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Determinant Role of Toll-like Receptor 4 (TLR4) on Angiotensin II in Isolated Umbilical Arteries from Normal and Gestational Diabetes Pregnant Women

Normal ve Gestasyonel Diyabetli Gebelerden Izole Edilen Umbilikal Arterlerde TLR4'ün Anjiyotensin II Üzerindeki Belirleyici Rolü

ABSTRACT

Objective: Gestational diabetes mellitus (GDM) is a common condition that occurs during pregnancy and results in cesarean section, pre-eclampsia, and neonatal morbidity. Angiotensin II is a potent vasoconstrictor and an important determinant of uteroplacental perfusion. Toll-like receptor 4 (TLR4) was found to contribute to diabetes progression. This study aimed to determine how TLR4 activation affects the contraction mediated by angiotensin II type 1 receptor in the isolated umbilical arteries of normal and GDM women.

Methods: Angiotensin II was applied with either a TLR4 agonist or TLR4 antagonists to isolated arteries from normal and GDM umbilical cords. The changes in the angiotensin II response were expressed as the maximal contraction percentage and pD2 values. The mRNA expression levels of TLR4 and angiotensin II type 1 receptor gene were measured via quantitative real-time polymerase chain reaction analysis.

Results: This study displayed an increased sensitivity to angiotensin II in the arteries of the GDM group compared with the normal group. The TLR4 agonist showed a synergistic effect with angiotensin II, while the TLR4 antagonists were not strongly determinant. In the GDM group, the TLR4 mRNA level is slightly higher than in the normal pregnancy group; however, no statistical difference was noted.

Conclusion: Toll-like receptor 4 may determine the vasoconstrictive effect of angiotensin II in normal and GDM umbilical arteries. Some studies showed a similar interaction as supportive. However, comprehensive animal and/or human studies are promising.

Keywords: Angiotensin II, gestational diabetes mellitus, lipopolysaccharides, TAK-242, toll-like receptor 4

ÖZET

Amaç: Gestasyonel diyabetes mellitus gebelikte sık karşılaşılan durumlardan biri olup sezaryen doğum, pre-eklampsi ve neonatal morbidite ile sonuçlanır. Anjiyotensin II güçlü bir vazokonstriktördür ve uteroplasental perfüzyonun önemli bir belirleyicisidir. Toll benzeri reseptör 4'ün diyabet ilerlemesine katkı sağladığı bulunmuştur. Bu çalışma, Toll benzeri reseptör 4 aktivasyonunun, normal ve gestasyonel diyabetli kadınlardan izole edilmiş umbilikal arterlerde anjiyotensin II tip 1 reseptöre bağlı kasılmayı nasıl etkilediğini belirlemeyi amaçlamıştır.

Yöntem: Normal ve gestasyonel diyabetli gebelerden izole edilen umbilikal arterlere anjiyotensin II varlığında TLR4 agonisti veya TLR4 antagonisti uygulandı. Anjiyotensin II etkisindeki değişiklikler maksimum kasılma yüzdesi ve pD2 değerleri olarak ifade edildi. Toll benzeri reseptör 4 ve anjiyotensin II tip 1 reseptör geninin mRNA ekspresyon düzeyleri kantitatif gerçek zamanlı zincir reaksiyon analizi yoluyla ölçüldü.

Bulgular: Bu çalışma, normal grupla karşılaştırıldığında gestasyonel diyabet grubunun arterlerinde anjiyotensin II'ye karşı duyarlılığın arttığını gösterdi. TLR4 agonisti, anjiyotensin II ile sinerjistik bir etki gösterirken, TLR4 antagonistleri güçlü bir şekilde belirleyici değildi. Gestasyonel diyabet grubunda TLR4 mRNA düzeyi normal gruba göre biraz daha yüksek olsa da istatistiksel bir fark gözlenmedi.

Sonuç: TLR4, normal ve gestasyonel diyabetli umbilikal arterlerde anjiyotensin II tip 1 reseptörünün vazokonstriktör etkisi üzerine belirleyici olabilir. Bazı destekleyici çalışmalar benzer bir etkileşimi göstermiştir. Ancak yapılacak kapsamlı hayvan ve/veya insan çalışmaları umut verici olacaktır.

Anahtar Kelimeler: Anjiyotensin II, gestasyonel diabetes mellitus, lipopolisakkaritler, TAK-242, toll benzeri reseptör 4

ORIGINAL ARTICLE
KLİNİK ÇALIŞMA

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Gestational diabetes mellitus (GDM) is a complication characterized by hyperglycemia, developed due to insulin dysfunction originating from the beta cells of the pancreas. It is known that patients with GDM experience dysfunction in the placenta and umbilical cord vessels due to prolonged hyperglycemia. Dysfunctions in the umbilical vessels, which facilitate oxygen and nutrient exchange between the mother and fetus, may affect fetal development. Consequently, the risk of adverse conditions such as pre-eclampsia, cesarean section, gestational hypertension, and fetal macrosomia is higher in diabetic pregnant women than in normal pregnant women.[1]

Umbilical vessels do not possess autonomic innervation. Autocrine mechanisms, such as the release of humoral vasoactive substances, play a critical role in regulating the tone of these vessels. Angiotensin II (AII) is a potent vasoconstrictor involved in blood pressure regulation. Studies have demonstrated that AII is more effective than other vasoconstrictors in maintaining placental vascular tone,[2] and that AII levels increase in pregnant rats.[3] In another study, AII was found to induce more contraction in aortic tissue from rats with GDM.[4]

The expression and endogenous ligand of toll-like receptor 4 (TLR4), an immune system receptor, is increased in type 1 diabetic mice.[5] It has also been reported that these receptors play a role in the development of type 2 diabetes and its complications in rats.[6] Furthermore, studies have shown that TLR4 mediates pathological events such as hypertension and atherosclerosis induced by increased AII levels. A TLR4 blocker has been shown to decrease mean arterial pressure and inhibit cardiac inflammatory factors in AII-induced hypertensive rats.[7] Another study demonstrated that TAK-242, a TLR4 antagonist, reduced mean arterial pressure in AII-induced hypertensive rats.[8]

The relationship between AII and TLR4 in regulating umbilical vascular tone in women with GDM has not been previously studied. This study investigates the effects of a TLR4 agonist and antagonists on the vasoconstrictor responses to AII in umbilical arteries from normal and GDM pregnant women and examines TLR4 mRNA expression levels in both groups of arteries.

Materials and Methods

Sample Collection

The Necmettin Erbakan University Ethical Review Committee of Non-Drug and Medical Device Research approved this study (Approval Number: 2020/2400, Date: 03.04.2020), and the study was conducted in accordance with the Helsinki Declaration. Umbilical artery rings were isolated from umbilical cords obtained from seven normal and seven GDM pregnant women admitted to the Obstetrics Clinic of the Faculty of Medicine, Necmettin Erbakan University.

Since waste materials from patients were used, informed consent was not applicable as previously noted.[9] Patients with any chronic diseases, especially Type II diabetes mellitus, were excluded from the study.

Isolated Organ Bath Studies

Umbilical cords were transferred to the laboratory in Krebs-Henseleit Solution (KHS) on dry ice. Krebs-Henseleit Solution was prepared as described previously.[9] Umbilical arteries were isolated, and surrounding tissues were removed. Arteries were cut into small pieces (3-4 mm wide), and ring preparations were obtained. The rings were placed into isolated organ baths, and to mimic their vital environment, 10 mL of KHS was added to each bath. The baths were continuously aerated with a mixture of 95% O_2 and 5% CO_2 at 37°C. The rings were then stretched to a basal tension of 1 g and allowed to equilibrate for 60 minutes in the KHS, with the solution being replaced every 15 minutes.

Following the equilibration period, the bath was filled with KCl (80 mM). After the maximal contraction responses were obtained with KCl, the tissues were washed with KHS at 15-minute intervals and rested for 60 minutes. Then, cumulative doses of AII (10^{-9}-10^{-4} M) were added to the bath, and the concentration response was recorded.

The experiment groups are categorized into five: AII, AII and N(gamma)-nitro-L-arginine methyl ester (L-NAME), AII and Lipopolysaccharides (LPS), AII and TAK-242, and lastly, AII and Berberine (BBR). L-NAME was incubated at a concentration of 10^{-4} M for 20 minutes.[9] LPS were applied at 0.3 µg/mL for 90 minutes.[10] TAK-242 was incubated at a concentration of 10^{-6} M for 20 minutes,[11] and BBR at 10^{-5} M for 30 minutes [12] before the addition of cumulative AII.

A transducer (BIOPAC MP36, USA) recorded the changes in the contraction responses isometrically (Commat, Türkiye) once they reached the resting period. The contraction responses mediated by the angiotensin II type 1 receptor (AGTR1) were calculated as the ratio to 80 mM KCl. Measurements were calculated and presented for each artery as a percentage of the maximal AII response (Emax) and the negative logarithm of the drug concentration that produces 50% of the maximal relaxation (pD2).

Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Samples from isolated arteries, weighing 25-50 mg, were cut into small pieces. RNA isolation was performed using an RNA isolation kit (SanPrep microRNA Kit, SK8811, Bio Basic, United States of America). cDNAs were amplified with Master Mix (Blastaq qPCR MasterMix, G891, ABM, Canada) using SYBR® Green and the primers mentioned in Table 1. LightCycler® 96 (Roche) was used for qRT-PCR analysis of the expression levels of the target genes AGTR1 and TLR4. Amplicons were normalized to the GAPDH housekeeping gene. Analysis was repeated twice and data is presented as the $2^{-\Delta\Delta Ct}$ value.

ABBREVIATIONS

AGTR1	Angiotensin II type 1 receptor
AII	Angiotensin II
BBR	Berberine
GDM	Gestational diabetes mellitus
KHS	Krebs-Henseleit Solution
L-NAME	N(gamma)-nitro-L-arginine methyl ester
LPS	Lipopolysaccharides
TLR4	Toll-like receptor 4

Chemicals

Human angiotensin II (17150) was purchased from Cayman Chemical. Other chemicals were bought from Sigma-Aldrich. TAK-242 (614316) and BBR (B3251) were dissolved in dimethyl sulfoxide. This concentration had no pharmacological effect.[13] LPS (L3129), L-NAME (N5751), and the others dissolved in distilled water.

These chemicals were selected because LPS, derived from Gram-negative organisms, is an exogenous agonist of TLR4. TAK-242 is a non-competitive inhibitor of TLR4.[14] Another substance that inhibits the TLR4 signaling pathway is BBR, a herbal alkaloid.[15] TAK-242 and BBR were used as antagonists of the TLR4 receptor. Nitric oxide (NO), produced by endothelial nitric oxide synthase present in vascular endothelial cells, plays an important role in maintaining vascular tone in pregnant women.[16] L-NAME is an inhibitor of endothelial nitric oxide synthase and consequently inhibits NO production.

Statistical Analysis

One-way analysis of variance was used to evaluate the values of the percentage of the maximal AII response (Emax) and the negative logarithm of the drug concentration that achieves 50% of the maximal relaxation (pD2). Furthermore, post hoc Dunnett's test was used for significant differences. For the evaluation of qRT-PCR analysis, the Mann-Whitney U test was employed. GraphPad Prism version 5.0 software (GraphPad Software Inc.; San Diego, CA, USA) was used to perform all analyses. A p-value below 0.05 was accepted as significant.

Results

Isolated Organ Bath Studies

AII produced dose-dependent contractile responses in umbilical artery rings taken from normal and GDM pregnant women. There was no difference in the Emax values of AII between the normal and GDM umbilical arteries ($P > 0.05$) (Figure 1). When pD2 values were compared, it was determined that there was an increase in sensitivity to AII in the GDM group ($P < 0.05$) (Figure 2).

Incubation of tissues taken from normal pregnant women with L-NAME and LPS significantly increased both the Emax and pD2 values of AII, but only a significant increase in the Emax value was observed in the GDM group (Figures 1 and 2).

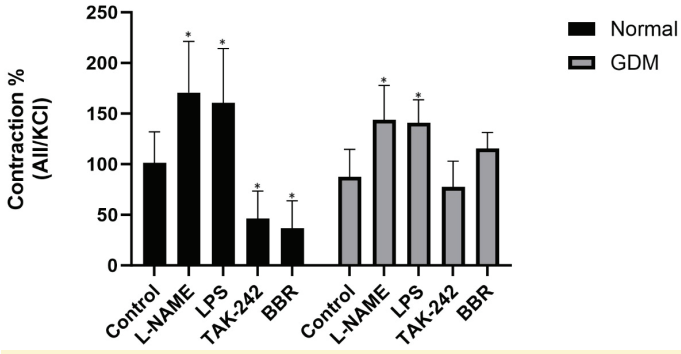

Figure 1. Emax values of angiotensin II (AII) in the normal and gestational diabetes mellitus (GDM) groups.

***P < 0.05 (compared to each group's own control values).**

Incubation of arteries taken from normal pregnant women with TAK-242 decreased the Emax value of AII ($P < 0.05$), but no significant difference was found in the pD2 values ($P > 0.05$). In the GDM group, incubation with TAK-242 showed a decreasing tendency in the Emax value of AII, but it was not significant ($P > 0.05$); however, it significantly reduced the pD2 value ($P < 0.05$) (Figures 1 and 2).

Incubation of tissues with BBR decreased the Emax value of AII and increased pD2 in normal pregnant women. In the GDM group, contraction responses mediated by AGTR1 and the pD2 value did not change (Figures 1 and 2).

qRT-PCR Analysis of TLR4 and AGTR1

Reverse transcription polymerase chain reaction analysis was used to examine the expression levels of mRNA of TLR4 and AGTR1. Toll-like receptor 4 mRNA expression level showed a relative increase in the GDM group compared with the normal group (Figure 3). However, the difference failed to reach significance

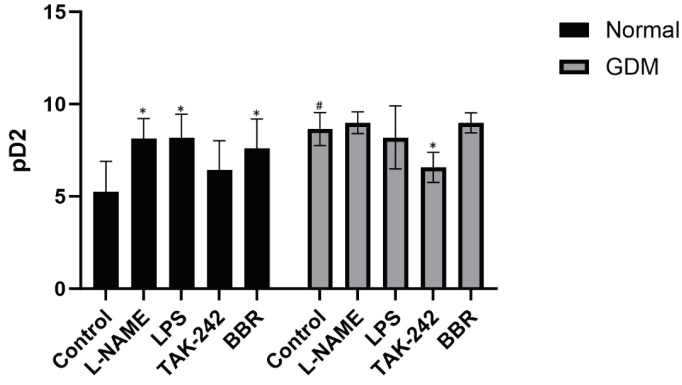

Figure 2. pD2 values of AII in the normal and GDM groups.

***P < 0.05 (compared to each group's own control values), #P < 0.05 (comparison between control groups of the normal and GDM groups).**

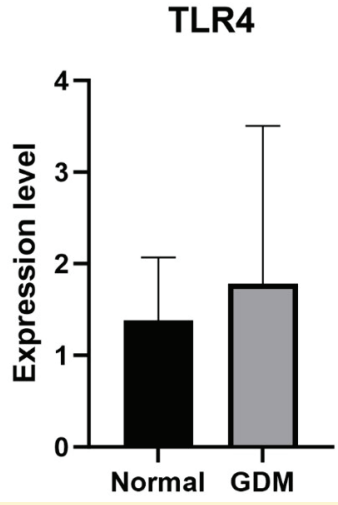

Figure 3. Toll-like receptor 4 (TLR4) mRNA expression levels in the normal and GDM groups. Measurements were repeated twice ($n = 7$, $P > 0.05$).

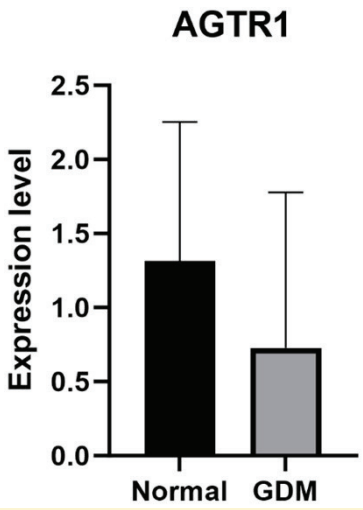

Figure 4. Angiotensin II type 1 receptor (AGTR1) mRNA expression levels in the normal and GDM groups. Measurements were repeated twice (n = 7, P > 0.05).

(P > 0.05). The result for AGTR1 expression also did not reach significance (P > 0.05) (Figure 4).

Discussion

In this study, the maximum contraction responses mediated by AGTR1 in arteries with GDM were not different from those in arteries of normal pregnant women. However, increased sensitivity to AII was observed in the GDM group. Incubation with LPS and L-NAME increased the maximum contraction and sensitivity to AII in the normal pregnant group, and only increased the maximum contraction responses mediated by AGTR1 in the GDM group. TAK-242 and BBR reduced the maximum contraction responses mediated by AGTR1 in the normal pregnant group, and BBR incubation caused an increase in its sensitivity. In the GDM group, TAK-242 and BBR did not change the maximum response to AII, but incubation with TAK-242 decreased the sensitivity of tissues to AII.

Studies in diabetic pregnant women and animal models have shown that endothelial and vascular dysfunction develops.[17,18] Razak et al.[19] in 2018 investigated the effects of U46619 (a thromboxane A2 analog) and adenosine on the chorionic arteries of women with GDM. U46619 did not change contractions in the chorionic arteries but decreased contraction responses in the chorionic veins. In the same study, adenosine reduced contractions in both chorionic arteries and veins in GDM. In another study, contraction mediated by the AGTR1 decreased in the thoracic aorta of rats with GDM while it increased in the abdominal aorta.[20] In our study, the contractile responses mediated by AGTR1 in the umbilical arteries of women with GDM did not differ compared to those in normal pregnant arteries, but the sensitivity of the arteries to AII increased in the GDM group.

L-NAME increased the contractile responses to AII in umbilical arteries from normal pregnant women. This finding suggests that contractile responses to AII in normal pregnant arteries can be modulated by endothelial NO. In women with GDM, circulating NO levels are reduced.[16] That incubation with L-NAME did not affect contractile responses to AII in arteries from pregnant women with GDM may be due to the development of diabetes-related endothelial dysfunction.[18]

A study has shown that TLR4 signaling mediates events such as hypertension and nephropathy that develop due to increased AII.[21] In a study in mice, AII infusion increased TLR4 mRNA levels in aortic tissue, and this increase was reduced by treatment with an anti-TLR4 antibody.[22] In another study, TLR4 expression was increased in hypertensive rat aorta and vascular smooth muscle cells, and the AII receptor blocker losartan prevented this increase.[23] It has also been suggested that TLR4 plays a role in the pathology of diabetes and diabetes-related complications.[5,6]

Our study investigated the relationship between TLR4 and contractile responses mediated by AGTR1 in umbilical arteries from pregnant women with GDM and normal pregnancies using LPS, TAK-242, and BBR.

Incubation of the umbilical arteries with LPS increased the contractile responses mediated by AGTR1 in both groups. Additionally, increased sensitivity to AII was also observed in arteries from the normal pregnant group. These results suggest a role for TLR4 receptor activation in the contractile responses mediated by AGTR1 in the umbilical artery.

In our study, incubating normal pregnant umbilical arteries with TAK-242 and BBR reduced maximal contractions mediated by AGTR1. Our findings suggest that TLR4 receptors play a role in umbilical artery contractile responses to AII in normal pregnant women. However, contrary to our expectations, incubation with TAK-242 and BBR did not affect the maximum contractile responses to AII in GDM arteries. Furthermore, the effects of TAK-242 and BBR on the sensitivity of tissues to AII were found to be different in this study. TAK-242 did not change the sensitivity of tissues to AII in normal pregnant umbilical arteries, but sensitivity to AII was decreased in arteries with GDM. While increased sensitivity to AII was observed in normal pregnant arteries incubated with BBR, sensitivity did not change in arteries with GDM. This may be due to the effect of BBR on mechanisms other than TLR4 antagonism in vascular structures. In a study on diabetic rats, BBR treatment increased the relaxation responses to acetylcholine in middle cerebral arteries.[24] In the study by Kang et al.[25] in 2002, intravenous berberine injection inhibited angiotensin-converting enzyme activity and produced nitric oxide-mediated relaxation in phenylephrine-contracted aortic tissues. In another study, it was reported that BBR caused contraction by increasing intracellular calcium levels in rat-isolated ventricular muscle strips.[26]

In vascular structures, the contractile effects of AII are mediated by AGTR1, while the relaxing effects are mediated by AGTR2.[3] One study showed that the contractile effects of AII and AGTR1 expression were increased in the aorta of diabetic pregnant rats compared to nondiabetic pregnant rats.[4] In another study, contraction mediated by AGTR1 was decreased in the thoracic aorta of rats with GDM while it was increased in the abdominal aorta. In addition, the mRNA expression of AGTR1 was increased in the thoracic and abdominal aorta of rats with GDM.[20] In our study, AGTR1 mRNA expression level in the GDM group was

found to be similar to the normal pregnant group. Additionally, TLR4 mRNA expression in the GDM group, despite an increasing tendency, showed no significant difference compared to normal pregnant women.

Limitations

The high standard deviation values, which are a limitation of this study, may be partly due to the difference in the effects of AII at the functional or receptor level in umbilical arteries due to GDM. Another limitation of our study is the small number of subjects. These factors limit the certainty of the study findings.

Conclusion

This study demonstrated that sensitivity to vasoconstriction mediated by AGTR1 increases significantly in GDM. It can also be concluded that the TLR4 agonist and L-NAME augment the vasoconstriction mediated by AGTR1, while TLR4 antagonists have the opposite effect. Thus, a positive correlation between TLR4 and AII in vasoconstriction may be inferred. However, these findings need to be supported by further comprehensive studies.

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Ethics Committee Approval: Our study was approved by the Necmettin Erbakan University University Ethical Review Committee of Non-Drug and Medical Device Research Approval Number: 2020/2400, Date: 03.04.2020).

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