<br>English | Glucose in values normal in pretreatment < 130mg/dL |

**Table 1. Data synthesis and Meta-analysis**

## Table 2: Studies characteristic with dose and time of treatment show difference between pre and post BMI.

| | Treatment | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Study** | Pre | Post | **Subgroup** | **dose/day** | **Time** | **age** | | **pre % BMI** | | **post % BMI** | |
| Hsieh et al., 2007[19] | 24 | 24 | MetforminSR | SR Metformin 1000-2000mg 1/d | 14 weeks | 58 | ±1,4 | 25,30 | ±0,8 | 25,00 | ±0,8 |
| Hsieh et al., 2007 [19] | 23 | 23 | MetforminRR | RR Metformin 500-1000mg 2/d | 14 weeks | 56,6 | ±1,7 | 25,60 | ±0,7 | 25,50 | ±0,7 |
| Bulcao et al., 2007[20] | 21 | 21 | Metformin | Metformin 850mg 2/d | 16 weeks | 48,6 | ±9,1 | 36,10 | ±0,8 | 37,30 | 1,1 |
| Chakraborty et al., 2011[21] | 110 | 110 | Metformin | Metformin 850-2000mg/d 1 week and 2/d 2 weeks | 4 weeks | 42,5 | ±17,68 | 27,00 | ±2,4 | 23,00 | ±3,9 |
| de Jager et al., 2005[22] | 196 | 182 | Metformin | Metformin 850mg 3/d | 16 weeks | 63 | ±9 | 29,60 | ±13,9 | 29,50 | ±14,1 |
| de Jager et al., 2014[23] | 196 | 131 | Metformin | Metformin 850mg 1-3/d | 16 weeks | 59,1 | ±11 | 30 | ±5 | ND | ND |
| Derosa et al., 2013[24] | 85 | 82 | Metformin | Metformin | 52 weeks | 56,7 | ±7,3 | 31,7 | ±1,5 | 30,9 | ±1 |
| Dolasik et al., 2013 [24] | 60 | 60 | Metformin | Metformin | 25 weeks | 53 | ±9 | 27,8 | ±3,6 | ND | ND |
| Fidan et al., 2011[25] | 20 | 20 | Metformin | Metformin 850 mg 1-3/d | 12 weeks | 52,6 | ±7,2 | 30,1 | ±5,3 | 29,3 | ±5,1 |
| Genovese et al., 2013 [26] | 29 | 26 | Metformin | Metformin | 16 weeks | 56,4 | ±7,9 | 31,70 | ±3,6 | ND | ND |
| Glintborg et al., 2014[27] | 19 | 19 | Metformin | Metformin | ND | 31 | ±6,36 | 25,90 | ±3,9 | 24,90 | ±6,6 |
| Kim et al., 2007[28] | 56 | 56 | Metformin | Metformin 1000mg 1/d | 12 weeks | 57,6 | ±9,4 | 25,80 | ±3,3 | 25,63 | ±2,3 |
| Kumar et al., 2013[29] | 30 | 30 | Metformin | Metformin 500mg 2/3/d | 12 weeks | ND | ND | 27,23 | ±2,77 | 26,86 | ±12,69 |
| Mao et a., 2009[30] | 43 | 30 | Metformin | Metformin 1750mg/d | 8 weeks | 54,12 | ±8,86 | 27 | ±2,01 | 26,67 | ±2,02 |
| Pradhan et al., 2009[31] | 126 | 124 | Metformin | Metformin | ND | 53,8 | ±11,5 | 36,20 | ±8,1 | ND | ND |
| Stocker et al., 2007[32] | 47 | 38 | Metformin | Metformin 850mg 1/d for 2 weeks, 1700mg 1/d for more 2 weeks (4 weeks) | 4 weeks | 65 | ±10 | 29,72 | ±0,74 | ND | ND |
| Tousoulis et al., 2011[33] | 17 | 17 | Metformin | Metformin 850mg 1/d | 12 weeks | 53,88 | ±11,06 | ND | ND | ND | ND |
| Derosa et al., 2010[34] | 473 | 175 | Metf+Other2 | Metformin 2000±500mg + Acarbose | 12 weeks | 57 | ±6 | 26,86 | ±0,7 | 26,97 | ±0,8 |
| Derosa et al., 2010[34] | 473 | 175 | Metf+Other1 | Metformin 2000±500mg + Pioglitazone | 12 weeks | 56 | ±7 | 26,86 | ±0,7 | 26,18 | ±0,6 |
| Derosa et al., 2007[35] | 48 | 48 | Metf+Other1 | Metformin + Rosiglitazone 2.250±750mg/d | 52 weeks | 56 | ±4 | 28,10 | ±1,6 | 26,00 | ±1,2 |
| Derosa et al., 2007[35] | 48 | 48 | Metf+Other | Metformin + Pioglitazone 2.250±750mg/d | 52 weeks | 55 | ±5 | 28,20 | ±1,7 | 26,60 | ±1,1 |
| Derosa et al., 2010[36] | 76 | 68 | Metf+Other | Metformin 850mg + Pioglitazone 15mg 2/d | 52 weeks | 58 | ±6 | 27,7 | ±1,3 | 26,7 | ±0,7 |
| Derosa et al., 2013[24] | 85 | 81 | Metf+Other | Metformin + Exenatide | 52 weeks | 57,3 | ±7,7 | 31,9 | ±1,7 | 29,6 | ±0,5 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Kumar et al., 2013[29] | 30 | 30 | Metf+Other | Metformin + garlic 500mg 2/3/d | 12 weeks | ND | ND | 27,72 | ±1,74 | 25,97 | ±10,7 |
| Pradhan et al., 2009[31] | 126 | 124 | Metf+Other | Metformin + insulin + glargine | ND | 54 | ±11,7 | 35,60 | ±7,9 | ND | ND |
| Tousoulis et al., 2011[33] | 18 | 18 | Metf+Other | Metformin 850mg/d + Atorvastatin 10mg/d | 12 weeks | 52,53 | ±9,57 | ND | ND | ND | ND |
| | 2006 | 1760 | | | | | | | | | |

ND, non-describe.

**Figure 1.**

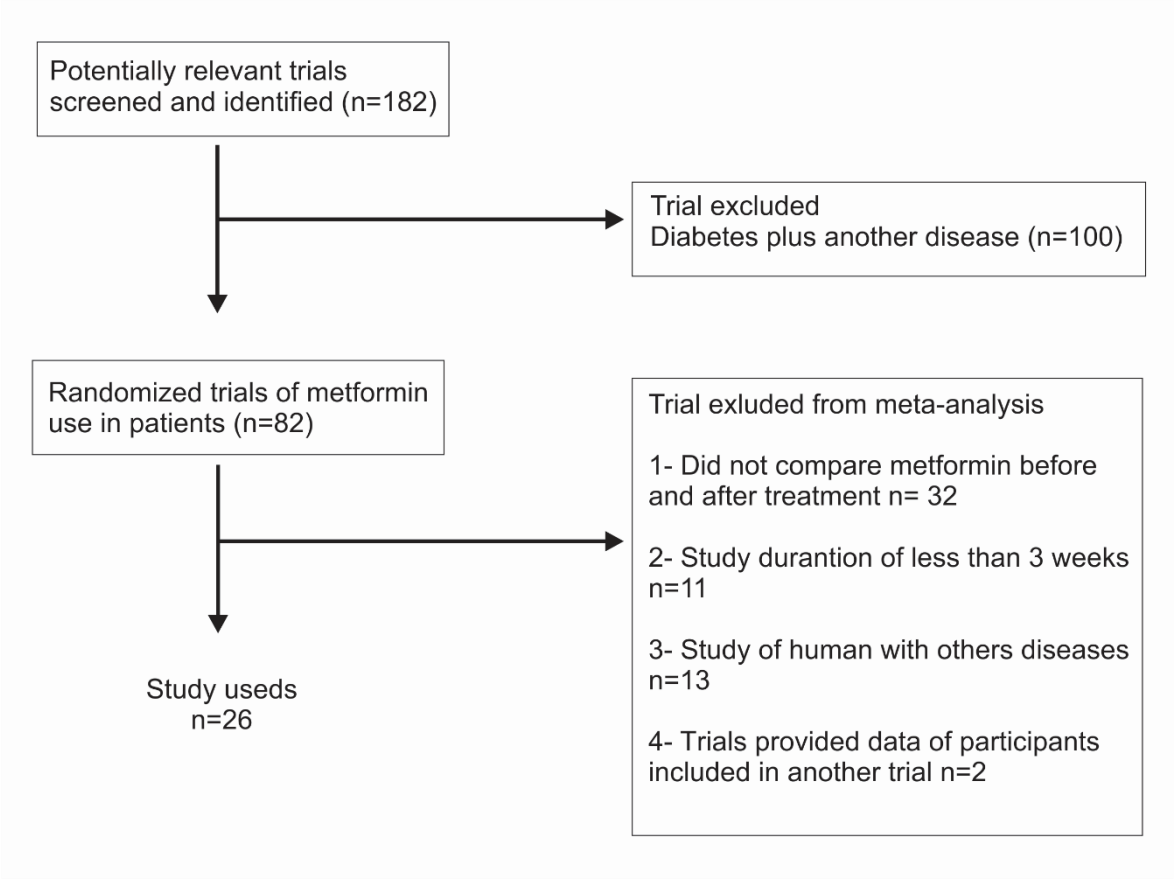
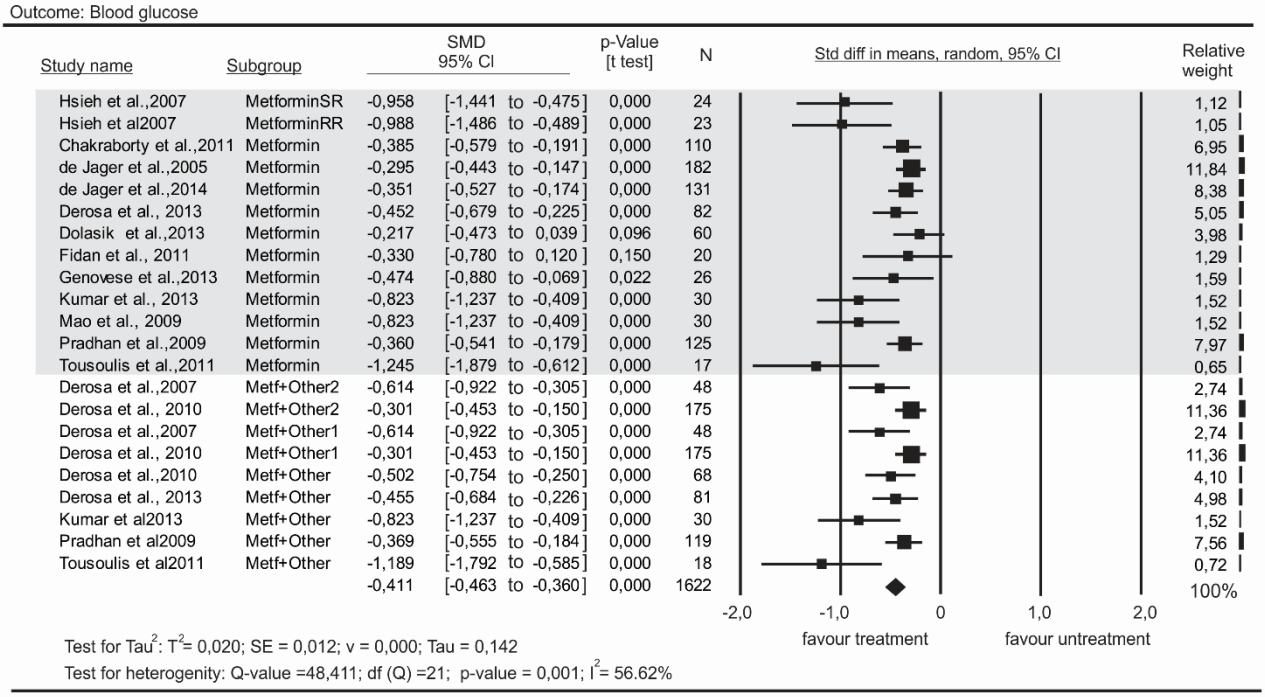



**Figure 1. Flow card of trial research.**

**Figure 2**

A




B

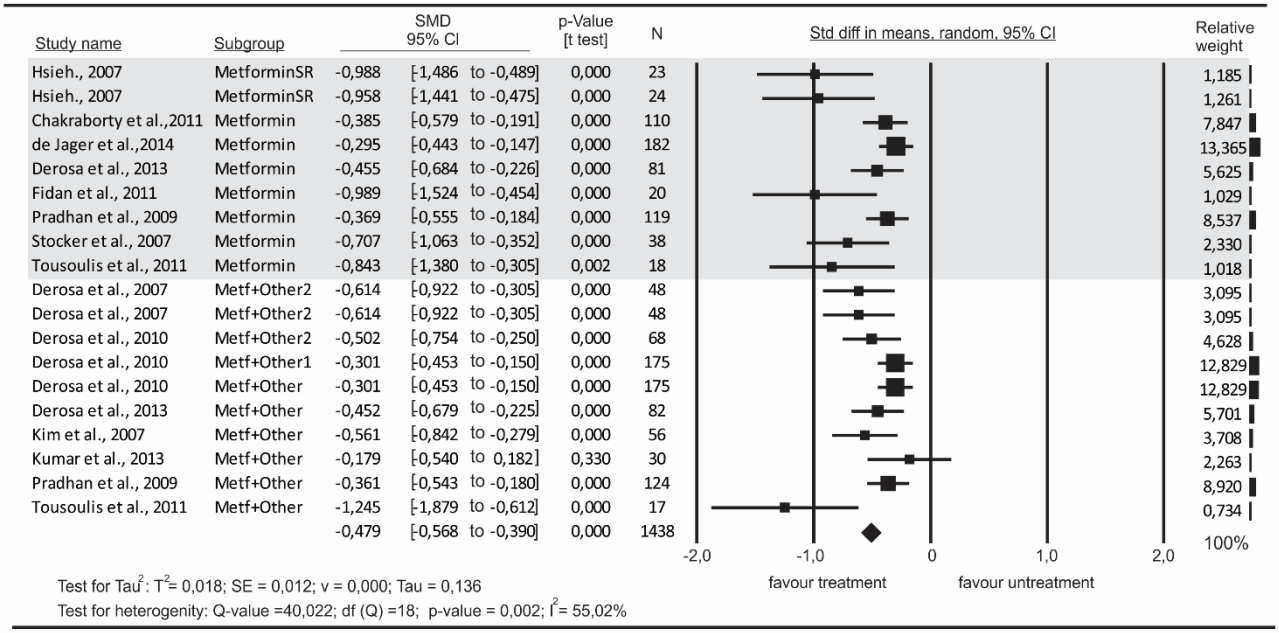



**Figure 2. Effect of post treatment with metformin on blood glucose level and glycated hemoglobin (HbA1c) in diabetic subjects.**
Forest plot from the meta-analysis of metformin on hematological parameters. Gray frame corresponds to metformin monotherapy and, the white frame corresponds metformin associated with another drug.

**Figure 3.**

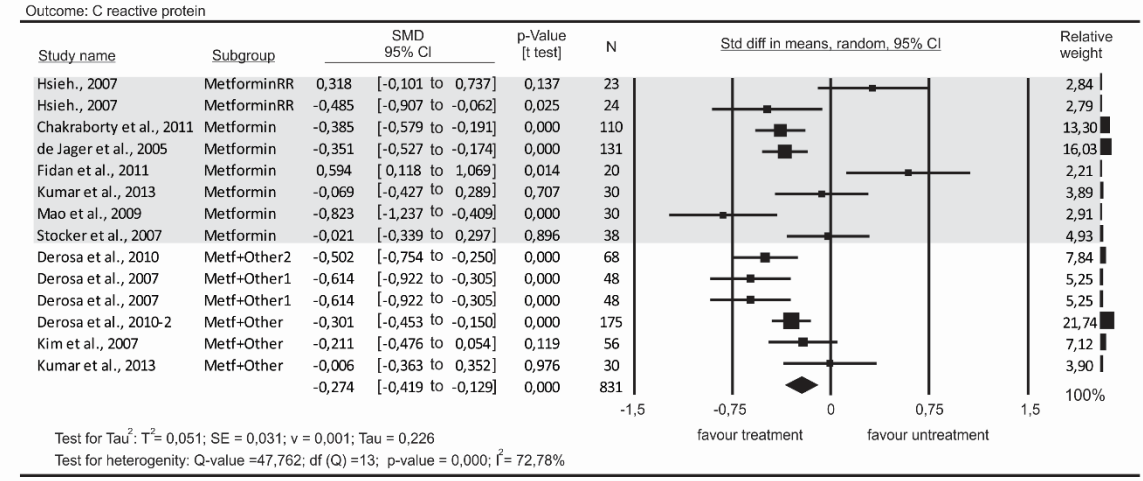



**Figure 3. Forest plot from the meta-analysis of metformin evaluating C-reactive protein (CRP) in diabetic subjects post metformin treatment.**
Gray frame corresponds to metformin monotherapy and the white frame corresponds metformin associated with another drug.

**Figure 4.**

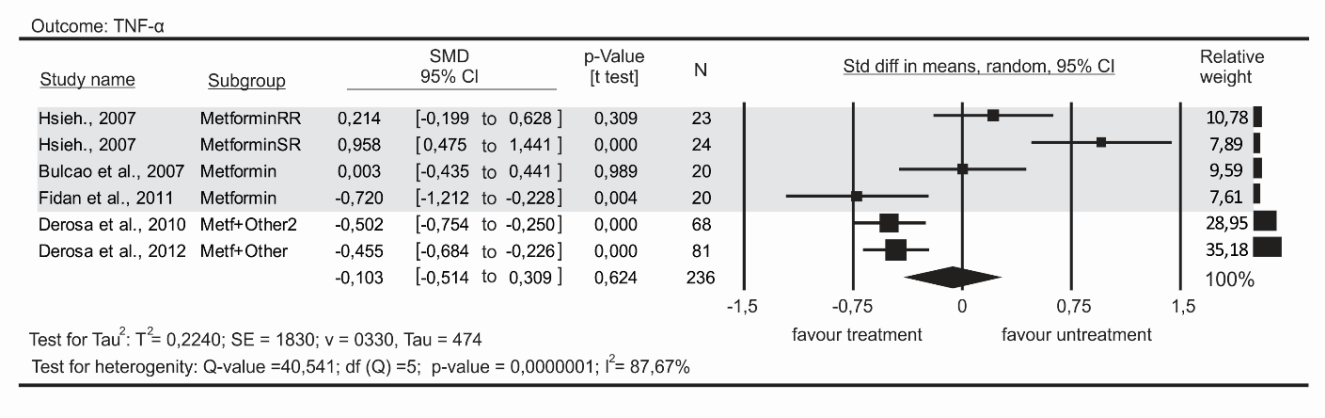



**Figure 4. Forest plot from the meta-analysis of metformin on tumor necrosis factor (TNFα) in diabetic subjects.**
Gray frame corresponds to metformin monotherapy and the white frame corresponds metformin associated with another drug.

**Figure 5.**

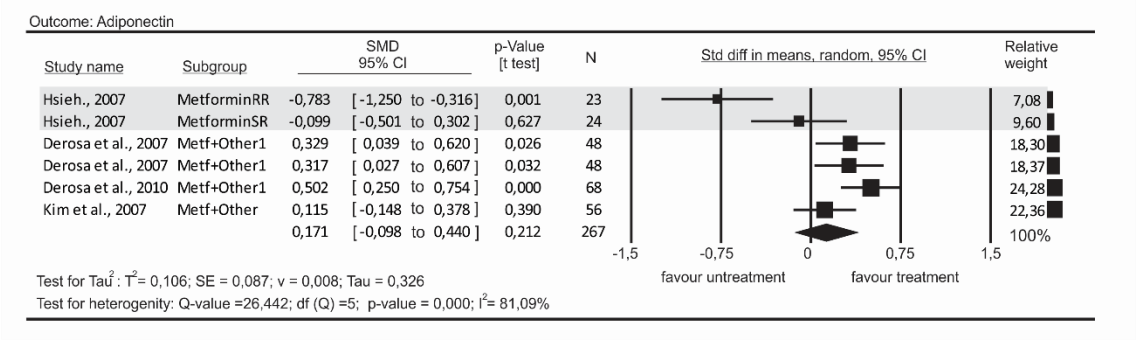



**Figure 5. Effect of the treatment with metformin on adiponectin in diabetic subjects.**
Gray frame corresponds to metformin monotherapy and the white frame corresponds metformin associated with another drug.

**Chapter 3 – paper 2**

Submits to Journal of Biochemistry & Pharmacology

# Anti-inflammatory and CaMKKβ/AMPK/Myosin V pathway effects of metformin in skeletal muscle ameliorates insulin resistance in diabetic rats

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**Abstract**

**Background:** Metformin increases insulin sensitivity by decreasing hepatic glucose production and increasing glucose disposal in skeletal muscle. However, modulation of inflammatory response and CaMKKβ/AMPK/Myosin V activation in gastrocnemius muscle by metformin treatment has not been demonstrated in hypoinsulinemic diabetic rats.

**Objective:** The present study investigated how the metformin improve insulin sensitivity in skeletal muscle of hypoinsulinemic diabetic rats.

**Methods:** Diabetes was induced by streptozotocin (45 mg/kg, intraperitoneally) 10 days prior treatments. On 11$^{th}$ day, diabetic rats were treated with metformin (500 mg/kg, oral gavage), insulin (2U at 08:00 h and 4U at 17:00 h, subcutaneously) or untreated. After 20 days, glycemia was measured and insulin sensitivity was determined by KITT. Serum Insulin, GLUT4, IRS*thr*, inflammatory markers (NF-κB, IκB, TNF-α and p-JNK) and CAMKK, AMPK and Myosin V in gastrocnemius muscle were determined by ELISA.

**Results:** As expected, insulin and metformin improved the insulin sensitivity. Besides, metformin treatment promoted reduction in inflammatory response mediated by NF-κB, IκB, TNF-α and p-JNK, and that was accompanied by increased CaMKKβ/AMPK/Myosin V/GLUT4 pathway activity in gastrocnemius muscle of diabetic rats.

**Conclusion:** Our findings suggest that metformin induces significant reductions in several inflammatory markers in skeletal muscle of diabetic rats. Metformin-induced increase in CaMKKβ/AMPK/Myosin V/GLUT4 pathway activity was associated with higher insulin sensitivity.

**Key words:**

Inflammatory pathway, insulin sensitivity, insulin pathway, hypoinsulinemic rat.

## Introduction

Skeletal muscle constitutes the largest insulin-sensitive tissue in the body and is the primary site for insulin-stimulated glucose uptake. Skeletal muscle resistance to insulin is fundamental to the development of the metabolic syndrome [1] and diabetes mellitus [2].

It is well documented that higher plasma glucose induces the expression of tumor necrosis factor-α (TNF-α) in skeletal muscle. TNF-α is a potent activator of nuclear factor-κβ (NF-κB), which may increase inflammatory cytokines [3-5]. NF-κB is found in all cell types [6-8] and its activity is controlled by regulatory proteins, called inhibitors of NF-κB (IκB) [9]. IκB exists in two phosphorylation states: a hyperphosphorylated state in unstimulated cells, and a hypophosphorylated newly synthesized state in LPS stimulated cells [10]. The phosphorylation of IκB and its proteolytic degradation, allowing the action of NF-κB [9]. Inflammatory mediators lead to phosphorylation ofc-Jun-N-terminal kinase (JNK) [5, 11] *which may decrease* insulin sensitivity. However, there are few data in the literature about NF-κB/JNK effects on insulin resistance in skeletal muscle of hypoinsulinemic diabetic rats.

Metformin is an effective hypoglycemic drug [12] that decreases glycemia by decreasing hepatic glucose production and increasing glucose disposal in skeletal muscle [13]. This drug activate AMP-activated protein kinase (AMPK) and decrease NF-κB and TNF-α signaling in endothelial cell [14]. The incubation of isolated epitrochlearis muscles with metformin promotes increase in the activity of catalytic subunits of AMPK [15]. AMPK activity in vastus lateralis biopsies of subjects with Type 2 Diabetes increased after 4 weeks and 10 weeks of treatment with metformin [13]. In addition, metformin decrease JNK activation and increase insulin receptor substrate (IRS1) concentration in skeletal muscle *in vitro* [16], which can increase translocation of glucose transporter 4 (GLUT4) in cardiac myocytes [17].

Reduced GLUT4 protein expression plays a significant role to promote decrease in insulin sensitivity of skeletal muscle, which has been described in murine models of insulinopenic diabetes [18]. Furthermore, the improvement in insulin action of skeletal muscle contributes to the therapeutic effects of metformin, mainly resulting in increased non-oxidative glucose disposal in these cells [19]. Myosin V, an unconventional motor protein family, participates in GLUT4 translocation in L6 muscle cells [20]. However, at this time, the myosin V expression induced by metformin treatment has not been evaluated. Although there is a lot of evidence of the role of metformin in intracellular signaling pathway, it is unclear how this drug improves insulin sensibility in skeletal muscle of hypoinsulinemic diabetic rats. In this report we tested the hypothesis that activation of CaMKKβ/AMPK/Myosin V pathway in skeletal muscle cells mediates the beneficial metabolic effects of metformin. The present study was also designed to determine the role of metformin in the regulation of inflammatory response in skeletal muscle of hypoinsulinemic diabetic rats.

## Material and Methods

*Animals*

Male Wistar rats (12 weeks, 181–223g) were maintained under standard conditions (22±1°C, humidity 60±5%, 12 h light/dark cycle) with food and water *ad libitum*. All procedures for handling, use and euthanasia of the animals followed the guidelines proposed by the Brazilian Society of Laboratory Animal Science and by the Ethics Committee for Animal Research of the Federal University of Uberlandia, Brazil (CEUA/UFU 053/13).

*Induction of experimental diabetes*

Animals were fasted 24h and rendered diabetic by a single injection of streptozotocin (STZ) (60 mg $kg^{-1}$, intraperitoneally). STZ was dissolved in 0.01 M citrate buffer (pH 4.5). Non-diabetic rats received a placebo dose (0.01 M citrate buffer). After 10 days, the fasting plasma glucose level was monitored by tail vein puncture using reactive strips (Biocheck Glucose Test Strip; Bioeasy, Belo Horizonte, MG, Brazil). Diabetes was defined as non-fasting glycaemia >250 mg/dL.

*Groups and treatments*

The animals were randomly divided into four groups (n=12 rats/group). Non-Diabetic (ND), Diabetic (D), Diabetic rats treated with NPH insulin - 6U/day divided in two subcutaneous injections: 2U at 08:00 h and 4U at 17:00h (D+I), and Diabetic rats treated with oral metformin - 500 mg $kg^{-1}$day by gavage (D+M). Treatments were performed for 20 days. After that period, rats were anesthetized with xylazine (10 mg $kg^{-1}$) and ketamine (100 mg $kg^{-1}$) for blood collection and euthanized for the removal of gastrocnemius skeletal muscle.

*Intravenous insulin tolerance test*

Tail blood samples were collected from animals fed *ad libitum* before (0 min) and 4, 8, 12, and 16 min after i.v. injection of regular insulin (0.75 U kg-1 BW). The constant rate of plasma glucose disappearance during insulin tolerance test (KITT) was calculated based on a linear regression of the Neperian logarithm of the plasma glucose concentrations (test strips, Advantage, Roche). The tests were performed from 09:00 to 11:00 h [21].

*Muscle homogenization*

Gastrocnemius muscle (0.1 g, removed before intravenous insulin tolerance test) was homogenized in buffer (25 mM Tris-HCl pH 7.4, cocktail protease inhibitor, 10 mM EDTA and 10 mM EGTA) for 3 min on ice. The homogenate was centrifuged at 15.000xg for 40 min. at 4°C. The pellet was resuspended in the same homogenization buffer. The supernatant was used for following analysis (GLUT4, CAMKKβ, p-CAMKKβ, IRS 1 thr, AMPK, p-MAPK, NF-κB, IκB, p-JNK) and pellet to (Myosin V) by *Western blotting* (WB) and ELISA methods.

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*ELISA*

Gastrocnemius muscles from all groups were diluted to a final concentration of 0.1 µg $mL^{-1}$ in coating buffer (100 mM bicarbonate/carbonate, pH 9.6). The microplate wells were coated with these samples (100 µL – 1 µg total protein well-1) overnight at 4°C and washed 3 times with PBS-T. Then, the wells were blocked with 200 µL of blocking buffer (2% BSA in PBS-T) for 4h at RT. 100 µL well-1 of the antibodies (anti-GLUT4, anti- CAMKKβ, anti- CAMKKβ phosphorylate, anti-IRS1 tyrosine, anti-AMPK, anti-AMPK phosphorylate, anti-myosin V, anti-NF-κB, anti-IκB, anti-JNK phosphorylate) diluted 1:1000 were added for 2h at RT and washed 3 times with PBS-T. 100 µL well-1 of RP-conjugated antibodies diluted 1:5000 were added for 2h at RT and washed 3 times with PBS-T. The secondary antibody (rabbit and mouse – conjugated HRP) was detected by adding 100 µL of the OPD-substrate at 20 min and read at 490 nm.

*SDS–PAGE and Western blotting*

The *Western blotting* was used to confirm the protein expression. Protein samples (10 µg) were applied to 5–22% polyacrylamide gradient gels under denaturing conditions [22] and stained with Coomassie brilliant blue. For immunoblotting, proteins were transferred to nitrocellulose membranes in Tris–glycine buffer[22]. The membranes were incubated with 5% dried milk in Tris-buffered saline (TBS-T) (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and probed with antibodies (anti- CAMKKβ, anti- CAMKKβ phosphorylate, anti-IRS1 tyrosine, anti-AMPK, anti-AMPK phosphorylate, anti-myosin V, anti-NF-κB, anti-IκB, anti-JNK phosphorylate) diluted to 0.2 µg/mL in TBS-T. The reaction was detected using an ECL kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions, and Armesham Imager 600 was used for exposure. The intensities of the protein bands were analyzed and compared using the ImageQuant TL software, version 8.1 (GE Healthcare Life Science).

*Insulin measurement*

Plasma insulin was analyzed by an enzyme immunoassay (EIA) using an insulin (mouse/rat) EIA kit (Bertin Pharma, Montigny-le-Bretonneux, France).

*Statistical analysis*

Prior to the statistical analysis, the data were tested for normality using the Shapiro-Wilk test. For the statistical analysis, data were normalized to the non-diabetic group, and the mean of each group was compared with the mean of the non-diabetic rats (positive control) and diabetic rats (negative control), using ANOVA followed by a Bonferroni test for multiple comparison. A Student's t test was used to compare the values of the intravenous insulin tolerance test of diabetic rats and diabetic rats treated with metformin. The significance level for all analyses was $p<0.05$. The results are shown as the means ±SEM.

**Results**

Glycemic characteristics of the animals are shown in Figure 1. Compared with non-diabetic rats (ND), diabetic (D) and metformin-treated diabetic (D+M) rats presented hyperglycemia ($p<0.001$). As expected, insulin treatment reduced ($p<0.05$) plasma glucose in diabetic rats. The metformin treatment did not change ($p>0.05$) plasma glucose in diabetic rats during removal of samples (Figure 1.A). For analysis of acute metformin treatment in diabetic rats (Figure 1.B), plasma glucose was evaluated 2h, 4h, 6h and 12h after a single administration by oral gavage. Metformin treatment of diabetic (D+M) rats induced a similar plasma glucose reduction ($p<0.05$) from 2h to 6h of treatment (Figure 1B).

As expected, the insulin sensitivity in diabetic (D) rats was lower ($p<0.05$) than non-diabetic (ND)rats. In addition, the insulin sensitivity was higher in both insulin-treated diabetic (D+I) and metformin-treated diabetic (D+M) rats, compared to diabetic (D) rats (Figure 2.A). Figure 2.B shows that diabetes decreased ($p<0.05$) the GLUT4 protein expression in gastrocnemius muscle. Insulin- (D+I) and metformin-treated (D+M) rats were able to increase ($p<0.01$) the GLUT4 protein expression as compared to diabetic condition level.

Low plasma insulin levels ($p<0.05$) were observed in diabetic (D) and metformin-treated diabetic (D+M) rats compared to non-diabetic (ND) rats. Increased insulin levels ($p<0.05$) were found in insulin-treated diabetic (D+I) rats compared with non-diabetic (ND) rats (Figure 3.A). The diabetes did not change IRS1*thr* expression in gastrocnemius muscle. As expected, chronic (20 days) treatment of insulin resulted in the upregulation of IRS1*thr* in gastrocnemius muscle of diabetic rats as compared to non-diabetic (ND) and to diabetic (D) rats. Furthermore, the chronic (20 days) metformin treatment also increases ($p<0.05$) IRS1*thr* expression in this tissue compared to diabetic (D) rats (Figure 3.B).

To assess the role of insulin and metformin treatment on the activation of CAMKKβ/AMPK/Myosin V pathway under diabetic conditions, these proteins were analyzed in gastrocnemius muscle. In diabetic (D) rats CAMKKβ phosphorylation was decreased ($p<0.05$) compared to the non-diabetic (ND) rats. Compared with non-diabetic rats (ND), 20-day diabetic rats (D) showed no significant changes in AMPK phosphorylation and myosin V content. After 20 days of insulin treatment, the effect of diabetes on CAMKKβ phosphorylation and myosin V content were reversed in gastrocnemius muscles. Compared with non-diabetic (ND) and diabetic (D) rats, insulin-treated diabetic (D+I) rats showed no significant changes in AMPK phosphorylation (Figure 4.A-4.C). As shown in Fig. 4., metformin treatment increased CAMKKβ phosphorylation ($p<0.05$), AMPK phosphorylation ($p<0.05$) and myosin V protein content ($p<0.05$) than non-diabetic (ND) and diabetic (D) rats.

To determine the kinetics of inflammatory response, we next tested if the insulin and metformin treatment would impact NF-κB and other related factors like IκB, TNF-α and p-JNK in skeletal muscle of diabetic rats (Figure 5.A-5.D). We found that diabetic (D) animals had higher ($p>0.05$) NF-κB and TNF-α expression compared to non-diabetic (ND) animals. Besides, the JNK phosphorylation was also increased ($p<0.05$) in diabetic (D) rats compared to non-diabetic (ND) rats. In gastrocnemius muscle of insulin-treated diabetic (D+I) rats, decreased ($p<0.05$) NF-κB expression, TNF-α expression and JNK phosphorylation were observed as

compared to diabetic (D) rats. IκB expression was increased ($p<0.05$) in insulin-treated diabetic (D+I) rats as compared to diabetic (D) rats. Metformin-treated diabetic (D+M) rats overexpressing ($p<0.05$) the NF-κB inhibitory protein, IκB, associated with reduction ($p<0.05$) in NF-κB protein expression than non-diabetic (ND) rats. TNF-α expression and JNK phosphorylation significantly decreased ($p<0.05$) in metformin-treated diabetic rats (D+M) than diabetic (D) rats.

## Discussion

The present study investigated the effects of metformin or insulin and their association with CaMKKβ/AMPK/Myosin V/GLUT4 pathway and inflammatory response in gastrocnemius muscle of hypoinsulinemic diabetic rats. To the best of our knowledge, the CaMKKβ/AMPK/Myosin V/GLUT4 pathway associated with inflammatory markers (NF-κB, IκB, TNF-α and p-JNK) has never been directly measured in hypoinsulinemic diabetic ratsdespite its critical role in regulating insulin resistance, which is linked to the development of the diabetes. The present results show evidence of increased CaMKKβ/AMPK/Myosin V/GLUT4 pathway activity in metformin-treated diabetic rats compared with diabetic rats. Furthermore, metformin reduced the inflammatory response mediated by NF-κB, IκB, TNF-α and p-JNK in skeletal muscle of diabetic rats. These effects were associated with reduction in insulin resistance.

The terminal half-life of metformin after oral administration occurs in 6h - 7h at doses of 100 and 200 mg/kg, respectively [23]. These data are consistent with our findings which indicate that metformin decreases glycaemia between 2 to 6hs after the oral administration, returning to baseline blood glucose after 12h. In the present study, the diabetic state of the animals was similar to that described in previous studies, and effect of insulin upon glycaemic control was also similar [24-26]. In addition, we showed that metformin treatment increases the plasma glucose disappearance rate (KITT) compared to non-treated diabetic rats indicating an improving the insulin sensitivity.

Metformin has been used in different pathologies [27-30], but, its major application is to decrease insulin resistance in the liver [31]. On the other hand, skeletal muscle is the predominant site of insulin-mediated glucose uptake. Therefore, the reduction of damage caused by proinflammatory cytokines in this tissue is essential for glucose homeostasis. Diabetes is associated with systemic inflammation and metabolic disorders [32, 33], increasing the level of TNF-α [5, 32, 33] and decreasing the GLUT4 translocation [34, 35]. It is well documented that TFN-α induce insulin resistance in skeletal muscle [5] by increase of phosphorylation of JNK and IRS1*ser*. Studies show that metformin decrease TNF-α via AMPK activation and inhibit NF-κB activation in vascular endothelial cells [36, 37]. Here, we have found that metformin have a similar mechanism of action in skeletal muscle of hypoinsulinemic diabetic rats. Our findings suggest that treatment with metformin improve insulin sensibility by maintenance of NF-κB/IκB complex, decrease of TNF-α expression, JNK phosphorylation associated with the activation of CaMKKβ/AMPK pathway in skeletal muscle. This could indicate that metformin regulates proinflammatory pathway in skeletal muscle by activation of

CAMKKβ/AMPK pathway. However, metformin is not specific AMPK activators. This drug activate AMPK by the upstream kinases serine/threonine kinase 1 (LKB1) in liver. The deletion of LKB1 in the liver did not impair AMPK activation in culture of muscle tissue [38], but deficiency of LKB1 in skeletal muscle prevents AMPK activation even in the presence of the AMP-mimetic agent, 5-aminoimidazole-4-carboxamide riboside (AICAR), the antidiabetic drug phenformin, or by muscle contraction [39]. Higher metformin dose induce AMPK phosphorylation independent of CAMKKβ in arterial smooth muscle [40]. On the other hand, other studies show that CAMKKα, regulates glucose uptake independent of AMPK and AKt activation, and that the CAMKKβ has a critical role in the regulation of contraction-induced glucose uptake in mouse skeletal muscle [41]. CAMKKβ activate AMPK in response to increase of intracellular calcium [42]. So we suggest that metformin can activate AMPK both as CaMKK independently.

Furthermore, intracellular calcium promotes GLUT4 translocation by CAMKKβ and AMPK activation [43]. Thus, CAMKK activity depends of the calcium intracellular level and can activate AMPK independently of changes in AMP [44]. Although we did not evaluate the release of calcium from the sarcoplasmic reticulum, we show for the first time an increase of CAMKKβ in skeletal muscle of rats treated with metformin. More studies are needed to understand how metformin activates the CAMKKβ in skeletal muscle. Additionally, we show that metformin decrease p-JNK and increase CaMKKβ/AMPK/Myosin V pathway IRS1*thr*. Our results is consistent with a previous study which suggested that metformin decrease JNK activation and increase IRS1 concentration in skeletal muscle *in vitro* [16]. Furthermore, to our knowledge, we also demonstrate for the first time that metformin treatment increase myosin V expression in skeletal muscle of hypoinsulinemic diabetic rats. The molecular mechanism involved in this process is due to activation of AS160 by p-AMPK that can occurs independently of the insulin signal [45]. In addition, study shows the co-localization of Myosin V and RAB8a in the GLUT4 vesicle exocytosis of muscle cells [46]. These finding indicated that metformin treatment is able to increase expression of myosin V involved in GLUT4 translocation independent of the insulin signal. Thus, we propose a metformin-induced pathway leading to GLUT4 translocation into the plasma membrane of muscle cells in gastrocnemius muscle (Fig. 6).

In summary, we show that metformin induces significant reductions in several inflammatory markers as NF-κB, IκB, TNF-α and p-JNK of skeletal muscle in hypoinsulinemic diabetic rats. Additionally, in this tissue, metformin treatment was followed by increased CaMKKβ/AMPK/Myosin V/GLUT4 pathway. The degree of pro-inflammatory markers and CaMKKβ/AMPK/Myosin V/GLUT4 pathway activation in skeletal muscle cells are related with the insulin sensitivity in metformin-treated diabetic rats compared with diabetic rats. Collectively, these novel findings may provide a significant step toward the elucidation of the metabolic and inflammatory signaling pathways regulating metformin-dependent skeletal muscle glucose uptake.

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## Author contributions

Conceived and designed the experiments: L.G.P., R.R.T., F.S.E. Performed the experiments: L.G.P., R.R.T., N.B.B., H.L.M., D.C.C., Analyzed the data: L.G.P., R.R.T., D.D.V., R.S.S. Wrote the paper: L.G.P., R.R.T., Participated in interpretation and revised the manuscript for publication: RS-S. Supervised the entire project: F.S.E.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## Reference


1. Stump CS, Henriksen EJ, Wei Y, Sowers JR. The metabolic syndrome: role of skeletal muscle metabolism. Ann Med. 2006;38(6):389-402. Epub 2006/09/30. doi: 10.1080/07853890600888413. PubMed PMID: 17008303.
2. Kainulainen H, Komulainen J, Joost HG, Vihko V. Dissociation of the effects of training on oxidative metabolism, glucose utilisation and GLUT4 levels in skeletal muscle of streptozotocin-diabetic rats. Pflugers Arch. 1994;427(5-6):444-9. Epub 1994/07/01. PubMed PMID: 7971142.
3. Roberts CK, Hevener AL, Barnard RJ. Metabolic syndrome and insulin resistance: underlying causes and modification by exercise training. Compr Physiol. 2013;3(1):1-58. Epub 2013/05/31. doi: 10.1002/cphy.c110062. PubMed PMID: 23720280; PubMed Central PMCID: PMCPMC4129661.
4. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. Science. 1996;271(5249):665-8. Epub 1996/02/02. PubMed PMID: 8571133.
5. Plomgaard P, Bouzakri K, Krogh-Madsen R, Mittendorfer B, Zierath JR, Pedersen BK. Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. Diabetes. 2005;54(10):2939-45. Epub 2005/09/28. PubMed PMID: 16186396.
6. You M, Ku PT, Hrdlickova R, Bose HR, Jr. ch-IAP1, a member of the inhibitor-of-apoptosis protein family, is a mediator of the antiapoptotic activity of the v-Rel oncoprotein. Mol Cell Biol. 1997;17(12):7328-41. Epub 1997/12/31 23:35. PubMed PMID: 9372964; PubMed Central PMCID: PMCPMC232589.
7. Stehlik C, de Martin R, Kumabashiri I, Schmid JA, Binder BR, Lipp J. Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. J Exp Med. 1998;188(1):211-6. Epub 1998/07/07. PubMed PMID: 9653098; PubMed Central PMCID: PMCPMC2525542.
8. Lee HH, Dadgostar H, Cheng Q, Shu J, Cheng G. NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. Proc Natl Acad Sci U S A. 1999;96(16):9136-41. Epub 1999/08/04. PubMed PMID: 10430908; PubMed Central PMCID: PMCPMC17745.
9. Baeuerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. Science. 1988;242(4878):540-6. Epub 1988/10/28. PubMed PMID: 3140380.
10. Rao P, Hayden MS, Long M, Scott ML, West AP, Zhang D, et al. IkappaBbeta acts to inhibit and activate gene expression during the inflammatory response. Nature. 2010;466(7310):1115-9. Epub 2010/08/27. doi: 10.1038/nature09283. PubMed PMID: 20740013; PubMed Central PMCID: PMCPmc2946371.
11. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. Gastroenterology. 132. United States2007. p. 2169-80.
12. Strack T. Metformin: a review. Drugs Today (Barc). 2008;44(4):303-14. Epub 2008/06/10. PubMed PMID: 18536788.
13. Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, et al. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. Diabetes. 2002;51(7):2074-81. Epub 2002/06/28. PubMed PMID: 12086935.
14. !!! INVALID CITATION !!!
15. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest. 2001;108(8):1167-74. Epub 2001/10/17. doi: 10.1172/jci13505. PubMed PMID: 11602624; PubMed Central PMCID: PMCPmc209533.
16. Kumar N, Dey CS. Metformin enhances insulin signalling in insulin-dependent and-independent pathways in insulin resistant muscle cells. Br J Pharmacol. 2002;137(3):329-36. Epub

2002/09/19. doi: 10.1038/sj.bjp.0704878. PubMed PMID: 12237252; PubMed Central PMCID: PMCPMC1573500.
17. Fischer Y, Thomas J, Rosen P, Kammermeier H. Action of metformin on glucose transport and glucose transporter GLUT1 and GLUT4 in heart muscle cells from healthy and diabetic rats. Endocrinology. 1995;136(2):412-20. Epub 1995/02/01. doi: 10.1210/endo.136.2.7835271. PubMed PMID: 7835271.
18. Dombrowski L, Roy D, Marette A. Selective impairment in GLUT4 translocation to transverse tubules in skeletal muscle of streptozotocin-induced diabetic rats. Diabetes. 1998;47(1):5-12. Epub 1998/01/08. PubMed PMID: 9421368.
19. Johnson AB, Webster JM, Sum CF, Heseltine L, Argyraki M, Cooper BG, et al. The impact of metformin therapy on hepatic glucose production and skeletal muscle glycogen synthase activity in overweight type II diabetic patients. Metabolism. 1993;42(9):1217-22. Epub 1993/09/01. PubMed PMID: 8412779.
20. Ishikura S, Klip A. Muscle cells engage Rab8A and myosin Vb in insulin-dependent GLUT4 translocation. Am J Physiol Cell Physiol. 2008;295(4):C1016-25. Epub 2008/08/15. doi: 10.1152/ajpcell.00277.2008. PubMed PMID: 18701652.
21. Okamoto MM, Anhe GF, Sabino-Silva R, Marques MF, Freitas HS, Mori RC, et al. Intensive insulin treatment induces insulin resistance in diabetic rats by impairing glucose metabolism-related mechanisms in muscle and liver. J Endocrinol. 2011;211(1):55-64. Epub 2011/07/13. doi: 10.1530/joe-11-0105. PubMed PMID: 21746792.
22. Laemmli UK, Favre M. Maturation of the head of bacteriophage T4. I. DNA packaging events. J Mol Biol. 1973;80(4):575-99. Epub 1973/11/15. PubMed PMID: 4204102.
23. Choi YH, Kim SG, Lee MG. Dose-independent pharmacokinetics of metformin in rats: Hepatic and gastrointestinal first-pass effects. J Pharm Sci. 2006;95(11):2543-52. Epub 2006/08/29. doi: 10.1002/jps.20744. PubMed PMID: 16937336.
24. Freitas HS, D'Agord Schaan B, da Silva RS, Okamoto MM, Oliveira-Souza M, Machado UF. Insulin but not phlorizin treatment induces a transient increase in GLUT2 gene expression in the kidney of diabetic rats. Nephron Physiol. 2007;105(3):p42-51. Epub 2007/01/06. doi: 10.1159/000098442. PubMed PMID: 17204838.
25. Sabino-Silva R, Freitas HS, Lamers ML, Okamoto MM, Santos MF, Machado UF. Na+-glucose cotransporter SGLT1 protein in salivary glands: potential involvement in the diabetes-induced decrease in salivary flow. J Membr Biol. 2009;228(2):63-9. Epub 2009/02/25. doi: 10.1007/s00232-009-9159-3. PubMed PMID: 19238474.
26. David-Silva A, Freitas HS, Okamoto MM, Sabino-Silva R, Schaan BD, Machado UF. Hepatocyte nuclear factors 1alpha/4alpha and forkhead box A2 regulate the solute carrier 2A2 (Slc2a2) gene expression in the liver and kidney of diabetic rats. Life Sci. 2013;93(22):805-13. Epub 2013/10/26. doi: 10.1016/j.lfs.2013.10.011. PubMed PMID: 24157454.
27. Li J, Benashski SE, Venna VR, McCullough LD. Effects of metformin in experimental stroke. Stroke. 2010;41(11):2645-52. Epub 2010/09/18. doi: 10.1161/strokeaha.110.589697. PubMed PMID: 20847317; PubMed Central PMCID: PMCPmc2994809.
28. Lv WS, Wen JP, Li L, Sun RX, Wang J, Xian YX, et al. The effect of metformin on food intake and its potential role in hypothalamic regulation in obese diabetic rats. Brain Res. 2012;1444:11-9. doi: 10.1016/j.brainres.2012.01.028. PubMed PMID: 22325091.
29. Di Pietro M, Parborell F, Irusta G, Pascuali N, Bas D, Silvia Bianchi M, et al. Metformin regulates ovarian angiogenesis and follicular development in a female Polycystic Ovary Syndrome rat model. Endocrinology. 2015:en20141765. Epub 2015/01/16. doi: 10.1210/en.2014-1765. PubMed PMID: 25590243.
30. Yin M, van der Horst IC, van Melle JP, Qian C, van Gilst WH, Sillje HH, et al. Metformin improves cardiac function in a nondiabetic rat model of post-MI heart failure. Am J Physiol Heart Circ Physiol. 2011;301(2):H459-68. Epub 2011/05/17. doi: 10.1152/ajpheart.00054.2011. PubMed PMID: 21572014.

31. Nagi DK, Yudkin JS. Effects of metformin on insulin resistance, risk factors for cardiovascular disease, and plasminogen activator inhibitor in NIDDM subjects. A study of two ethnic groups. Diabetes Care. 1993;16(4):621-9. Epub 1993/04/01. PubMed PMID: 8462390.
32. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444(7121):860-7. doi: 10.1038/nature05485. PubMed PMID: 17167474.
33. Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. Diabetes Care. 2004;27(3):813-23. PubMed PMID: 14988310.
34. Gibbs EM, Stock JL, McCoid SC, Stukenbrok HA, Pessin JE, Stevenson RW, et al. Glycemic improvement in diabetic db/db mice by overexpression of the human insulin-regulatable glucose transporter (GLUT4). J Clin Invest. 1995;95(4):1512-8. Epub 1995/04/01. doi: 10.1172/jci117823. PubMed PMID: 7706456; PubMed Central PMCID: PMCPmc295634.
35. Zisman A, Peroni OD, Abel ED, Michael MD, Mauvais-Jarvis F, Lowell BB, et al. Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. Nat Med. 2000;6(8):924-8. Epub 2000/08/10. doi: 10.1038/78693. PubMed PMID: 10932232.
36. Hattori Y, Suzuki K, Hattori S, Kasai K. Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells. Hypertension. 2006;47(6):1183-8. Epub 2006/04/26. doi: 10.1161/01.hyp.0000221429.94591.72. PubMed PMID: 16636195.
37. Huang NL, Chiang SH, Hsueh CH, Liang YJ, Chen YJ, Lai LP. Metformin inhibits TNF-alpha-induced IkappaB kinase phosphorylation, IkappaB-alpha degradation and IL-6 production in endothelial cells through PI3K-dependent AMPK phosphorylation. Int J Cardiol. 2009;134(2):169-75. Epub 2008/07/04. doi: 10.1016/j.ijcard.2008.04.010. PubMed PMID: 18597869.
38. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science. 2005;310(5754):1642-6. Epub 2005/11/26. doi: 10.1126/science.1120781. PubMed PMID: 16308421; PubMed Central PMCID: PMCPmc3074427.
39. Sakamoto K, McCarthy A, Smith D, Green KA, Grahame Hardie D, Ashworth A, et al. Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. EMBO J. 24. England2005. p. 1810-20.
40. Pyla R, Osman I, Pichavaram P, Hansen P, Segar L. Metformin exaggerates phenylephrine-induced AMPK phosphorylation independent of CaMKKbeta and attenuates contractile response in endothelium-denuded rat aorta. Biochem Pharmacol. 2014;92(2):266-79. Epub 2014/09/03. doi: 10.1016/j.bcp.2014.08.024. PubMed PMID: 25179145; PubMed Central PMCID: PMCPmc4252840.
41. Witczak CA, Jessen N, Warro DM, Toyoda T, Fujii N, Anderson ME, et al. CaMKII regulates contraction- but not insulin-induced glucose uptake in mouse skeletal muscle. Am J Physiol Endocrinol Metab. 298. United States2010. p. E1150-60.
42. Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, et al. Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. Cell Metab. 2. United States2005. p. 9-19.
43. Li Q, Zhu X, Ishikura S, Zhang D, Gao J, Sun Y, et al. Ca(2)(+) signals promote GLUT4 exocytosis and reduce its endocytosis in muscle cells. Am J Physiol Endocrinol Metab. 307. United States: 2014 the American Physiological Society.; 2014. p. E209-24.
44. Suter M, Riek U, Tuerk R, Schlattner U, Wallimann T, Neumann D. Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. J Biol Chem. 281. United States2006. p. 32207-16.
45. Kramer HF, Witczak CA, Fujii N, Jessen N, Taylor EB, Arnolds DE, et al. Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. Diabetes. 2006;55(7):2067-76. Epub 2006/06/29. doi: 10.2337/db06-0150. PubMed PMID: 16804077.

46. Sun Y, Chiu TT, Foley KP, Bilan PJ, Klip A. Myosin Va mediates Rab8A-regulated GLUT4 vesicle exocytosis in insulin-stimulated muscle cells. Mol Biol Cell. 2014;25(7):1159-70. Epub 2014/01/31. doi: 10.1091/mbc.E13-08-0493. PubMed PMID: 24478457; PubMed Central PMCID: PMCPmc3967978.

## Figures and Legends

### Figure 1.

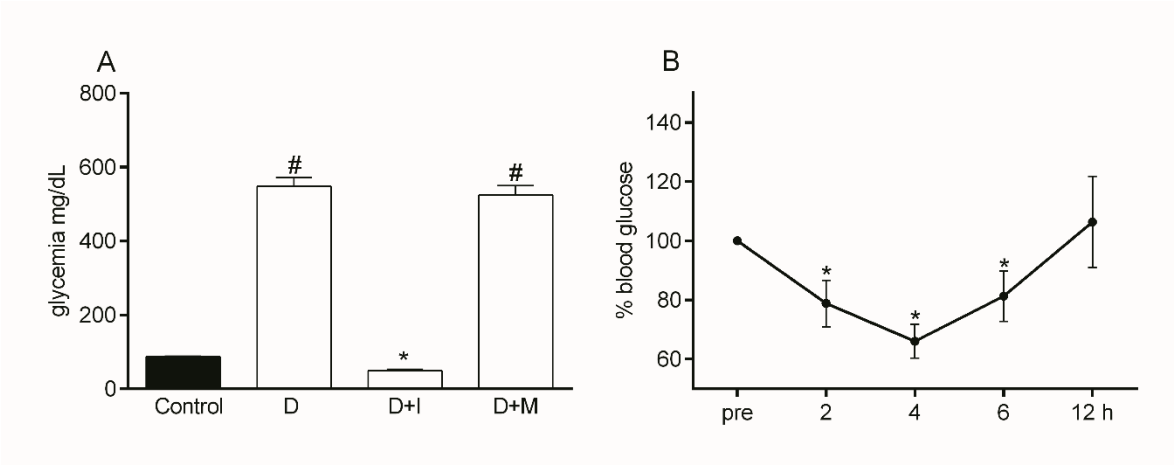



**Figure 1. Fasting plasma glucose in non-diabetic (ND), untreated diabetic (D), and insulin (D+I)- or metformin (D+M)- treated diabetic rats.**
(A) Blood glucose levels. (B) Daily blood glucose variation in rats treated with metformin. Data expressed as mean ± SEM; # vs. non-diabetic; * vs. diabetic; $p<0.05$; n = 8–10 per group.

**Figure 2.**

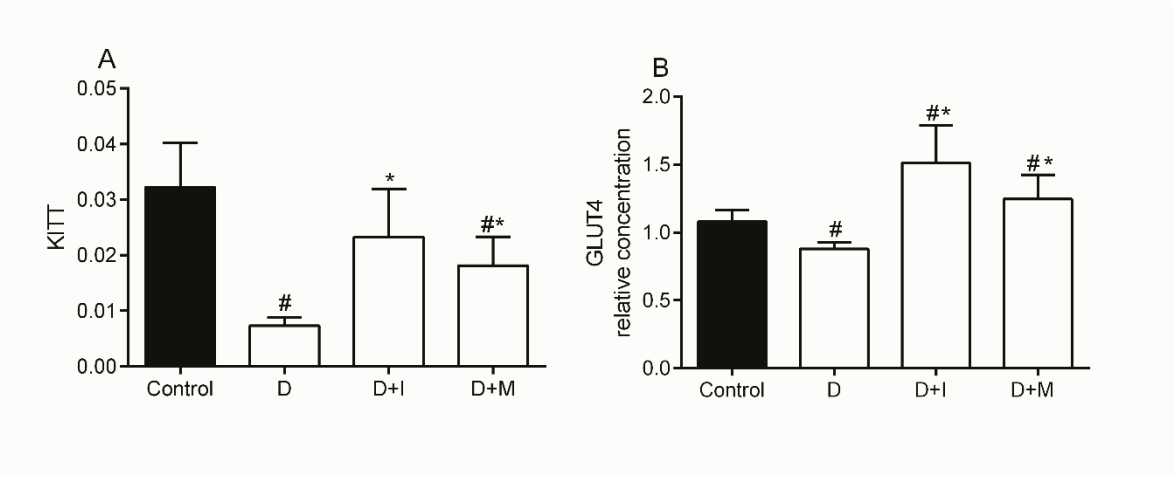



**Figure 2. Insulin sensitivity (A) and GLUT4 expression in gastrocnemius (B) muscles of on-diabetic (ND), untreated diabetic (D), and insulin (D+I)- or metformin (D+M)- treated diabetic rats.**

Data expressed as mean ± SEM; # vs. non-diabetic; * vs. diabetic; $p<0.05$; n = 8–10 per group.

**Figure 3.**

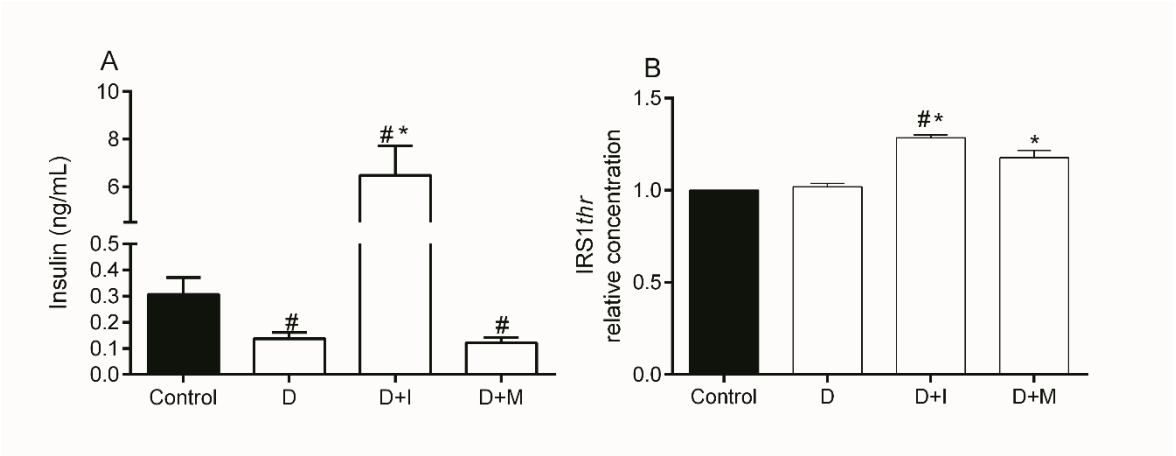



**Figure 3. Basal plasma insulin and IRS1thr expression in gastrocnemius (B) muscles of on-diabetic (ND), untreated diabetic (D), and insulin (D+I)- or metformin (D+M)- treated diabetic rats.**
Data expressed as mean ± SEM; # vs. non-diabetic; * vs. diabetic; $p<0.05$; n = 8–10 per group.

**Figure 4.**

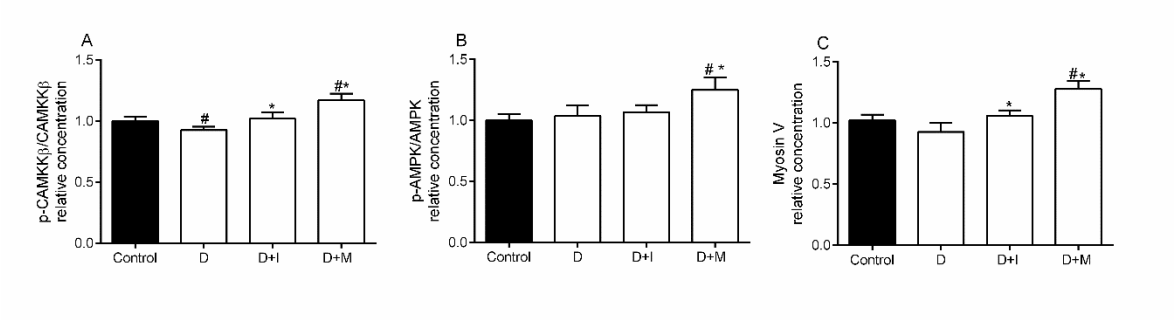



**Figure 4. Intracellular signal triggered by metformin action.**
(A) p-CAMKKβ/CAMKKβ concentration; (B) p-AMPK/AMPK concentration; (C) Myosin V concentration. Non-diabetic (ND), untreated diabetic (D), and insulin (D+I)- or metformin (D+M)-treated diabetic rats**.** Data expressed as mean ± SEM; # vs. non-diabetic; * vs. diabetic; $p<0.05$; n = 8–10 per group.

**Figure 5.**

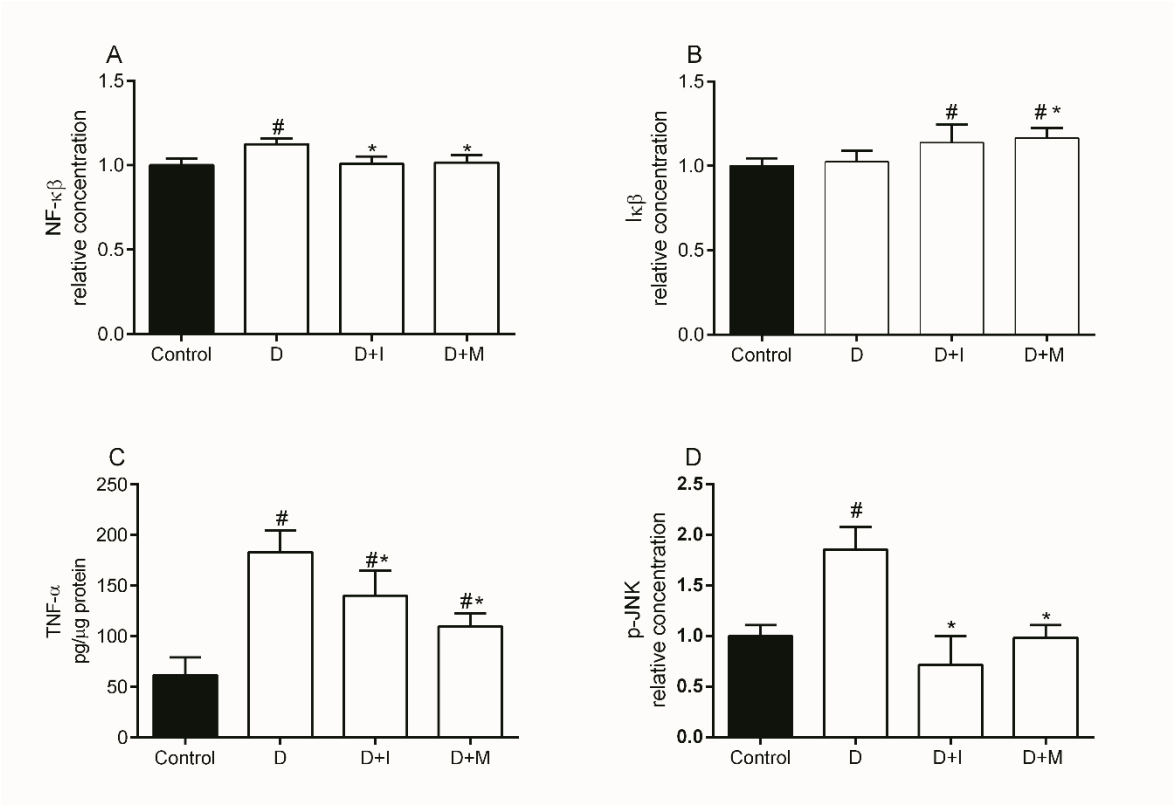



**Figure 5. Metformin role on inflammatory pathway.**
(A) NF-κB concentration; (B) IκB concentration; (C) TNF-α concentration; (D) p-JNK concentration. Non-diabetic (ND), untreated diabetic (D), and insulin (D+I)- or metformin (D+M)-treated diabetic rats**.** Non-diabetic (ND), untreated diabetic (D), and insulin (D+I)- or metformin (D+M)-treated diabetic rats**.** Data expressed as mean ± SEM; # vs. non-diabetic; * vs. diabetic; $p<0.05$; n = 8–10 per group.

**Figure 6.**

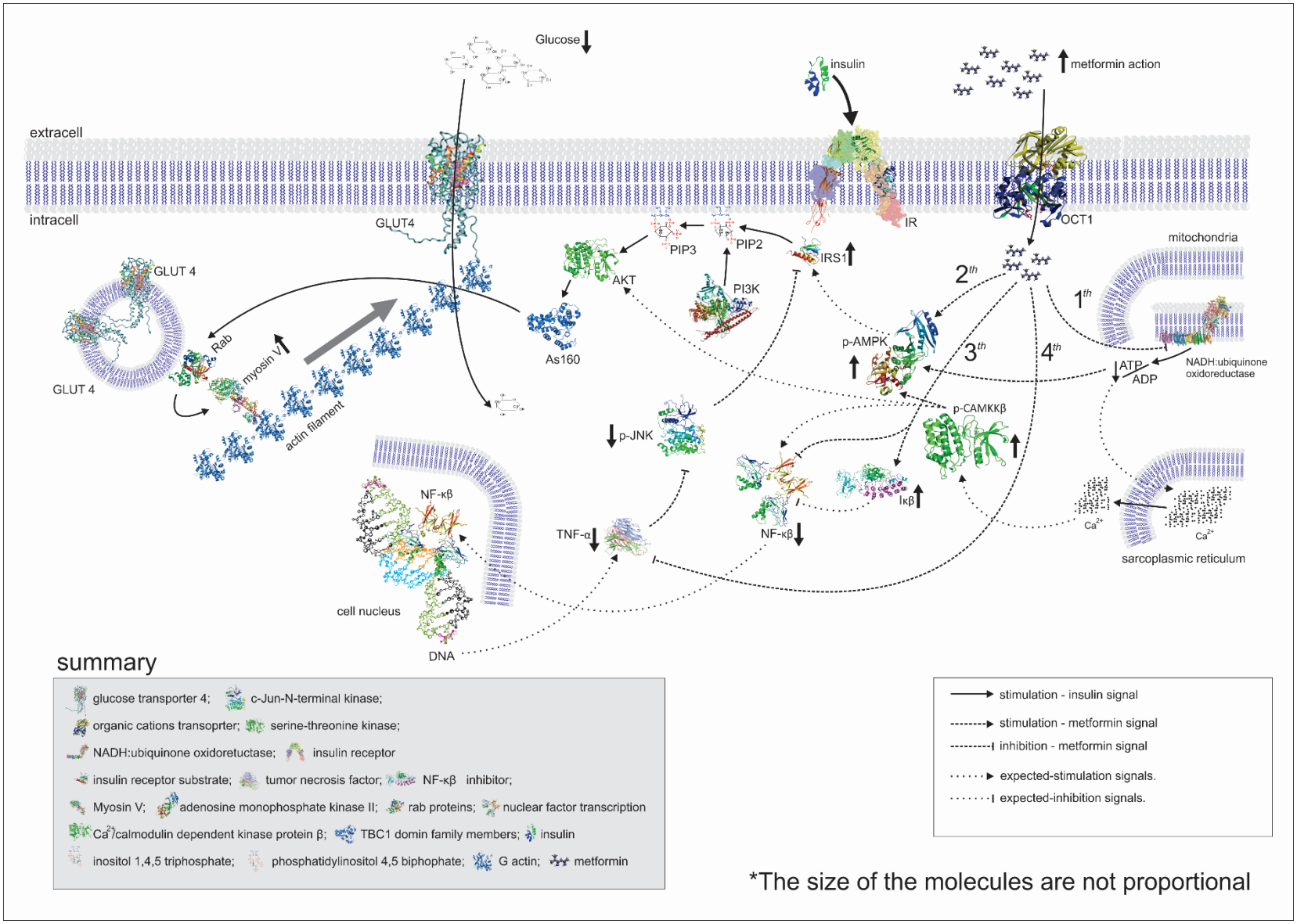
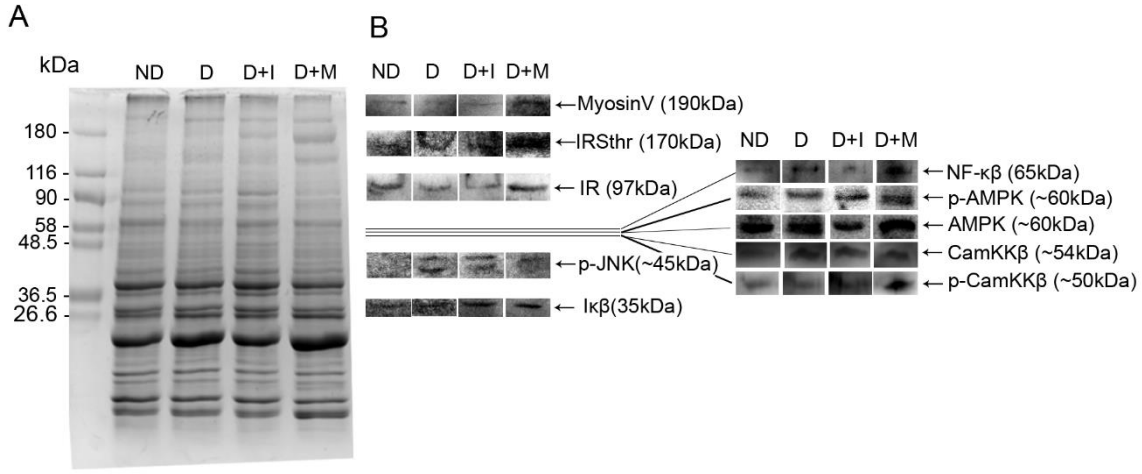

Glucose
insulin
metformin action
extracell
intracell
GLUT4
IR
OCT1
PIP3
PIP2
IRS1
AKT
PI3K
mitochondria
GLUT 4
Rab
myosin V
actin filament
As160
GLUT 4
2th
1th
3th
4th
p-AMPK
ATP
ADP
NADH:ubiquinone oxidoreductase
p-CAMKKβ
p-JNK
NF-κβ
Iκβ
TNF-α
NF-κβ
Ca2+
Ca2+
sarcoplasmic reticulum
cell nucleus
DNA
summary
glucose transporter 4;
c-Jun-N-terminal kinase;
organic cations transoprter;
serine-threonine kinase;
NADH:ubiquinone oxidoreductase;
insulin receptor
insulin receptor substrate;
tumor necrosis factor;
NF-κβ inhibitor;
Myosin V;
adenosine monophosphate kinase II;
rab proteins;
nuclear factor transcription
Ca2+/calmodulin dependent kinase protein β;
TBC1 domin family members;
insulin
inositol 1,4,5 triphosphate;
phosphatidylinositol 4,5 biphophate;
G actin;
metformin
stimulation - insulin signal
stimulation - metformin signal
inhibition - metformin signal
expected-stimulation signals.
expected-inhibition signals.
*The size of the molecules are not proportional

**Figure 6: Metformin role on skeletal muscle.**
Schematic draw of a proposed pathway for the molecular mechanism of metformin on skeletal muscle. Arrows indicate the modulations (↑ increase, ↓ decrease) of each parameter in insulin (D+I)- or metformin (D+M)-treated diabetic rats. Scheme shows the molecular mechanism of metformin on skeletal muscle by different signaling pathways: 1$^{th}$ inhibition of complex I mitochondrial, 2$^{th}$ activation AMPK, 3$^{th}$ maintenance of NF-κB/IκB complex and 4$^{th}$ inhibition of TNFα. Indirectly, the metformin active AMPK and CaMKKβ, which are regulatory points. In addition, metformin increases IκB and decrease NF-κB concentration and consequently inhibits TNFα expression and p-JNK concentration. Furthermore, metformin increases IRS*thr*, Myosin V and GLUT4 concentrations. Taken together, metformin improve glucose uptake decrease inflammatory pathway and increase GLUT4 translocate.

**Supplementary figure**




**Supplementary figure.**
**Representative figure of inflammatory signaling pathways induced by hyperglycemia of gastrocnemius muscle of hypoinsulinemic diabetic rats.** A- SDS-PAGE 5-22% stained with coomassie blue (b- before; a- after). B- WB of inflammatory pathway. Antibody uses: anti-myosin V, anti-IRS*thr*, anti-IR, anti-NF-κB, anti-p-AMPK, anti-AMPK, anti, p-JNK, anti-CAMKKII, anti-p-CAMKKII and, anti-IκB.

**Chapter 4 – paper 3**

Submits to Journal of Diabetic Research

# The effects of metformin on inflammatory and apoptotic pathways in soleus muscle of diabetic rats.

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Running title: Anti-inflammatory mechanism of metformin in soleus muscle

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**Abstract**

Diabetes is characterized by a proinflammatory state which can activate TLR2 and TLR4, and these receptors could induce NF-κB and JNK activation in skeletal muscle. In this study, we investigated the inflammatory and apoptotic signaling pathways triggered by TLRs/NF-κB and JNK activation in skeletal muscle of diabetic rats treated with metformin before and after an insulin tolerance test. Metformin treatment decreased p-JNK and NF-κB, and increased IκB concentrations. This attenuation leads to a decrease of TNFα and CXCL1/KC, and an increase of p-AMPK, BAX and Bcl2 concentration. Furthermore, KITT revealed an improvement of the insulin sensitivity in the diabetic rats treated with metformin. In addition, metformin was not capable of attenuating the changes in the inflammatory pathway triggered by insulin injection as the increase of TNFα and TLR4 in metformin treated rats, and IκB, CXCL1/KC, TNFα and p-AMPK increase in the untreated group. Taken together, these results point out that metformin may attenuate the activation of the inflammatory pathway TLRs/NF-κB/TNFα/CXCL1/KC and the apoptotic signaling BAX/Bcl2/p-JNK, which could be accompanied by a reduction of the inflammatory damage caused by hyperglycemia and an improvement of insulin sensitivity in diabetic rats.

**Key words:**

Insulin resitance, toll like receptor, NF-κB/IκB pathway, skeletal muscle

## Introduction

Diabetes is characterized by a proinflammatory state and high blood glucose levels induces the activation of several inflammatory pathways [1-3], besides being associated with disorders of the immune system [4]. Inflammation is pivotal in the development of insulin resistance in part via the activation of the toll-like receptors (TLRs), as TLR4 and TLR2 [5, 6]. TLR4 and TLR2 expression are increased in diabetes [7] and the activation of these receptors contributes to insulin resistance in muscle [8].

Upon activation, TLR4 and TLR2 recruit the transcription factor nuclear factor-κβ (NF-κB) and the c-Jun NH2-terminal kinase (JNK), which initiates the transcription of proinflammatory cytokine genes [9-12], as chemokine ligand 1 (CXCL1/KC) and tumor necrosis factor (TNFα). These chemokines are involved with decreased insulin sensitivity [13]. In skeletal muscle, both JNK [14] and NF-κB [15, 16] signal transduction pathways impair insulin signaling. In addition, JNK may induces the phosphorylation of serine residues in insulin receptor substrate (IRS-1) [17-19] while NF-κB induces, by transcriptional activation, the production of proinflammatory cytokines, including TNFα, IL-6, IL-1β, and resistin[20, 21]. These cytokines further activates JNK and NF-κB pathways through a feed-forward mechanism [22].

JNK and NF-κB are activated and inhibited by different mechanisms. Classically, JNK can be activated by bothMKK4 and MKK7. These enzymes can phosphorylate Thr-Pro-Tyr residues induced by several stress stimuli and it is inhibited by JNK interacting protein (JIP) [23]. NF-κB in turn, is activated by the phosphorylation of inhibitor κβ (IκB), which liberates NF-κB to translocate into the cell nucleus stimulating the transcription of inflammatory mediators[24]. The lack of inhibition of these molecules can stimulate apoptotic processes, otherwise trigger inflammation. However, the mechanism of the interactions among these mediators remains unclear.

Evidence indicates that both JNK and NF-κB also participate of pro-apoptotic and anti-apoptotic pathways, respectively. JNK activation also promotes BAX translocation to mitochondria [25] and the phosphorylation of Bcl2, suppressing its function [26]. NF-κB also activates Bcl-2 expression [27]. BAX and Bcl2 are members of Bcl-2 family of proteins, which can be divided into pro-apoptosis (BAX, Bid, Bad, Bak, Bic, Bok, Bcl-XS, and Hrk) or anti-apoptosis (Bcl2, Bcl-XL, Bcl-W, A1 and Mcl-1) proteins [28].

Metformin is a drug that has a hypoglycemiant action, broadly used in the treatment of diabetes, and with a variety of other applications[29]. For example, in the inflammatory pathway, metformin inhibited the nuclear translocation of NF-κB and the phosphorylation of STAT3 in cancer stem cells [30]. Moreover, this drug promotes the inhibition of TNFα-induced IκB kinase phosphorylation and IL-6 production in endothelial cells through PI3K-dependent AMP-activated protein kinase (AMPK) phosphorylation [31, 32] as well as, suppresses TLRs of the myocardial infarction in rat [33]. In apoptotic signal, metformin activates AMPK,

which stimulates JNK1–Bcl-2 signaling and disrupts the Beclin 1–Bcl-2 complex, normalizing the cardiac autophagy and attenuating high glucose–induced apoptosis in H9c2 cultured cells [34]. These findings suggest that metformin may attenuate the inflammatory damage caused by hyperglycemia and improve insulin sensitivity in skeletal muscle.

Thus, the aim of the present study was to investigate the metformin role on both inflammatory and apoptotic signaling pathway in skeletal muscles of streptozotocin-diabetic rats before and after insulin tolerance test.

## Material and methods

*Animals*

Male Wistar rats (7 weeks and ~230 g of body weight) were kept in standard conditions (22±1 °C, humidity 60%±5 and 12h light/12h darkness cycle) with *ad libitum* food and water. The procedures for handling and use of animals followed the resolutions proposed by the Brazilian Society of Laboratory Animal Science and by the Ethics Committee for Animal Research of the Federal University of Uberlandia, Brazil (CEUA / UFU 107/14).

*Diabetes Induction*

Animals were fasted 12 hours and rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ) (45 mg/kg BW). Diabetes was defined as a non-fasting glucose > 250 mg/dL in tail vein blood (Accu-Chek Performa) 10 days after STZ injection.

*Groups and treatment*

Diabetic animals were divided randomly into 2 groups (n=6): untreated diabetic (D) and diabetic treated with metformin (DM). Metformin ((metformin hydrochloride, Merck) was given daily by oral gavage at 500 mg/kg body weight. Ten days after diabetes induction, DM rats were subjected to a 7 day treatment with metformin and blood samples and soleus muscles were obtained from anesthetized rats (sodium thiopental, 60 mg/kg, ip) before and 16 min after intravenous injection of regular insulin (0.75U/kg BW) to intravenous insulin tolerance test. Then, animals were euthanized by sodium thiopental overdoses (80 mg/kg body weight).

*Intravenous insulin tolerance test*

Glucose levels were measured on samples obtained from the tail vein in *ad libitum* fed animals before (0min) and 4, 8, 12, and 16min after intravenous injection of regular insulin (0.75U/kg BW). The constant rate for blood glucose disappearance during insulin tolerance test (KITT) was calculated based on the linear regression of the Neperian logarithm of blood glucose concentrations (test strips, Advantage, Roche). The tests were performed between 0900 to 1100h [35].

*Sample collection and Tissue Preparation*

The soleus muscles were quickly removed before (right side) and 16 min after (left side) regular insulin injection to intravenous insulin tolerance test. After that, the

tissues were washed with chilled saline (NaCl 0.9%) and immersed in liquid nitrogen. For ELISA and western blotting analyses, soleus muscles were homogenized in buffer (25 mM Tris-HCl pH 7.4, cocktail protease inhibitor, 10 mM EDTA and 10 mM EGTA) during 3 min. The homogenized was centrifuged at 15.000xg for 40 min. at 4°C. The pellet was resuspended in the same homogenization buffer. The supernatant were used for the WB analysis.

*ELISA*

CXCL1/KC, TNFα proteins detection was performed with commercially available sandwich ELISA kits with capture and detection antibodies according to manufacturer's instructions (R&D Systems, Minneapolis, MN). Each sample and standard was analyzed in triplicate. The plates were read on a microplate reader (Molecular Devices, Sunnyvale, CA) and analyzed (SOFTmax software; Molecular Devices).

*SDS–PAGE and Western blotting*

Protein samples (10 µg) were applied to 5–22% polyacrylamide gradient gels under denaturing conditions [36] and stained with Coomassie brilliant blue. For immunoblotting, proteins were transferred to nitrocellulose membranes in Tris–glycine buffer as described[37] . The membranes were incubated with 5% dried milk in Tris-buffered saline (TBS-T) (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) and immunodetected using primary antibodies (anti-TLR4, anti-TLR2, anti NF-κB, anti-p-AMPK, anti, p-JNK, anti-Bcl2 and, anti-BAX ), diluted to 0.2 µg/mL in TBS-T [37]. The reaction was detected using an ECL kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions, and Armesham Imager 600 was used for exposure. The intensities of the protein bands were analyzed and quantified using the ImageQuant TL software, version 8.1 (GE Healthcare Life Science), and the results were expressed as a percentage.

*Statistical analysis*

All results were presented as mean ± SD. The Shapiro-Wilk test was used to evaluate the normality of data distribution. Data were normalized with untreated diabetic group before regular insulin injection as 1.00. Comparisons of means were performed by unpaired Student's t-test and One-way ANOVA, Student–Newman–Keuls *post hoc* test. Differences were considered significant at $p<0.05$.

## Results

Table 1. Morphometric and metabolic parameters of diabetic rats untreated and treated with metformin.

Metformin doses was 500 mg/kg body weight/day. Basal glycemia was measured in blood collected at the beginning (time 0) of the insulin tolerance test. Data are mean±SD. of 6 rats. One-way ANOVA, Student–Newman–Keuls *post hoc* test. # Statistical difference between untreated and treated with metformin. * Statistical difference between before and after insulin injection $p<0.05$.

| | untreated | | metformin treatment | |
|---|---|---|---|---|
| | **initial** | **final** | **initial** | **final** |
| **Body weight** | 265.3±23.8 | 283.9±27.9 | 244.9±14.2 | 312.6±17.9 *# |
| **Soleus muscle weight** | 0.150±0.009 | 0.146±0.012 | 0.149±0.012 | 0.145±0.02 |
| | **before insulin** | **after insulin** | **before insulin** | **after insulin** |
| **Blood glucose** | 465.8±68.5 | 443.3±25.9 | 360.2±55.8 # | 284±20.5 *# |

Table 1 shows that the untreated diabetic rats have a less weight gain compared to metformin treatment and, no change was observed in soleus muscle weights. As expected, higher blood glucose level was observed in untreated diabetic rats and metformin treatment decrease the glycaemia. In addition, after insulin injection we observed a decrease of blood glucose level in metformin treatment compared with each other groups.

*Representative figure of the role of metformin and insulin in soleus muscle of diabetic rats.*

Supplementary figure 1 shows the protein profile and representative images of apoptotic and pro-inflammatory proteins WB in soleus muscle of diabetic rats untreated and treated with metformin as well as, before and 16 min after regular insulin injection. The statistical analyses of the quantification of these proteins are demonstrated below.

*Metformin role on inflammatory markers expression in soleus muscle of diabetic rats*

To verify whether metformin treatment decreases inflammatory signal in skeletal muscle of diabetic rats and to analyze if inflammatory pathway is dependent of insulin, soleus muscles were removed from rats untreated and treated with metformin before, and after (16min), regular insulin injection. Figure 1 shows that metformin treatment suppressed both TLR2 and TLR4 concentration in soleus muscle of diabetic rats. However, only TLR4 concentration was increased 16 min after regular insulin injection in rats treated with metformin (Figure 1.A and 1.B). In addition, metformin treatment decreased NF-κB and increased IKβ concentrations. After regular insulin injection, NF-κB decreased and IκB increased in untreated diabetic rats. These changes in NF-κB and IκB parameters were not observed in insulin-treated rats (Figure 1.C and 1.D).

Then we checked if the metformin alters the expression of two inflammatory cytokines (TNFα and CXCL1/KC) in soleus muscle and the role of insulin in this process. Metformin treatment decreased TNFα and CXCL1/KC concentrations in soleus muscle compared with untreated diabetic group. Moreover, the insulin injection promoted the increase of TNFα in both untreated and treated with metformin diabetic rats (Figure 1.E and 1.F). However, CKCL1/CK increased only

in untreated diabetic group after insulin injection (Figure 1.F). Furthermore, metformin treatment decreased p-JNK concentration (Figure 1.G) before regular insulin injection. As expected, metformin treatment increased p-AMPK expression prior to insulin administration (Figure 1.H). After regular insulin injection, p-AMPK increased in untreated diabetic rats and these changes were not observed in metformin-treated rats.

*Metformin role on insulin sensitivity of diabetic rats*

To verify whether metformin treatment improves the insulin sensibility, decay glucose and plasma glucose disappearance rate were measured. Metformin treatment increase decay glucose and increase the KITT compared to untreated diabetic rats. The slope of plasma glucose concentration during 0-16min. after insulin administration in the metformin-treated group was higher than the untreated group, indicating an improving in the insulin sensitivity (Figure 2).

*Metformin role on apoptotic markers expression in soleus muscle of diabetic rats*

To understand how metformin treatment alters the dynamic of apoptotic signaling in the skeletal muscle and whether this mechanism is dependent of insulin, we probe the same samples described previously. Metformin treatment increased BAX concentration before regular insulin injection compared to untreated group (Figure 3.A) and, interestingly, similar results were observed for Bcl2 concentration. However, after insulin injection an increase of Bcl2 was verified only in the untreated group (Figure 3.B). No difference was observed on BAX:Bcl2 ratio in both untreated and treated diabetic rats, before and after insulin injection (Figure 3.C).

## Discussion

A large number of evidences suggest that metformin acts on different signaling pathways [38]. However, there are few data of this action on inflammatory/apoptotic signaling pathway in skeletal muscle. This tissue has a central role on peripheral glucose uptake and the role of inflammation and metformin treatment in skeletal muscle, especially in soleus, remains undetermined. Hyperglycemia is associated with an increase of the pro-inflammatory pathway [1-3] and disorders of the innate immune system [4]. Studies in other tissues showed that metformin acts on TLRs activity [33] and NF-κB/IκB signal [31, 39] attenuating the transcription of pro-inflammatory regulators. In the regulation of apoptotic pathway, AMPK activation by metformin also stimulate JNK-Bcl2 signaling [34]. These findings suggest that metformin may attenuate the inflammatory injuries promoted by hyperglycemia. To our knowledge, we demonstrate for the first time that metformin treatment suppresses inflammatory pathways (TLR2/TLR4 and NF-κB/ IκB/ p-JNK, TNFα and CXCL1/KC) as well as, attenuates apoptotic signaling (BAX:Bcl2 ratio) independent of p-AMPK in soleus muscle of diabetic rats.

In the inflammatory pathway, TLRs can initiate a complex signaling cascade that activates inflammatory markers leading to cellular injury (more details [40]). It is

know that diabetes stimulates the TLRs activation [41, 42] and that TLR2 and TLR4 are expressed in insulin target tissues [40, 43]. Here, we show a higher concentration of these receptors in soleus muscle of untreated diabetic rats compared to metformin treatment. Some studies have shown that metformin treatment suppressed TLRs pathway [33] and decreased the TLR4 concentration by attenuating the TLR4 mRNA [44]. This finding in other tissues corroborate with the present results in soleus muscle, which showed the attenuation of both TLR4 and TLR2 concentrations in diabetic rats treated with metformin. We also observed an increase of TLR4 concentration after regular insulin injection, this is in agreement with other study which showed the role of insulin in the transcription of TLRs [45].

Furthermore, TLR2 and TLR4 are central to the regulation of inflammatory responses through activation of NF-κB [9-12]. Some previous studies suggested that metformin may inhibit NF-κB via activation of IκB in vascular endothelial cells [39] and suppress the expression of NF-κB induced by high blood glucose level via AMPK activation in glomerular mesangial cells *in vitro* [46, 47]. As expected, metformin treatment increased phosphorylated AMPK in soleus muscle of diabetic rats. The reduction of phosphorylated AMPK in soleus after acute effects of insulin (16 minutes) are in agreement with previous studies that suggest that insulin, via activation of Akt, inhibits AMPK activity by phosphorylation of Ser 485 of α-AMPK [48, 49]. The present set of data confirmed that metformin was also able to reduce NF-κB associated with the increase in IκB concentration in soleus muscles of diabetic rats. Besides, the association of metformin with acute effects (16 minutes) of insulin do not show additional effects on NF-κB and IκB concentrations. To our knowledge, the present study showed for the first time that acute effects of insulin (16 minutes) promotes decrease in NF-κB concentration and increase in IκB concentration.

NF-κB role in transcriptional activation to production of inflammatory cytokines is well established [20, 21]. Several evidences indicate that TNFα is a cytokine involved in systemic inflammation. Other studies showed that metformin inhibited TNFα expression in bladder smooth muscle cells [50] as well as, inhibit TNFα-induced mRNA expression and NF-κB activation via AMPK in endothelial cells [31, 39]. However, this mechanism of inhibition has not yet been described in skeletal muscle. In the present study, we demonstrate that metformin treatment suppressed TNFα expression in skeletal muscle of diabetic rats which may be associated with decreased of NF-κB [51]. Surprisingly, insulin treatment promotes an increase in TNFα levels in soleus of diabetic untreated rats and diabetic metformin-treated rats. We suggest that these acute effects (16 minutes) of insulin was reduced due to metformin treatment. In addition, we showed a decrease of CXCL1/KC after metformin treatment. This chemokine is involved in the recruitment and activation of neutrophils [52] linked with the increase of TNFα [53, 54]. However, the mechanisms by which metformin inhibit this cytokine inflammatory response remains unclear.

Metformin also attenuate the activation of JNK in Alzheimer's disease-like neuropathology [55]. In addition, JNK can be activated by TNFα [56, 57] which is

increased in hyperglycemic condition [58] impairing insulin pathway. These results are consistent with our findings that showed a decrease of p-JNK levels related with the reduction in TNFα levels after metformin treatment. However, the regulation of these proteins is not correlated after acute effects of insulin in soleus muscle of diabetic rats. Some studies also showed that JNK pathway impairs insulin signal transduction directly through IRS inhibitory phosphorylation [17-19]. Although our study has not analyzed IRS substrate, we verified that metformin treatment improves glucose uptake as demonstrated by the increase of insulin sensitivity. This evidence indicated that metformin promotes the reduction of the inflammatory markers in muscle cells, which improves insulin pathway and contributes to glucose homeostasis.

Recently was reported that metformin also inhibit JNK phosphorylation in rat hepatocyte against oxidative stress-induced apoptosis [59]. Inflammatory pathway and apoptotic signaling may be interconnected by JNK/ AMPK-induced activation [60, 61]. It is well documented that JNK promotes BAX translocation to mitochondria releasing cytochrome c, which leads to apoptosis [25], as well as suppress the function of the anti-apoptotic protein - Bcl2 [26]. The ratio of BAX:Bcl2 is detrimental to the life/death signaling following apoptotic stimuli which was significantly increased in metformin-treated tumors. The antitumor effect of metformin appears to be dependent on the inhibition of NF-κB and mTOR signaling pathways [62]. Our results are in agreement with those presented in other studies, once we observed an increase in BAX concentration after the treatment with metformin. However, we also show that metformin decreases p-JNK concentration and increases the concentration of Bcl2 independent of p-AMPK. Other studies have also observed that the treatment with metformin decreased the JNK concentration, modulating the expression of Bcl2 and attenuating apoptotic processes [63, 64]. Considering that BAX:Bcl2 ratio was unchanged and p-JNK decreased after metformin treatment, the present data also revealed an attenuation of the apoptotic signaling in vivo.

Taken together, our data point out that metformin may attenuate the activation of the inflammatory pathway TLRs/NF-κB/TNFα/CXCL1/KC and the apoptotic signaling BAX:Bcl2 ratio/p-JNK independent of p-AMPK, which could be accompanied by a reduction of the inflammatory damage caused by hyperglycemia in skeletal muscle of diabetic rats. These alterations may explain the improvement of insulin sensitivity observed in diabetic rats treated with metformin. Furthermore, the treatment with metformin was not capable of attenuating the changes in the inflammatory pathway triggered by insulin injection.

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## Author contributions

Conceived and designed the experiments: L.G.P., R.R.T., R.S.S., F.S.E. Performed the experiments: L.G.P., R.R.T., D.D.V., L.N.B., S.R.D., Analyzed the data: L.G.P., R.R.T., R.S.S., F.S.E., Wrote the paper: L.G.P., L.N.B., R.R.T., R.S.S., Supervised the entire project: F.S.E.

## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## References


1. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006;444(7121):860-7. doi: 10.1038/nature05485. PubMed PMID: 17167474.
2. Pickup JC. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. Diabetes Care. 2004;27(3):813-23. PubMed PMID: 14988310.
3. Plomgaard P, Bouzakri K, Krogh-Madsen R, Mittendorfer B, Zierath JR, Pedersen BK. Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. Diabetes. 2005;54(10):2939-45. Epub 2005/09/28. PubMed PMID: 16186396.
4. Beyan H, Buckley LR, Yousaf N, Londei M, Leslie RD. A role for innate immunity in type 1 diabetes? Diabetes Metab Res Rev. 2003;19(2):89-100. Epub 2003/04/04. doi: 10.1002/dmrr.341. PubMed PMID: 12673777.
5. Kim JJ, Sears DD. TLR4 and Insulin Resistance. Gastroenterol Res Pract. 2010;2010. Epub 2010/09/04. doi: 10.1155/2010/212563. PubMed PMID: 20814545; PubMed Central PMCID: PMCPMC2931384.
6. Galbo T, Perry RJ, Jurczak MJ, Camporez JP, Alves TC, Kahn M, et al. Saturated and unsaturated fat induce hepatic insulin resistance independently of TLR-4 signaling and ceramide synthesis in vivo. Proc Natl Acad Sci U S A. 110. United States2013. p. 12780-5.
7. Devaraj S, Dasu MR, Rockwood J, Winter W, Griffen SC, Jialal I. Increased toll-like receptor (TLR) 2 and TLR4 expression in monocytes from patients with type 1 diabetes: further evidence of a proinflammatory state. J Clin Endocrinol Metab. 2008;93(2):578-83. Epub 2007/11/22. doi: 10.1210/jc.2007-2185. PubMed PMID: 18029454; PubMed Central PMCID: PMCPmc2243229.
8. Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, et al. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes. 57. United States2008. p. 2595-602.
9. Takeuchi O, Akira S. Toll-like receptors; their physiological role and signal transduction system. Int Immunopharmacol. 1. Netherlands2001. p. 625-35.
10. Lin HY, Tang CH, Chen JH, Chuang JY, Huang SM, Tan TW, et al. Peptidoglycan induces interleukin-6 expression through the TLR2 receptor, JNK, c-Jun, and AP-1 pathways in microglia. J Cell Physiol. 2011;226(6):1573-82. Epub 2010/10/15. doi: 10.1002/jcp.22489. PubMed PMID: 20945380.
11. Wang H, Wu Y, Ojcius DM, Yang XF, Zhang C, Ding S, et al. Leptospiral hemolysins induce proinflammatory cytokines through Toll-like receptor 2-and 4-mediated JNK and NF-kappaB signaling pathways. PLoS One. 7. United States2012. p. e42266.
12. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4(7):499-511. Epub 2004/07/02. doi: 10.1038/nri1391. PubMed PMID: 15229469.
13. Caricilli AM, Nascimento PH, Pauli JR, Tsukumo DM, Velloso LA, Carvalheira JB, et al. Inhibition of toll-like receptor 2 expression improves insulin sensitivity and signaling in muscle and white adipose tissue of mice fed a high-fat diet. J Endocrinol. 2008;199(3):399-406. Epub 2008/09/13. doi: 10.1677/joe-08-0354. PubMed PMID: 18787058.
14. Henstridge DC, Bruce CR, Pang CP, Lancaster GI, Allen TL, Estevez E, et al. Skeletal muscle-specific overproduction of constitutively activated c-Jun N-terminal kinase (JNK) induces insulin resistance in mice. Diabetologia.

2012;55(10):2769-78. Epub 2012/07/27. doi: 10.1007/s00125-012-2652-8. PubMed PMID: 22832498; PubMed Central PMCID: PMCPmc3590919.
15. Andreasen AS, Kelly M, Berg RM, Moller K, Pedersen BK. Type 2 diabetes is associated with altered NF-kappaB DNA binding activity, JNK phosphorylation, and AMPK phosphorylation in skeletal muscle after LPS. PLoS One. 2011;6(9):e23999. Epub 2011/09/21. doi: 10.1371/journal.pone.0023999. PubMed PMID: 21931634; PubMed Central PMCID: PMCPmc3172218.
16. Zhang J, Wu W, Li D, Guo Y, Ding H. Overactivation of NF-kappaB impairs insulin sensitivity and mediates palmitate-induced insulin resistance in C2C12 skeletal muscle cells. Endocrine. 2010;37(1):157-66. Epub 2010/10/22. doi: 10.1007/s12020-009-9283-y. PubMed PMID: 20963565.
17. Aguirre V, Uchida T, Yenush L, Davis R, White MF. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J Biol Chem. 2000;275(12):9047-54. Epub 2000/03/18. PubMed PMID: 10722755.
18. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, et al. A central role for JNK in obesity and insulin resistance. Nature. 2002;420(6913):333-6. Epub 2002/11/26. doi: 10.1038/nature01137. PubMed PMID: 12447443.
19. Werner ED, Lee J, Hansen L, Yuan M, Shoelson SE. Insulin resistance due to phosphorylation of insulin receptor substrate-1 at serine 302. J Biol Chem. 279. United States2004. p. 35298-305.
20. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med. 1997;336(15):1066-71. Epub 1997/04/10. doi: 10.1056/nejm199704103361506. PubMed PMID: 9091804.
21. Baker RG, Hayden MS, Ghosh S. NF-kappaB, inflammation, and metabolic disease. Cell Metab. 13. United States: A 2011 Elsevier Inc; 2011. p. 11-22.
22. Shoelson SE, Herrero L, Naaz A. Obesity, inflammation, and insulin resistance. Gastroenterology. 132. United States2007. p. 2169-80.
23. Vlahopoulos S, Zoumpourlis VC. JNK: a key modulator of intracellular signaling. Biochemistry (Mosc). 2004;69(8):844-54. Epub 2004/09/21. PubMed PMID: 15377263.
24. Sun Z, Andersson R. NF-kappaB activation and inhibition: a review. Shock. 2002;18(2):99-106. Epub 2002/08/09. PubMed PMID: 12166787.
25. Tsuruta F, Sunayama J, Mori Y, Hattori S, Shimizu S, Tsujimoto Y, et al. JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. EMBO J. 23. England2004. p. 1889-99.
26. Liu J, Lin A. Role of JNK activation in apoptosis: a double-edged sword. Cell Res. 2005;15(1):36-42. Epub 2005/02/03. doi: 10.1038/sj.cr.7290262. PubMed PMID: 15686625.
27. Heckman CA, Mehew JW, Boxer LM. NF-kappaB activates Bcl-2 expression in t(14;18) lymphoma cells. Oncogene. 2002;21(24):3898-908. Epub 2002/05/29. doi: 10.1038/sj.onc.1205483. PubMed PMID: 12032828.
28. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 1999;13(15):1899-911. Epub 1999/08/13. PubMed PMID: 10444588.
29. Strack T. Metformin: a review. Drugs Today (Barc). 2008;44(4):303-14. Epub 2008/06/10. PubMed PMID: 18536788.
30. Hirsch HA, Iliopoulos D, Struhl K. Metformin inhibits the inflammatory response associated with cellular transformation and cancer stem cell growth.

Proc Natl Acad Sci U S A. 2013;110(3):972-7. Epub 2013/01/02. doi: 10.1073/pnas.1221055110. PubMed PMID: 23277563; PubMed Central PMCID: PMCPmc3549132.
31. Huang NL, Chiang SH, Hsueh CH, Liang YJ, Chen YJ, Lai LP. Metformin inhibits TNF-alpha-induced IkappaB kinase phosphorylation, IkappaB-alpha degradation and IL-6 production in endothelial cells through PI3K-dependent AMPK phosphorylation. Int J Cardiol. 2009;134(2):169-75. Epub 2008/07/04. doi: 10.1016/j.ijcard.2008.04.010. PubMed PMID: 18597869.
32. Steinberg GR, Michell BJ, van Denderen BJ, Watt MJ, Carey AL, Fam BC, et al. Tumor necrosis factor alpha-induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. Cell Metab. 2006;4(6):465-74. Epub 2006/12/05. doi: 10.1016/j.cmet.2006.11.005. PubMed PMID: 17141630.
33. Soraya H, Farajnia S, Khani S, Rameshrad M, Khorrami A, Banani A, et al. Short-term treatment with metformin suppresses toll like receptors (TLRs) activity in isoproterenol-induced myocardial infarction in rat: are AMPK and TLRs connected? Int Immunopharmacol. 2012;14(4):785-91. Epub 2012/11/06. doi: 10.1016/j.intimp.2012.10.014. PubMed PMID: 23122726.
34. He C, Zhu H, Li H, Zou MH, Xie Z. Dissociation of Bcl-2-Beclin1 complex by activated AMPK enhances cardiac autophagy and protects against cardiomyocyte apoptosis in diabetes. Diabetes. 2013;62(4):1270-81. doi: 10.2337/db12-0533. PubMed PMID: 23223177; PubMed Central PMCID: PMCPMC3609561.
35. Okamoto MM, Anhe GF, Sabino-Silva R, Marques MF, Freitas HS, Mori RC, et al. Intensive insulin treatment induces insulin resistance in diabetic rats by impairing glucose metabolism-related mechanisms in muscle and liver. J Endocrinol. 2011;211(1):55-64. Epub 2011/07/13. doi: 10.1530/joe-11-0105. PubMed PMID: 21746792.
36. Laemmli UK, Favre M. Maturation of the head of bacteriophage T4. I. DNA packaging events. J Mol Biol. 1973;80(4):575-99. Epub 1973/11/15. PubMed PMID: 4204102.
37. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. Biotechnology. 1992;24:145-9. Epub 1992/01/01. PubMed PMID: 1422008.
38. Isoda K, Young JL, Zirlik A, MacFarlane LA, Tsuboi N, Gerdes N, et al. Metformin inhibits proinflammatory responses and nuclear factor-kappaB in human vascular wall cells. Arterioscler Thromb Vasc Biol. 26. United States2006. p. 611-7.
39. Hattori Y, Suzuki K, Hattori S, Kasai K. Metformin inhibits cytokine-induced nuclear factor kappaB activation via AMP-activated protein kinase activation in vascular endothelial cells. Hypertension. 2006;47(6):1183-8. Epub 2006/04/26. doi: 10.1161/01.hyp.0000221429.94591.72. PubMed PMID: 16636195.
40. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol. 21. United States2003. p. 335-76.
41. Dasu MR, Devaraj S, Park S, Jialal I. Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects. Diabetes Care. 33. United States2010. p. 861-8.
42. Cejkova P, Nemeckova I, Broz J, Cerna M. TLR2 and TLR4 expression on CD14 and CD14 monocyte subtypes in adult-onset autoimmune diabetes. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2015. Epub 2015/05/06. doi: 10.5507/bp.2015.016. PubMed PMID: 25942429.

43. Boyd JH, Divangahi M, Yahiaoui L, Gvozdic D, Qureshi S, Petrof BJ. Toll-like receptors differentially regulate CC and CXC chemokines in skeletal muscle via NF-kappaB and calcineurin. Infect Immun. 2006;74(12):6829-38. Epub 2006/09/20. doi: 10.1128/iai.00286-06. PubMed PMID: 16982839; PubMed Central PMCID: PMCPmc1698076.
44. Soraya H, Clanachan AS, Rameshrad M, Maleki-Dizaji N, Ghazi-Khansari M, Garjani A. Chronic treatment with metformin suppresses toll-like receptor 4 signaling and attenuates left ventricular dysfunction following myocardial infarction. Eur J Pharmacol. 2014;737:77-84. Epub 2014/05/21. doi: 10.1016/j.ejphar.2014.05.003. PubMed PMID: 24842192.
45. Ghanim H, Mohanty P, Deopurkar R, Sia CL, Korzeniewski K, Abuaysheh S, et al. Acute modulation of toll-like receptors by insulin. Diabetes Care. 31. United States2008. p. 1827-31.
46. Gu J, Ye S, Wang S, Sun W, Hu Y. Metformin inhibits nuclear factor-kappaB activation and inflammatory cytokines expression induced by high glucose via adenosine monophosphate-activated protein kinase activation in rat glomerular mesangial cells in vitro. Chin Med J (Engl). 2014;127(9):1755-60. Epub 2014/05/06. PubMed PMID: 24791887.
47. Giannarelli R, Aragona M, Coppelli A, Del Prato S. Reducing insulin resistance with metformin: the evidence today. Diabetes Metab. 29. France2003. p. 6S28-35.
48. Kovacic S, Soltys CL, Barr AJ, Shiojima I, Walsh K, Dyck JR. Akt activity negatively regulates phosphorylation of AMP-activated protein kinase in the heart. J Biol Chem. 2003;278(41):39422-7. Epub 2003/08/02. doi: 10.1074/jbc.M305371200. PubMed PMID: 12890675.
49. Horman S, Vertommen D, Heath R, Neumann D, Mouton V, Woods A, et al. Insulin antagonizes ischemia-induced Thr172 phosphorylation of AMP-activated protein kinase alpha-subunits in heart via hierarchical phosphorylation of Ser485/491. J Biol Chem. 2006;281(9):5335-40. Epub 2005/12/13. doi: 10.1074/jbc.M506850200. PubMed PMID: 16340011.
50. Wang Z, Cheng Z, Cristofaro V, Li J, Xiao X, Gomez P, et al. Inhibition of TNF-alpha improves the bladder dysfunction that is associated with type 2 diabetes. Diabetes. 61. United States2012. p. 2134-45.
51. Gupta S, Bi R, Kim C, Chiplunkar S, Yel L, Gollapudi S. Role of NF-kappaB signaling pathway in increased tumor necrosis factor-alpha-induced apoptosis of lymphocytes in aged humans. Cell Death Differ. 2005;12(2):177-83. Epub 2005/01/14. doi: 10.1038/sj.cdd.4401557. PubMed PMID: 15647756.
52. Luster AD. Chemokines--chemotactic cytokines that mediate inflammation. N Engl J Med. 1998;338(7):436-45. Epub 1998/02/12. doi: 10.1056/nejm199802123380706. PubMed PMID: 9459648.
53. Vieira SM, Lemos HP, Grespan R, Napimoga MH, Dal-Secco D, Freitas A, et al. A crucial role for TNF-alpha in mediating neutrophil influx induced by endogenously generated or exogenous chemokines, KC/CXCL1 and LIX/CXCL5. Br J Pharmacol. 158. England2009. p. 779-89.
54. Ehses JA, Lacraz G, Giroix MH, Schmidlin F, Coulaud J, Kassis N, et al. IL-1 antagonism reduces hyperglycemia and tissue inflammation in the type 2 diabetic GK rat. Proc Natl Acad Sci U S A. 106. United States2009. p. 13998-4003.

55. Li J, Deng J, Sheng W, Zuo Z. Metformin attenuates Alzheimer's disease-like neuropathology in obese, leptin-resistant mice. Pharmacol Biochem Behav. 2012;101(4):564-74. Epub 2012/03/20. doi: 10.1016/j.pbb.2012.03.002. PubMed PMID: 22425595; PubMed Central PMCID: PMCPmc3327803.
56. Davies C, Tournier C. Exploring the function of the JNK (c-Jun N-terminal kinase) signalling pathway in physiological and pathological processes to design novel therapeutic strategies. Biochem Soc Trans. 2012;40(1):85-9. Epub 2012/01/21. doi: 10.1042/bst20110641. PubMed PMID: 22260670.
57. Deng Y, Ren X, Yang L, Lin Y, Wu X. A JNK-dependent pathway is required for TNFalpha-induced apoptosis. Cell. 2003;115(1):61-70. Epub 2003/10/09. PubMed PMID: 14532003.
58. Moller DE. Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes. Trends Endocrinol Metab. 2000;11(6):212-7. Epub 2000/07/06. PubMed PMID: 10878750.
59. Conde de la Rosa L, Vrenken TE, Buist-Homan M, Faber KN, Moshage H. Metformin protects primary rat hepatocytes against oxidative stress-induced apoptosis. Pharmacol Res Perspect. 2015;3(2):e00125. Epub 2015/06/04. doi: 10.1002/prp2.125. PubMed PMID: 26038701; PubMed Central PMCID: PMCPmc4448984.
60. Kefas BA, Cai Y, Kerckhofs K, Ling Z, Martens G, Heimberg H, et al. Metformin-induced stimulation of AMP-activated protein kinase in beta-cells impairs their glucose responsiveness and can lead to apoptosis. Biochem Pharmacol. 2004;68(3):409-16. doi: 10.1016/j.bcp.2004.04.003. PubMed PMID: 15242807.
61. Kefas BA, Cai Y, Ling Z, Heimberg H, Hue L, Pipeleers D, et al. AMP-activated protein kinase can induce apoptosis of insulin-producing MIN6 cells through stimulation of c-Jun-N-terminal kinase. J Mol Endocrinol. 2003;30(2):151-61. Epub 2003/04/10. PubMed PMID: 12683939.
62. Chaudhary SC, Kurundkar D, Elmets CA, Kopelovich L, Athar M. Metformin, an antidiabetic agent reduces growth of cutaneous squamous cell carcinoma by targeting mTOR signaling pathway. Photochem Photobiol. 2012;88(5):1149-56. Epub 2012/05/01. doi: 10.1111/j.1751-1097.2012.01165.x. PubMed PMID: 22540890; PubMed Central PMCID: PMCPMC3476735.
63. Quentin T, Steinmetz M, Poppe A, Thoms S. Metformin differentially activates ER stress signaling pathways without inducing apoptosis. Dis Model Mech. 5. England2012. p. 259-69.
64. Arunachalam G, Samuel SM, Marei I, Ding H, Triggle CR. Metformin modulates hyperglycaemia-induced endothelial senescence and apoptosis through SIRT1. Br J Pharmacol. 2014;171(2):523-35. Epub 2014/01/01. doi: 10.1111/bph.12496. PubMed PMID: 24372553; PubMed Central PMCID: PMCPMC3904269.

## Figures and legends

## Figure 1. Metformin role on inflammatory pathway

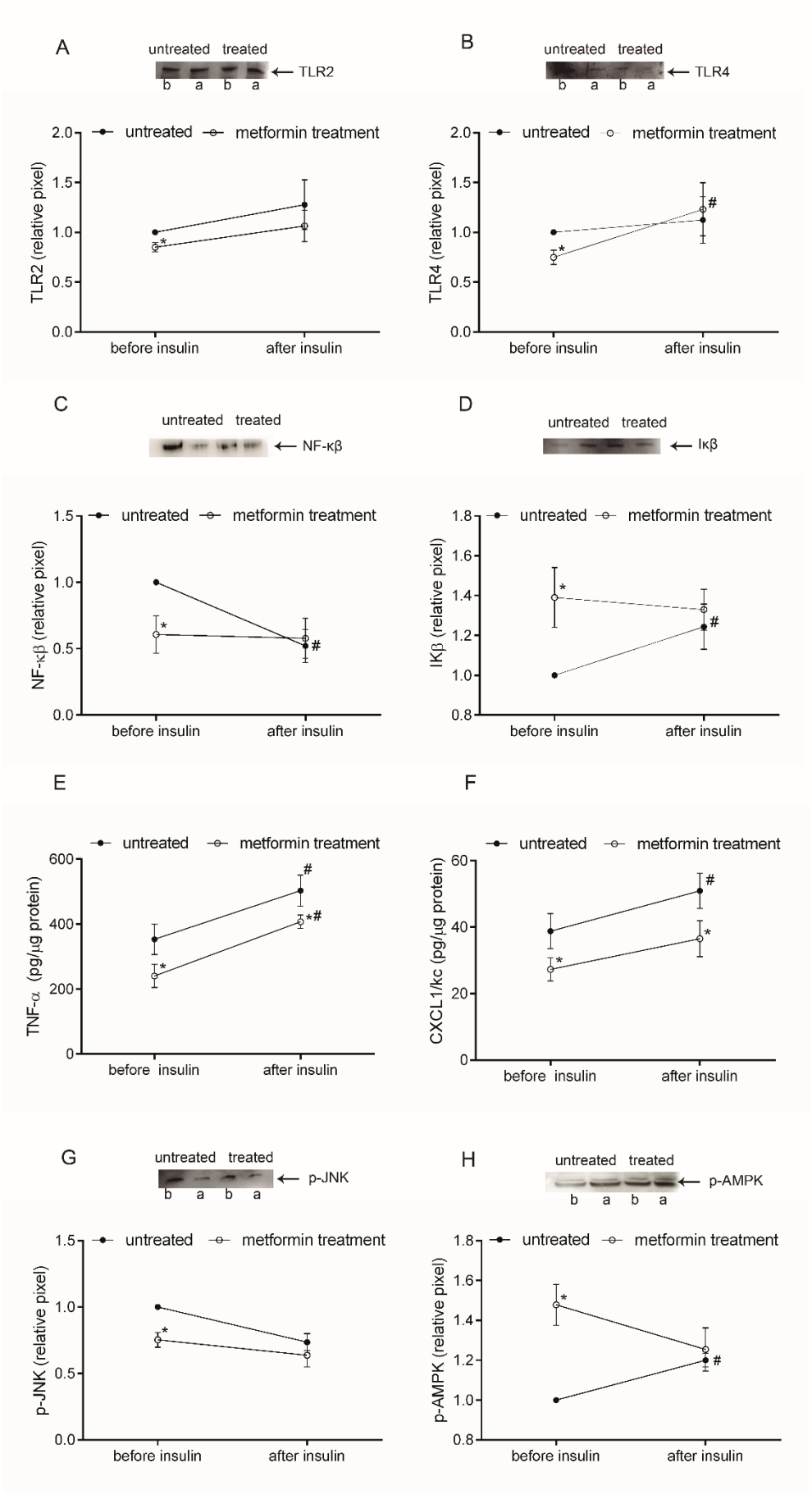


**Figure 1.** Effect of metformin on inflammatory signaling triggered by TLRs in soleus muscle. The tissue was collected before and 16 min after regular insulin injection (0.75 U/kg BW). Variation of **A**- TLR2; **B**- TLR4; **C**- NF-κB; **D**- lKβ, **E**-TNFα; **F**-CXCL1/KC; **G**- p-JNK; **H**- p-AMPK concentration before and after insulin injection. Results are means ± SD of 6 rats/group. *p<0.05 vs. untreated diabetic rats; # p<0.05 vs. before insulin injection; paired Student's.

**Figure 2. Glucose disappearance rate.**

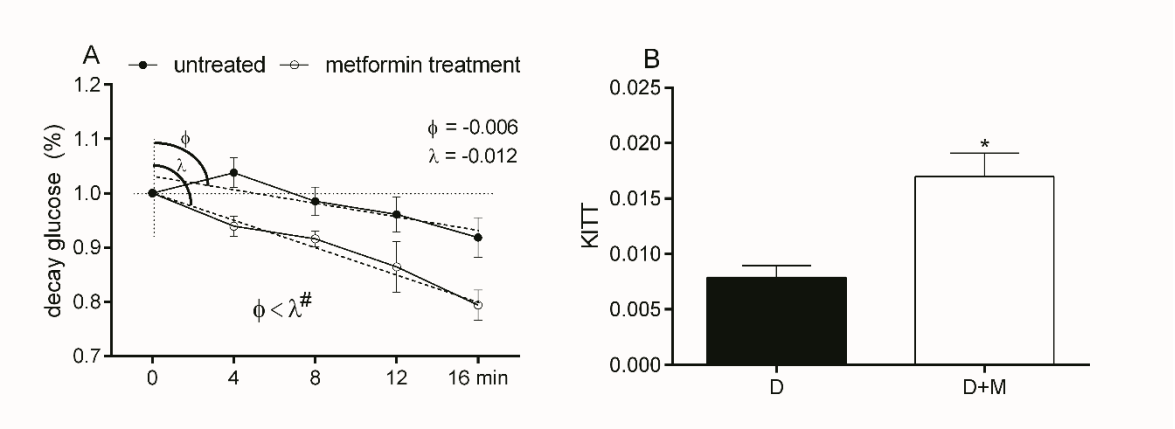



**Figure 2:** The effects of metformin on the dacay glucose (A) and plasma glucose disappearance rate (KITT) (B) in diabetic rats. Glycaemia was measured (0 min) and 4, 8, 12, and 16 min after i.v. injection of regular insulin (0.75 U/kg BW). Results represent the mean±SD (n=6), $p \leq 0.05$, (#) statistical difference before and 16 min after regular insulin injection using t-test.

**Figure 3. Metformin role on apoptotic pathway**

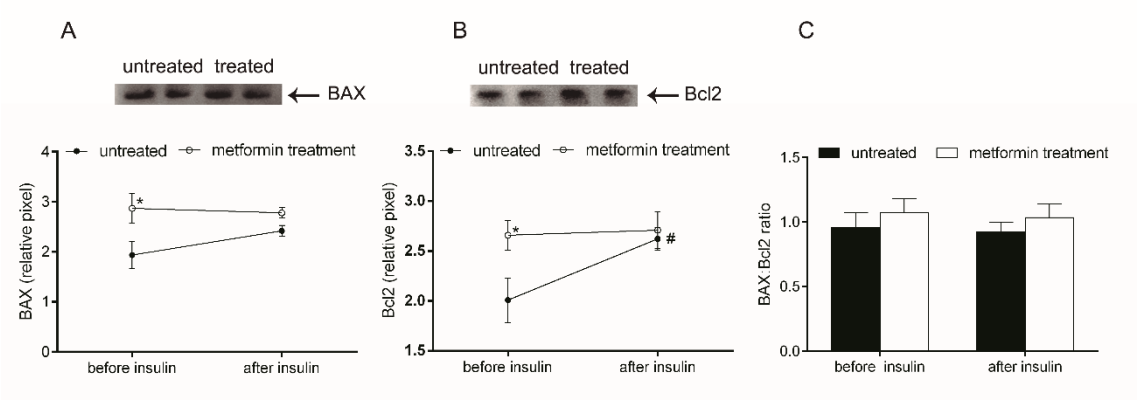
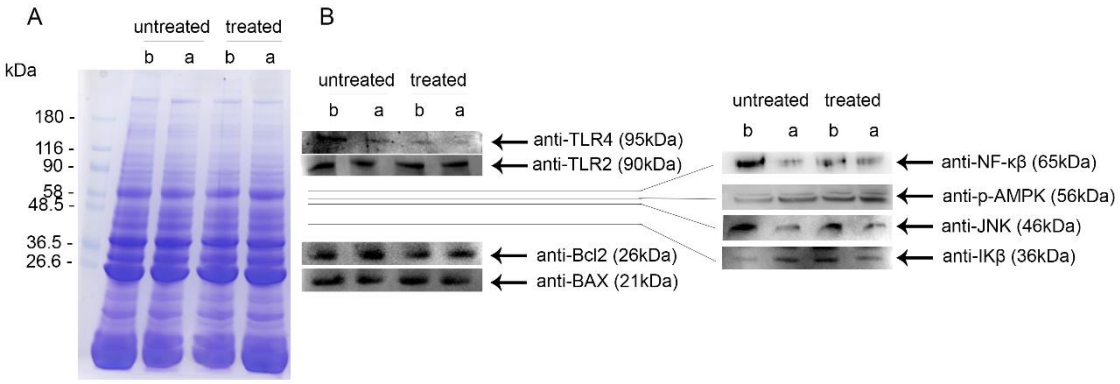



**Figure 3:** Effect of metformin on apoptotic pathway in soleus muscle. The muscle was collected before and 16 min after regular insulin injection (0.75U/kg BW). Dynamic of **A**-BAX; **B**- Bcl2; **C**- BAX:Bcl2 ratio; concentration. Results are means ± SD of 6 rats/group. *p<0.05 vs. untreated diabetic rats; # p<0.05 vs. before insulin injection; paired Student's.

**Supplementary figure. Summary of SDS PAGE and WB analysis.**




Supplementary figure: Apoptotic and pro-inflammatory signaling pathways of soleus muscle before and after insulin injection in diabetic rats untreated and treated with metformin. **A-** SDS-PAGE 5-22% stained with coomassie blue (b: before insulin injection; a: after insulin injection). **B**- WB of apoptotic and pro-inflammatory pathway.