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Effects of Metformin on TNF-α Release in Lipopolysaccharide-Induced Monocytes in Rats

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Research Article

History

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ABSTRACT

Objective: Inflammation which is a response of immune system was demonstrated in many disorders such as atherosclerosis, hypertension, diabetes, cancer and rheumatoid arthritis. Metformin, an oral antidiabetic drug, has anti-inflammatory effect apart from blood glucose regulatory effect. However, the mechanism of its anti-inflammatory effect is not clearly understood. In this study, the effect of metformin on the release of cytokines (TNF-α and IL-6) from LPS stimulated rat mononuclear blood cells was investigated.

Methods: Blood samples (5ml) were taken from healthy, male, 8-12 weeks old rats (n=5, 200-250g) through cardiac puncture under general anesthesia into sterile EDTA containing tubes. Monocytes were separated by centrifugation and were resuspended in RPMI 1640 media (3.3±0.2x105 /ml). Cells were then incubated with metformin (2.5 µM, 25 µM, 250 µM) for 2,5 hours followed by addition of LPS (100 ng/ml, 1µg/ml) for further 5 hours. After centrifugation, the supernatant was taken and TNF-α level was measured by ELISA.

Results: There was no statistically significant change in the amounts of TNF-α in the LPS + metformin groups compared to the 100 ng/ml LPS group (p>0.05). In LPS+metformin groups, compared to 1 µg/ml LPS, 2.5 µM and 250 µM metformin significantly increased TNF-α levels (p<0.05), while 25 µM metformin did not make a significant difference (p>0.05). The amount of IL-6 was not within measurable range in this study.

Conclusion: In summary, metformin increased the amount of released TNF-α rather than decreased in our study.

Keywords: Metformin, Cytokine, LPS, Inflammation, Mononuclear Rat Cells

Sıçanlarda Lipopolisakkarid ile İndüklenen Monositlerde Metforminin TNF-α Salınımı Üzerine Etkileri

Araştırma Makalesi

Süreç

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ÖZET

Amaç: İmmün sistem yanıtının bir sonucu olan inflamasyon, pek çok hastalıkta ortaya çıkan bir tablodur (ateroskleroz, hipertansiyon, diyabet, kanser, romatoid artrit gibi). Diyabet tedavisinde kullanılan metforminin, kan şekerini düzenleyici etkisinden bağımsız olarak, antienflamatuvar etkisininde olduğu bulunmuştur. Ancak etki mekanizması tam olarak açıklanamamıştır. Bu çalışmada, metforminin, lipopolisakkarit (LPS) ile inkübe edilmiş sıçan monositlerinden proinflamatuar sitokinler olan TNF-α ve IL-6 sentez ve salınımı üzerindeki etkisi araştırıldı.

Yöntem: Erkek 8-12 haftalık sağlıklı sıçanlardan (n=5, 200-250 g), genel anestezi (Xylazin 3 mg/kg + Ketamin 90 mg/kg) altında kardiyak puncture yöntemiyle 5 ml kan alındı ve steril EDTA'lı tüplere konuldu. Santrifuj (ficoll 3ml, 400xg, 30dk) yardımıyla mononükleer hücreler ayrıştırıldı. Hücreler RPMI 1640 media ile sulandırıldı (3.3±0.2x105 /ml). Hücreler önce metformin (2.5 µM, 25 µM, 250 µM) ile 2,5 saat inkübe edildi. Bu sürenin sonunda LPS (100 ng/ml, 1µg/ml) eklenerek 5 saat daha inkübe edildi. Daha sonra santrifuj (400xg, 20dk.) yapıldı, süpernatantlar -80°C'de, ELISA yöntemi ile TNF-α ve IL-6 düzeyleri ölçülene kadar saklandı.

Bulgular: Hücrelerin deneyler sonunda canlılık oranları %98'in üzerindeydi. TNF-α miktarları arasındaki fark, LPS 100 ng/ml ile, LPS + metformin (2.5µM, 25µM, 250µM) grupları karşılaştırıldığında (2179±359 ve 1613±437, 2915±572, 6059±948 pg/ml), istatistiksel olarak anlamlı bulunmadı (p>0.05). LPS 1µgr/ml ile, LPS+metformin (2,5µM, 25µM, 250µM) grupları karşılaştırıldığında (1752±553 ve 3023±745, 2344±598, 6238±841 pg/ml), 2.5µM (p0.05). IL-6 miktarları ELISA yöntemi ile ölçülecek miktarda bulunmadı. Bu konuda yapılan diğer çalışmalardan elde edilen sonuçlara göre, metforminin proinflamatuar sitokinlerin sentezini inhibe ettiği bulunmuştur.

Sonuç: Bu çalışmada, metformin, LPS ile indüklenen sıçan mononükleer hücrelerinden, TNF-α sentez ve salınımını, azaltmaktan ziyade artırmaya yönelik bir etki gösterdi.

Anahtar Kelimeler: Metformin, Sitokin, Lipopolisakkarit, Inflamasyon, Mononükleer Sıçan Hücresi

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Introduction

Inflammation is a tissue caused by physical, chemical and other factors (pathogens). It is a strong physiological response at the cellular and humoral level to cellular injury. The aim in inflammation is to eliminate the damaging factor and products, to remove the harmful confined to the ground, repair of damaged tissues and to ensure its renewal.[1] The chemical mediator mediating the inflammatory tissue response is histamine. Discoveries of these mediators are also increasing. Mediators are various chemical substances that originate from damaged tissue, cells or plasma. In addition to histamine, chemicals such as substance P, serotonin, nitric oxide and cytokines are among the mediators that play a role in inflammation. Some intermediates are involved in inflammation (eg cyclooxygenase products). Some endogenous chemical mediators are released from the area of injury. These substances, called cytokines, are molecules in the protein structure.[2] Cytokines are in polypeptide structure and the most important ones in inflammation are interleukins (IL) and tumor necrosis factor-alpha (TNF-α). In particular, IL-6 and TNF-α share many common biological properties. Both are synthesized by activated macrophages, lymphocytes and other cell types and are called proinflammatory cytokines.[3] The molecular weight of the mature form of IL-6, which is a multifunctional cytokine, varies between 22000-30000kDa and consists of 184 amino acids.[4,5] The IL-6 gene is on chromosome.[7] Mononuclear phagocytic cells are the most important source of IL-6. IL-6 is also synthesized by fibroblasts, endothelial cells, B and T lymphocytes, hepatocytes, keratinocytes, glial cells, and bone marrow stroma cells.[6] IL-6 plays an important role in the defense mechanism of the host by regulating the immune response, acute phase reactions and hematopoiesis.[4,7,8] TNF, IL-1, platelet-derived growth factor (PDGF), cytokines such as IFN-beta, antigens, mitogens and bacterial endotoxins (lipopolysaccharide) stimulate IL-6 formation in different cell types. IL-6 is an important mediator of the inflammatory response. Cells involved in host defense against infectious microorganisms and their products and secreted by damaged tissues. IL-6 and TNF alpha levels were found to be high in sepsis and especially in septic shock caused by Gram(-) bacteria.[5,9] IL-6 concentration is increased in bacterial meningitis, CSF and blood.[10,11] It has been shown that IL-6 is released from monocytes in HIV infection. During infection some cytokines affect each other. IL-1 and TNF directly affect the IL-6 gene and increase the production of IL-6.[12] Although IL-6 has antiviral activity, it stimulates the production of MHC1 class antigens with interferons.[13] Tumor necrosis factor alpha (TNF-α) is a cytokine mainly synthesized by T lymphocytes and macrophages, with a secretory form of 17 KD and a membrane form of 26 KD.[14,15] TNF-α is a potent paracrine and autocrine regulator that acts locally at low concentrations (10-9 M) in immunoinflammatory reactions. It also regulates growth and differentiation in many cell types. Especially its combination with interferon-gamma (IFN-γ) is cytotoxic. It is involved in necrosis of murine sarcomas in vivo. Studies show that TNF-α plays an important role in acute inflammation and antitumoral immunity. It manages adhesion and chemotaxis by stimulating neutrophil and endothelial cells. TNF-α is secreted from many cell types such as activated monocytes, macrophages and, to a lesser extent, activated T cells, B cells, mast cells, fibroblasts, keratinocytes, Kupffer cells, smooth muscle, synovial cover cells, and basophils. TNF-α-mediated proliferation of fibroblasts and endothelial cells is important in wound healing. In addition, TNF-α is an important stimulant in the synthesis of endothelial vascular cell adhesion molecule (VCAM). TNF-α production is inhibited by IL-10, TGF-β, PGE, cyclosporine A, dexamethasone, ibuprofen, methylprednisolone and pentoxifylline.[16,17]

Metformin (C(=NH)NHC(=NH)N(CH3)2HCl) is a biguanide derivative oral antidiabetic with a molecular weight of 165.62. Metformin is slowly absorbed from the gastrointestinal tract after oral administration. It is mainly absorbed from the small intestine. Foods reduce the absorption of metformin and prolong its absorption time. The absolute bioavailability of the drug is approximately 50-60% and metformin reaching its peak plasma concentration in 1 - 3 hours following oral administration. Its binding to plasma proteins is negligible. Depending on time, it also enters into erythrocytes. It reaches steady-state plasma concentrations within 24-48 hours. Metformin is not metabolized in the liver. 90% of the absorbed drug is excreted within the first 24 hours mainly by tubular secretion and urinary tract. The half-life is about 1.5 - 6 hours. Metformin pharmacokinetics are not altered in diabetic and nondiabetic subjects with normal renal function at clinical doses. In patients with impaired renal function, the plasma and blood half-life of metformin is prolonged. It decreases glucose production in the liver, decreases the absorption of glucose from the intestines, and increases insulin sensitivity (increases peripheral glucose uptake and use).[18] While fasting insulin levels and all-day plasma insulin response increase with metformin treatment, insulin secretion is not stimulated. Its mechanism of action is different from other oral antidiabetic agents. Since metformin does not stimulate insulin secretion, unlike sulfonylureas, it does not cause hypoglycemia or hyperinsulinemia either in patients with type 2 diabetes or in normal individuals. During metformin treatment, fasting insulin levels and all-day plasma insulin response increase, and insulin secretion does not change.[19] Metformin has positive effects on abnormal serum lipid levels in most type II diabetic patients. Metformin alone or in combination with a sulfonylurea reduces mean fasting serum triglyceride, total cholesterol and LDL cholesterol levels without adverse effects on other lipid levels.[20] Gram-negative bacteria are bacteria that do not retain the crystal violet stain during the Gram staining procedure. Many Gram-negative bacteria are pathogenic. That is, they have the ability to cause disease in humans. This disease-causing ability is mainly due to the lipopolysaccharide (LPS) content of the Gram-negative cell

wall. In vivo, gram-negative bacteria, externally added LPS, or other endotoxins elicit a series of immune system responses. The most studied toxin is LPS in gram negative bacteria. Lipid A component in the LPS structure is responsible for toxicity.[21] This antigenic structure and toxins stimulate circulating mononuclear phagocytic cells by binding to the CD14 receptor. Tumor necrosis factor (TNF-α), interleukin and platelet-activating factor (PAF) are released from monocytes.[21] In this study, in order to investigate whether metformin has anti-inflammatory activity, the effect of metformin on the release of cytokines (TNF-α and IL-6) from LPS stimulated rat monocytes was investigated.

Materials and Methods

Animals and Drugs

In the research, Male 200–250 g, 8–12 weeks old healthy rats were used and left to normal water and feeding in 12 hours light and 12 hours dark environment. Experiment protocols were approved by Sivas Cumhuriyet University Animal Ethics Committee. The animals were habituated to laboratory conditions prior to testing. All experiments were performed blindly between 10 and 15 hours. PBS (phosphate buffer saline), RPMI 1640 media (Sigma-Aldrich), Ficoll Histopaque 1083 (Sigma-Aldrich), E. Coli 0111:B4 lipopolysaccharide (LPS), Metformin HCL (Bilim Pharmaceutical Factory, Turkey), TNF- α rat ELISA were used in the study. Metformin used in the research was from Bilim Pharmaceuticals; LPS was obtained from sigma company, TNF-α kit was obtained from RayBio. Rats were obtained from Cumhuriyet University animal laboratory.

Protocol

Rats were anesthetized with xylazine + ketamine (xylazine 3 mg/kg subcutaneous, ketamine 90 mg/kg subcutaneous), blood samples were taken by cardiac puncture method and placed in sterile EDTA tubes with a 10 ml sterile EDTA syringe. Blood samples taken from rats were placed in sterile 15 ml tubes and diluted 1:1 with PBS. 3 ml of Ficoll histopaque was added to another 15 ml tube. Phosphate buffer saline and blood mixture were slowly added onto Ficoll histopaque without mixing, and centrifuged (400xg) for 20 minutes. The mononuclear cells that were clearly differentiated were transferred to a sterile 15 ml centrifuge tube with the help of a pasteur pipette and 10 ml of PBS was added. Cells were washed by gentle shaking. It was centrifuged for 10 minutes (100xg), the supernatant was discarded, washing was repeated by adding 10ml of PBS. After the second wash, the cells remaining at the bottom were diluted with RPMI 1640 media (10 ml) in which 2.5 U heparin, 100 U penicillin, 100 µg streptomycin. A 1 ml sample taken from the cell stock was spread on Thoma slide and counted under 10x magnification. The sample taken in the same way was examined morphologically and the definition of mononuclear cells was provided. Giemsa and Liscia - De Marchi staining methods were used to define the cells morphologically. The cells were spread on the slide and covered with a coverslip, stained using 1µl Trypan Blue from the space between the coverslip and their viability was examined under the microscope. Cells with disrupted cell membrane structure appeared in blue. Lipopolysaccharide (LPS) was prepared as 100 ng/ml and 1 µg/ml in 0.9% saline. Metformin was prepared as 2.5 µM, 25 µM, 250 µM in 0.9% saline. The prepared solutions were sterilized with 2-micron diameter injector filters.

Incubation of Cells with LPS and Metformin

Cells were taken into 10 eppendorf tubes (20 - 40x10^4 cells/ml) and the tubes were incubated in the incubator (Nuaire, NU 5500E, USA) with LPS and/or Metformin as indicated below in the Table 1.

In accordance with this protocol, cells were first incubated with metformin for 2.5 hours. At the end of this period, LPS was added and incubated for another 5 hours. At the end of the incubation period, all Eppendorf tubes were centrifuged for 20 minutes in a micro-centrifuge device (Sigma, 6-16K, Germany). (400xg). Supernatants were taken and stored at –80 ºC until ELISA was performed. TNF-α levels were measured using the ELISA kit (RayBiotech Inc. USA). Micro plate reader (450nM wavelength selected) was used in the measurement.

Table 1 The experimental groups

Groups	Drugs
1	Carrier only (20 µl sterile 0.9% saline)
2	Carriers and 100 ng LPS (1 ml)
3	Carriers and 1 µg LPS (1ml)
4	100ng LPS and 2.5 µM Metformin
5	100 ng LPS and 25 µM Metformin
6	100ng LPS and 250 µM Metformin
7	1 µg LPS and 2.5 µM Metformin
8	1µg LPS and 25 µM Metformin
9	1 µg LPS and 250 µM Metformin
10	Carrier and 250 µM Metformin were added

Statistics

Results were evaluated using the Student-T (paired) test (SPSS 14.0 for Windows). P<0.05 value was considered statistically significant.

Result and Discussion

Control of Cell Viability

To check the viability of the cells, the cells were spread on the slide, covered with a coverslip, and the slide was stained using 1µl Trypan Blue from the gap between the coverslips, and their viability was examined under the microscope. Cells with disrupted cell membrane structure appeared in blue color. Viability rates were found to be >98%.

The Effect of Metformin on TNF- α Levels

When 100 ng/ml LPS (2179±359 pg/ml) was compared with LPS + metformin (2.5µM, 25µM, and 250µM) (1613±437, 2915±57, 6059±948 pg/ml respectively), the difference between them was not statistically significant (p>0.05) (Table 2, figure 1). On the other hand, metformin did not affect TNF-α amounts when given alone.

Table 2. Amounts of TNF-α (pg/ml) released from mononuclear cells under the influence of LPS (100ng/ml) and LPS + Metformin (2.5, 25, 250µM)

LPS (100ng/ml) (n=5)	**LPS+Met** (2.5µM) (n=5)	**LPS+Met** (25µM) (n=5)	**LPS+Met**(250µM) (n=3)
TNF-α (pg/ml) 2179±359	TNF-α (pg/ml) 1613±437	TNF-α (pg/ml) 2915±572	TNF-α (pg/ml) 6059±948
p value	p>0.05	p>0.05	p>0.05

Mean± standard error, p values compared to LPS 100ng/ml. Met: Metformin, LPS: Lipopolysaccharide, V: Carrier

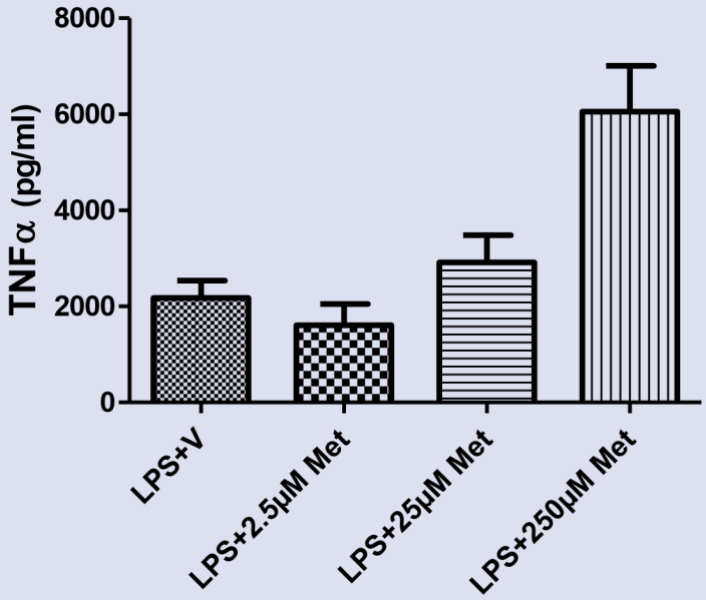

Figure 1: Amounts of TNF-α released from LPS (100 ng/ml)-induced mononuclear cells via LPS and LPS + Metformin (2.5, 25, 250µM) (pg/ml, mean±standard error). Met: Metformin, LPS: lipopolysaccharide, V: Carrier

Table 3:. Amounts of TNF-α (pg/ml) released from mononuclear cells under the influence of LPS (1µg/ml) and LPS + Metformin (2.5, 25, 250µM)

LPS (1µgr/ml) (n=5)	**LPS+Met** (2.5 µM) (n=5)	**LPS+Met** (25µM) (n=5)	**LPS+Met** (250µM) (n=5)
1752±553	3023±745	2344±598	6238±841
p value	p<0.05	p>0.05	p<0.05

Mean± standard error, p values compared to LPS 100ng/ml. Met: Metformin, LPS: Lipopolysaccharide, V: Carrier

When 1µg/ml LPS (1752±553 pg/ml) was compared to LPS + metformin (2.5µM, 25µM, and 250µM) (3023±745, 2344±598, and 6238±841 pg/ml respectively), metformin at doses of 2.5 µM and 250 µM (p<0.05) significantly increased TNF-α levels, while metformin at dose of 25 µM did not make a difference (p>0.05) (Table 3, figure 2). On the other hand, metformin did not affect TNF-α amounts when given alone.

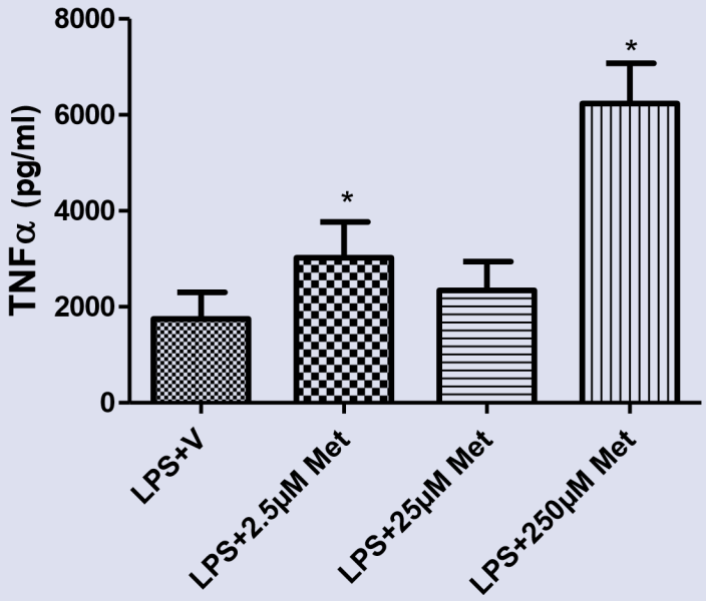

Figure 2: Amounts of TNF-α released from LPS (1μg/ml)-induced mononuclear cells via LPS and LPS + Metformin (2.5, 25, 250μM) (pg/ml, mean±standard error). Met: Metformin, LPS: lipopolysaccharide, V: Carrier

The Effect of Metformin on IL-6 Levels

The amounts of IL-6 were not measurable in all experiments.

The anti-inflammatory effects of metformin have been examined in liver, lung, endothelial cells, and macrophages in many studies. This study shows that metformin reduces liver triglyceride content in fat-rich fed rats and its anti-inflammatory effect is confirmed by the decrease in the amount of TNF-α and MMPs activity.[22] Additionally, it has been stated that metformin has an effect on improving liver dysfunction after partial hepatectomy, preventing neutrophil accumulation in the liver, and preventing the increase of IL-6 and interferon-γ.[21]

Studies in animal models reveal that metformin reduces hepatic inflammation in non-alcoholic steatohepatitis models and has an anti-inflammatory effect in conditions such as acute lung injury.[23,24,25] However, the effect of metformin on reducing the amount of TNF-α in the experiment conducted in this study differed from similar studies in the literature. It has been emphasized that high metformin concentrations may cause toxic effects and therefore should be used with caution. Although the results of the study differ from similar studies in the literature, it was emphasized that these differences may be due to factors such as species differences, experimental conditions and metformin concentrations used. At this point, the importance of studies evaluating the anti-inflammatory effects of metformin, especially at the doses used in clinical applications in humans, is emphasized. In addition to metformin's effects on glucose metabolism, the study noted that it reduced plasminogen activator inhibitor (PAI)-1, von-Willebrand factor, and smooth muscle cell contractility, and reduced inflammation markers in cases of polycystic ovary syndrome. These findings suggest that metformin may play a positive role not only in the treatment of diabetes but also in the modulation of inflammation. It has been stated that metformin increases AMP-mediated activated protein kinase (AMPK) activity and thus reduces the release of pro-inflammatory agents. This effect supports the idea that metformin may also be effective as an antiatherogenic drug in diabetic patients. According to the results of the study, the effect of metformin on reducing or increasing TNF-α release from rat mononuclear cells differed from similar studies in the literature. This difference may be due to differences in the cell type used, experimental conditions, and metformin concentrations. Therefore, it has been emphasized that in studies evaluating the anti-inflammatory effects of metformin, it is important to carefully select the species and experimental conditions.

Conclusion

At concentrations of 2.5 and 250 μM, Metformin exhibited an augmentation in the released TNF-α levels instead of a reduction, despite the induction being carried out with 1 μgr/ml LPS.

Conflict of Interest

Authors declare no conflict of interest.

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