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The Effect of P2X1 Receptor on Vascular Responses in the Diabetic Rat Model

Diyabetik Sıçan Modelinde Damar Yanıtları Üzerine P2X1 Reseptörünün Etkisi

ABSTRACT

Objective:

Although it is known that there are changes in the vascular purinergic system in diabetes, it is unknown whether P2X1-mediated vascular responses are affected. We aimed to investigate the vascular responses mediated by P2X1 receptor activation in the streptozotocin-induced diabetes model, in this study.

Method:

Animals were divided into two groups: diabetes and control. Diabetes was induced by 65 mg/kg single dose of streptozotocin. After 12 weeks, second branches of the mesenteric artery were isolated and placed into the wire myograph to evaluate the vascular responses to (ATP) and P2X1 receptor agonist. Vascular responses were also examined in the presence of endothelial nitric oxide synthase, cyclooxygenase, or K+ channel inhibitors, to determine the possible mechanism/s of relaxation responses.

Results:

In the diabetes group relaxation responses to ATP and P2X1 receptor agonists were lower compared to the control group. Vascular relaxation responses to P2X1 receptor agonists were significantly decreased in both groups in the presence of endothelial nitric oxide synthase inhibitor. Cyclooxygenase inhibitor and K+ channels inhibitors significantly blocked vascular relaxation responses in the diabetes group but not in control animals.

Conclusion:

The results of this study revealed that vascular P2X1 receptor-mediated relaxation responses are decreased in diabetes and the pathways mediating these responses were changed.

Key Words:

Diabetes, Purinergic signaling, Vasodilatation

ÖZ

Amaç:

Diyabette vasküler pürinerjik sinyalizasyonun değiştiği bilinmesine rağmen, P2X1 aracılı vasküler yanıtların etkilenip etkilenmediği bilinmemektedir. Bu çalışmada, streptozotosin ile oluşturulan diyabetik sıçan modelinde P2X1 reseptör aracılı vasküler yanıtları incelemeyi amaçladık.

Yöntem:
Hayvanlar kontrol ve diyabet olmak üzere iki gruba ayrıldı. Diyabet grubundaki sıçanlara 65 mg/kg streptozotosin, intraperitoneal yolla tek doz uygulandı. On iki hafta sonra mezenter arterin ikinci dalı alınarak, (ATP) ve P2X1 reseptör agonistine cevaben oluşan damar yanıtları, telli miyograf düzeneği kullanılarak değerlendirildi. Vasküler gevşeme yanıtlarına aracılık edebilecek olası mekanizmaların açığa çılarılması amacıyla, vasküler yanıtlar aynı zamanda endotelial nitrik oksit sentaz, siklooksijenaz ve K+ kanal inhibitörlerinin varlığında da incelendi.

Bulgular:
Diyabet grubunda ATP ve P2X1 reseptör agonistine verilen gevşeme yanıtları kontrol grubuna kıyasla önemli düzeyde düşük bulundu. P2X1 reseptör agonistine cevaben oluşan gevşeme yanıtları her iki grupta endotelial nitrik oksit sentaz inhibitörü varlığında önemli derecede azalma gösterdi. Siklooksijenaz inhibitörü ve K+ kanal inhibitörleri ise diyabet grubunda vasküler gevşeme yanıtlarını önemli düzeyde baskılarken, kontrol grubunda etkili bulunmadı.

Sonuç:
Bu çalışmanın sonuçları, diyabette vasküler P2X1 reseptör aracılı gevşeme yanıtlarının azaldığını ve bu yanıtlara aracılık eden yolakların değiştiğini ortaya koymuştur.

Anahtar Sözcükler:
Diyabet, Pürinerjik sinyalizasyon, Vazodilatasyon

INTRODUCTION

ATP and its metabolites are potent mediators of vasomotor tone in several vascular beds. Purinergic receptors, that can bind ATP and ATP degradation products, are present in many tissues in animals and humans. It is known that purinergic signaling has significant effects on vascular tonus and remodeling and plays important roles in various physiological and pathophysiological conditions in the cardiovascular system (1,2). Purinergic receptors are divided into two groups as P1 and P2 receptors and can cause contraction or relaxation of the vessel depending on the location in the vascular tissue and the signaling pathway they are paired with. Although P2 receptors are classified as P2X and P2Y, many subtypes have also been shown. The vasodilator actions of ATP are mediated mainly by P2Y receptors located on the endothelium. P2X receptors, which have seven subtypes, are ligand-gated cation channels and activated by ATP causing membrane depolarization (3). Arterial P2X receptors are usually described as smooth muscle receptors and mediate vasoconstriction (4-6). However, it's suggested by several studies that P2X receptors are also expressed on the endothelium, depending on vascular bed types (7-10). Previous studies have shown endothelial expression of P2X1 receptor in healthy rat mesenteric arteries and its activation with a specific ligand causes vasodilation of isolated mesenteric arteries (11). However, few and insufficient studies examine the role of P2X1 receptors in physiological and pathophysiological conditions compared to other purinergic receptors (1,6,8).

The chronic metabolic disorder diabetes mellitus (DM) is a disease characterized by chronic hyperglycemia. Progressive vascular dysfunction is inevitable as a result of chronic metabolic changes occurring in diabetes. On the other hand, DM contributes to the development of cardiovascular diseases such as hypertension, cardiomyopathy, and atherosclerosis. These complications accompanying diabetes are largely due to micro-and macro-vascular dysfunction. Although the underlying causes of vascular dysfunction are multifactorial, purinergic signaling is also altered in diabetes and contributes to vascular dysfunction development (12). Although studies examining how vascular signaling changes in diabetes have mostly focused on P2X7, P2X4, and P2Y1 receptor-mediated pathways, it is not yet clear whether P2X1 receptor-mediated signaling is affected (12-14). Therefore, the aim of the present study was to investigate whether P2X1 receptor-mediated purinergic signaling alters the vascular response in DM. Second, we examined the vasodilator factors that may mediate the P2X1 induced vascular responses. For this purpose, we blocked endothelial-derived vasoactive mediators, including nitric oxide (NO), prostacyclin (PGI2), or endothelium-derived hyperpolarizing factor (EDHF), independently.

MATERIAL and METHODS

Animals

Local Ethics Committee on Experimental Animal Research of Akdeniz University approved all animal procedures and experiments (ID: 628/ 2017.02.05). This research complies with all the relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration. Forty Wistar male rats (8 weeks old) were used in this study. The rats were housed in stainless steel cages with a constant room temperature at 23±2°C and on a 12:12-h light-dark cycle and had free access to rat chow and drinking water.

Grouping and induction of experimental diabetes model

The rats were randomly divided into two groups as follows: Group 1, Control (C, n=20) rats; Group 2, Streptozotocin (STZ)-induced diabetic rats (DM, n=20). Diabetes was induced in the diabetic group with a single dose of 65 mg/kg STZ in 0.1 mol/L freshly prepared citrate buffer (pH 4.5), intra-peritoneally. One week after injection of STZ, blood glucose level was measured and rats with blood glucose levels above 250 mg/dL were considered diabetic. C rats were given citrate buffer alone (pH = 4.5).

Body weights were assessed at the beginning and at the end of the experiment, fluid-food consumption and blood glucose levels were monitored regularly (daily and weekly, respectively). Blood sampling was performed from the tail vein and blood glucose was measured with the use of a glucometer.

The blood pressure (BP) of rats was measured by using a noninvasive tail-cuff method. Measurements were performed at the start and end of the experiment. Data were obtained with a MAY-BPHR 9610-PC unit and MP 150 data-acquisition system (BIOPAC Systems; Santa Barbara, CA).

Termination of the experiment

At the end of 12 weeks, all rats were killed by withdrawing the blood from the abdominal aorta under thiopental sodium (80 mg·kg−1, i.p.) anesthesia. The mesenteric vascular bed was excised and transferred to the dissecting dishes filled with ice-cold physiological saline solution (PSS) containing (in mM) 110 NaCl, 5 KCl, 24 NaHCO3, 1 KH2PO4, 1 MgSO4, 2.5 CaCl2, 0.02 EDTA, and 10 glucose.

Vascular ring preparation

A second-order branch of the superior mesenteric artery, approximately 230-250 µm in diameter, was isolated from the mesenteric vascular bed, cleaned off surrounding connective tissue, and cut into 2-mm-long segments under a dissecting microscope (SZ61, Olympus, Japan). Vessel rings were mounted in a wire myograph (Model 620M, Danish Myo Technology, Aarhus N, Denmark) with two tungsten wires (25 µm in diameter). Myograph bath solution (PSS) was maintained at 37°C and gassed continuously with 95% O2/5% CO_2 (pH 7.4). The vessel segments were rested for 15 min. prior to the normalization procedure. In the normalization procedure, the vessels were stretched to a length that yielded a circumference equivalent to 90% of that given by internal pressure of 90 mmHg. The isometric tension generated by the vessels was recorded using isometric force transducers (Danish Myo Technology, Aarhus, Denmark). Also, the diameter–tension graph was drawn and basal tension calculated with the help of computer software (LabChart Pro V7, ADInstruments, Bella Vista, Australia). After that arteries were left to rest for an hour at its calculated basal tension. The bath solution was changed every 15 min. during the resting period. After resting, the period vitalization procedure was performed by adding KCl (20 mM) and phenylephrine (Phe 10−7 M) to the bath solution, and this procedure was repeated three times, sequentially.

Three resistance arteries were obtained from each experimental animal and different protocols were applied simultaneously. The number of vessels used for each experimental protocol was between 8 and 10 (n= 8-10, each experimental protocol).

Determination of Vasodilator Responses

Dose-Response Curve to ATP and P2X1 Receptor Agonist

The dose–relaxation curve in response to ATP (10–6-10−4 M) and α,β-methylene ATP (α,β-meATP; 10–6-10−4 M), a highly selective agonist of P2X1 receptor was obtained in rings pre-contracted with Phe (10–6 M). Then 4,4',4",4"'-[Carbonyl-bis(imino-5,1,3-benzenetriyl-bis(carbonylimino))] tetrakis-1,3-benzenedisulfonic acid, octasodium salt (NF 449), a highly selective P2X1 receptor antagonist was added in the organ bath at the dose of 50 µM and after 20 min. the incubation period, the dose-response curve to ATP and α,β-meATP reevaluated. The dose–relaxation curve in response to ATP was performed at the presence of an ecto-ATPase inhibitor, 6-N, N-diethyl-D-beta, gamma-dibromomethylene ATP (ARL 67156; 10-4 M), to prevent degradation of ATP.

Assessment of Endothelium-dependent dilation

Vascular responses were also examined under four different conditions to reveal factors that may mediate vascular relaxation responses to α,β-meATP: (a) vasodilator responses were assessed in the presence of Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME; 10-4 M) in order to inhibit nitric oxide synthase (NOS). (b) vasodilator responses were assessed in the presence of indomethacin (INDO; 10-5 M) in order to inhibit cyclooxygenase (COX) and (c), vasodilator responses were assessed in the presence of apamin (APA, a calcium-activated K+ channel (KCa) blocker; (10-6 M) and (d) Tetraethylammonium (TEA, a non-specific K+ channel inhibitor; 10-6 M). Prior to obtaining the vascular responses vessels were incubated for 30 min. with inhibitors separately. In between all the consecutive protocols applied, the vascular segments were left to rest for 30 min.

All doses–relaxation curves were obtained in rings pre-contracted with Phe (10–6 M). The Phe-induced steady state of contraction was considered to be 100%, and the relaxation responses were calculated as percentages of this contraction response.

Statistical Analysis

All results are expressed as mean±SEM. Statistical analysis was performed by Paired t or unpaired Student t-test for observations between two groups. Two-way ANOVA for repeated measurements followed by the Dunnett test was used for comparison of the response curves (GraphPad Prism. 5 Software Inc, SanDiego, CA, USA). A value of $P<0.05$ was considered statistically significant.

RESULTS

Characteristics of experimental animals

Initial levels of blood glucose, body weight, food, and water intake were not different between the groups. Diabetic animals had higher blood glucose levels ($p<0.001$), food and water intake ($p<0.001$) but lower body weight than those of control rats at the final of the study ($p<0.001$). Blood glucose level, food, and water intake were higher ($p<0.001$), whereas body weight was lower ($p<0.01$) in diabetic animals at the end of the study compared to their initial values. Blood pressure levels were not different between the groups at the initial or end of the study (Table I).

Table I. Changes in blood glucose, body weight, food-water intake, and blood pressure during the experimental period

	C		DM	
	Initial	Final	Initial	Final
Blood glucose (mg/dl)	133.1±5.1	163.5±14.30	129.8±4.6	483.1±31.0***###
Body weight (g)	221.9±2.5	414.9±12.0	242.8±8.4	202.5±11.6***##
Food intake (g/day)	30.34±2.8	33.94±4.5	32.34±5.0	81.38±2.5***###
Water intake (ml/day)	51.02±3.4	48.52±5.9	52.45±4.9	128.8±6.7***###
Blood pressure (mm Hg)	136.6±4.3	142.8±3.7	145.9±3.5	144.8±4.5

C, Control; DM, Diabetes.
'Initial' and 'Final' represent the observation times of the first and twelve weeks of the experimental period, respectively.
Values are expressed as means ± SE.
***P<0.001 vs control; ##P<0.01, ###P<0.001 vs initial.

Dose-Response Curves of Small Mesenteric Arteries

The concentration-response curves to ATP and α,β-meATP in mesenteric resistance arteries from experimental groups have been shown in Figures 1a and 1b, respectively.

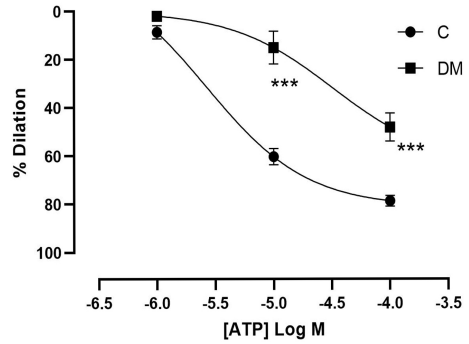

Figure 1. a. ATP-induced dilation in small mesenteric arteries of C and DM animals. C, Control; DM, diabetes. ***P<0.001 vs control.

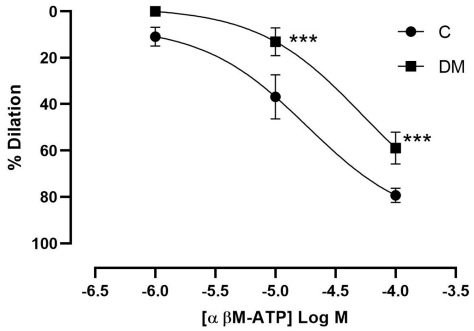

Figure 1. b. αβ-methylene ATP-induced dilation in small mesenteric arteries of C and DM animals. C, Control; DM, diabetes. ***P<0.001 vs control.

The relaxation responses to both agents significantly decreased in DM groups compared to C animals (p<0.001, p<0.001). Dilation responses to ATP (Figure 2a) and α,β-meATP (Figure 2b), were markedly blunted by the selective P2X1 receptor antagonist NF 449 (NF; 10−5 M) in both groups (p<0.001; p<0.001).

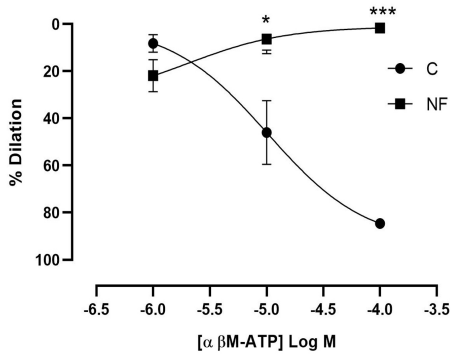

Figure 2. a. ATP-induced dilation responses in small mesenteric arteries of C and DM animals in the absence and presence of selective P2X1 receptor blocker (NF 449). C, Control; DM, diabetes; NF, NF 449. *P<0.05, *** P<0.001, vs control.

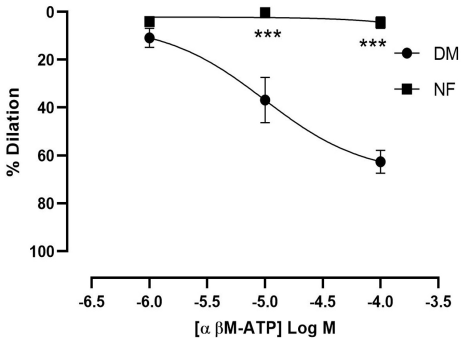

Figure 2. b. αβ-methylene ATP-induced dilation responses in small mesenteric arteries of C and DM animals in the absence and presence of selective P2X1 receptor blocker (NF 449). C, Control; DM, diabetes; NF, NF 449. ***P<0.001 vs diabetes.

Endothelium-dependent dilation

In order to examine the contribution of eNOS, COX, and EDHF pathways to the vascular responses, we also obtained dose-response curves in response to the selective P2X1 receptor agonist, in the presence of L-NAME, INDO, APA, and TEA in the organ bath. In C animals L-NAME significantly depressed the dilation responses to α,β-meATP (p<0.001), whereas INDO, TEA, and APA did not affect it (Figure 3).

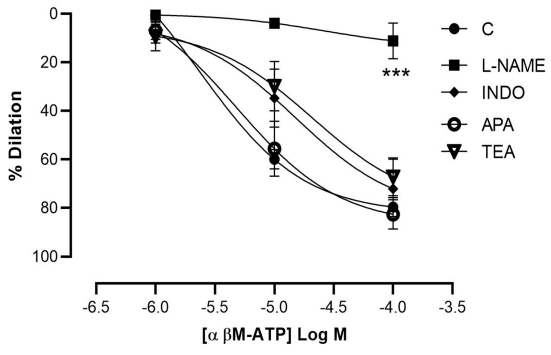

Figure 3. αβ-methylene ATP -induced dilation responses in small mesenteric arteries of C animals in the absence and presence of L-NAME, INDO, APA or TEA. C, Control; L-NAME, NOS blocker; INDO, Indomethasin; APA, Apamin; TEA, Tetraethylammonium ***P<0.001 vs control.

On the other hand, α,β-meATP -induced vascular dilation responses were significantly suppressed by all of the inhibitors excluding TEA in the DM group (p<0.001), (Figure 4).

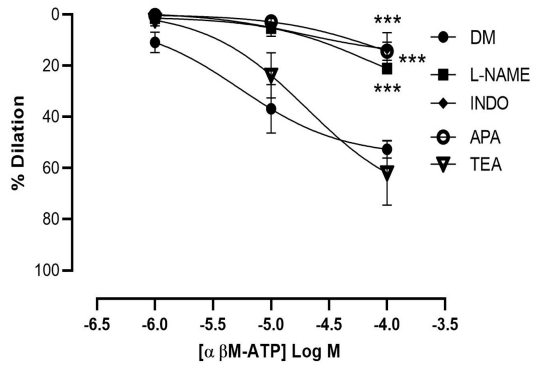

Figure 4. αβ-methylene ATP -induced dilation responses in small mesenteric arteries of DM animals in the absence and presence of L-NAME, INDO, APA or TEA. DM, diabetes. L-NAME, NOS blocker; INDO, Indomethasin; APA, Apamin; TEA, Tetraethylammonium. ***P<0.001 vs diabetes.

DISCUSSION

The relationship between diabetes and vascular complications in humans has been investigated in detail in numerous studies. It's well known that purinergic receptors are common in the vascular system and contribute to the regulation of vascular tone. The present study has investigated whether P2X1 receptors contribute to impaired vascular responses in the experimental diabetes model. According to the results of our study vascular dilation responses to ATP and a strong selective P2X1 receptor agonists, α,β-meATP, are impaired in the diabetes group. These relaxation responses were completely abolished in the presence of the specific P2X1 receptor antagonist. While ATP exerts its relaxant effect through the NOS pathway, it has been demonstrated that α,β-meATP achieves its relaxant effect primarily by NOS and partially by PGI2, a product of the COX pathway, and EDHF pathway.

In our study, STZ was used for induction of the diabetes model, and the blood glucose levels of STZ animals were found to be an average of 480 mg/dl at the end of the study. Additionally, increased food and water consumption and weight loss were detected in diabetic animals. All of these findings are in accordance with the literature that demonstrates the successful induction of the experimental diabetes model (15). Although hypertension would be expected to contribute to diabetes, measured blood pressure levels were found to be similar in C and DM groups in our study. This issue is controversial in the literature. Although some studies demonstrated the development of hypertension in STZ-induced diabetes model; other studies indicated that there is no change in blood pressure, in accordance with our findings (16,17). These different results may arise from the species and sex of the used experimental animals, the dose of STZ administered, and /or the duration of diabetes.

Besides being a neurotransmitter, ATP is an important extracellular signaling molecule and plays an important role in the regulation of purinergic signaling and vascular tone. The effects of ATP on vascular tone are mediated by purinergic receptors located on vascular smooth muscle cells and endothelium. The binding of ATP to purinergic receptors on the endothelium generally causes vasodilation mediated by NO, PGI2, or EDHF, while it is binding to its receptors in smooth muscle cells causes vasoconstriction. The common opinion is that ATP has a permanent and pronounced vasodilator effect after temporary vasoconstriction (1), in in-vitro conditions. We found a similar vasoactive pattern in mesenteric arteries in response to ATP (data not shown).

ATP exerts its effects via P2X and P2Y receptors found on vascular endothelium and smooth muscle cells. Studies investigating the effects of ATP on vascular tonus focused on endothelial P2X4, P2Y1, and P2Y2 receptors and several P2X receptors on smooth muscle cells. Although there are considerable evidence showing the expression of P2X mRNA and protein in endothelial cells, little is known about their functions. While the P2X1 receptor is expressed primarily in vascular smooth muscle cells, some studies identified its expression in some vascular endothelial cells including human mammary arteries, saphenous veins, rat mesenteric arteries, etc. Whether there are numerous studies investigating the role of P2X1 receptors found on smooth muscle, there is no study investigating the possible roles of P2X1 receptors expressed on endothelial cells.

In our study, both ATP and P2X1 agonists caused a permanent relaxation response followed by a transient and mild contraction response (data not shown) in the vessels obtained from C and DM group animals. On the other hand, it has been shown that P2X1 receptors are mostly located on vascular smooth muscle cells and cause vasoconstriction as a result of their activation (18). However, it was also shown that P2X1 receptors were also present on the endothelium of mesenteric arteries and when stimulated, they first caused temporary vasoconstriction and then permanent vasodilation (11,19). These findings coincide with the results of our study. In addition to these findings, our study has been demonstrated P2X1 receptor-mediated relaxation of small mesenteric arteries in diabetic animals for the first time. On the other hand, although both ATP and α,β-meATP generate dose-dependent relaxation responses in both groups, these responses were found to be lower in the DM group than those of C animals. In addition, these relaxation responses to both ATP and α,β-meATP were completely abolished in the presence of the P2X1 receptor antagonist. This finding confirms that relaxation responses to both ATP and α,β-meATP are predominantly mediated P2X1 receptor (20).

In our study, lower relaxation responses to ATP and α,β-meATP occurred in the diabetes group compared to the control group may be due to endothelial dysfunction common in diabetes (21). Hyperglycemia, insulin resistance, oxidant stress, decreased eNOS activity and NO bioavailability are the main factors in the development of endothelial dysfunction in diabetes (21-23). By stimulation of P2X1 receptors on endothelial cells release of endothelial-derived relaxant factors such as NO, PGI2, EDHF, has been shown before. Hence, diminished relaxation response in the DM group may cause by the decrement of these vasodilator mediators (18). Another aim of this study was to investigate the pathways involved in the relaxation responses elicited by P2X1. For this purpose, we examined the vascular relaxation responses to α,β-meATP, a P2X1 receptor agonist, in the presence of the endothelium-derived relaxant factor inhibitors in the organ bath. As a result of these experiments, it was determined that the relaxation responses to α,β-meATP

completely disappeared in the C group in the presence of L-NAME. However, inhibitors of other endothelium-derived relaxant factors, PGI2 and EDHF, did not cause any changes in the C group. In contrast to our results, another study supported that P2X1-related relaxation response is mediated mostly by EDHF in the mesenteric vascular bed of control animals (11). We demonstrated in the present study no contribution of EDHF to α,β-meATP mediated relaxation responses by using non-specific potassium channel blocker, TEA or KCa channel blocker, APA. It has been shown that the expression of P2X1 receptors decreases with age in vascular tissues (24). Therefore, these conflicting results may arise from the age differences of the rats that might cause altered receptor expression or signaling of vascular tissues in these studies. In the DM group, the vasodilation response created by α,β-meATP was inhibited not only with L-NAME but also in the presence of INDO and APA. The effect of TEA may not have been observed since it was a nonspecific potassium channel inhibitor. These results, which we obtained using K+ channel inhibitors, are compatible with the literature, and it has been reported that changes in vascular KCa activation occur in diabetes (25).

These results show that the NO, PGI2, and KCa pathways, all contribute to the P2X1 receptor-mediated dilation response in the STZ-induced diabetes rat model.

Although there is no similar study to compare the results of our study, the differences in relaxation responses to ATP and α,β-meATP, and the pathways they use, may be the cause of the changes in the vascular purinergic signaling in DM. It has even been suggested that these changes may contribute to diabetes induced vascular disorders (12,26). Impairment of purinergic receptor-NO signaling, altered purinergic receptor expression, and/or changes in purinergic receptor sensitivity are among the factors mediating endothelial dysfunction that occurs in diabetes (26).

CONCLUSION

In our study using the second branch of the mesenteric artery, α,β-meATP, the P2X1 receptor agonist caused relaxation in both healthy and diabetic animals, but this relaxation response was significantly reduced in diabetic conditions. Besides, the endothelium-derived factors that mediate the relaxation response were different in control and diabetic animals. While only the NOS pathway contributes to the relaxation response in control animals, in diabetic animals all three of the NO, PGI2, and KCa channels have been shown to contribute to the P2X1 receptor-mediated dilatation responses. Considering all the results of our study, it can be suggested that P2X1-mediated vascular purinergic signaling changes in diabetes. However, considering severe vascular complications accompany diabetes, further studies are needed to elucidate vascular purinergic signaling.

Ethics Committee Approval:

This research complies with all the relevant national regulations, institutional policies and in accodance with the tenets of the Helsinki Declaration. Local Ethics Committee on Experimental Animal Research of Akdeniz University approved all animal procedures and experiments (ID: 628/ 2017.02.05).

Author Contributions:

Concept - G.A., N.Ö., P.Ü., F.B.; Design - G.A., N.Ö., P.Ü., F.B.; Supervision - P.Ü., F.B.; Resources – Akdeniz University BAP; Materials - G.A., N.Ö., F.B.; Data Collection and/or Processing - G.A., N.Ö.; Analysis and/ or Interpretation - F.B.; Literature Search - G.A., F.B.; Writing Manuscript - F.B.; Critical Review - P.Ü., F.B.

Conflict of Interest:

The authors have no conflict of interest to declare.

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