A2B Adenosine Receptor Agonist Improves Erectile Function in Diabetic Rats

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Diabetes is an important risk factor for erectile dysfunction (ED). Recent studies have indicated that A2B adenosine receptor (ADORA2B) signaling is essential for penile erection. Thus, we hypothesize that diabetic ED may be attributed to impaired A2B adenosine signaling. To test this hypothesis, we generated diabetic rats by injecting streptozocin as animal model. After 12 weeks, immunohistochemistry staining was used to localize the expression of ADORA2B. Western Blot and guantitative PCR were employed to determine ADORA2B expression level. Intracavernosal pressure (ICP) measurement was used to evaluate erectile function. Diabetic rats received a single intravenous injection of BAY 60-6583, an ADORA2B agonist, or vehicle solution, at 60 min before the ICP measurement. The results showed that ADORA2B expressed in the nerve bundle, smooth muscle, and endothelium in penile tissue of control mice. Western Blot and quantitative PCR results indicated that the expression levels of ADORA2B protein and mRNA were significantly reduced in penile tissues of diabetic rats. Functional studies showed that the erectile response induced by electrical stimulation was remarkably decreased in diabetic rats, compared with age-matched control rats. However, at 60 min after BAY 60-6583 treatment, the erectile function was improved in diabetic rats, suggesting that enhancement of ADORA2B signaling may improve erectile function in diabetic ED. This preclinical study has revealed a previously unrecognized therapeutic possibility of BAY 60-6583 as an effective and mechanism-based drug to treat diabetic ED. In conclusion, we propose that impaired A2B adenosine signaling is one of the pathological mechanisms of diabetic ED.

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Introduction

Erectile dysfunction (ED) is defined as the inability to achieve and maintain the erection sufficient to permit satisfactory sexual intercourse. ED may be caused by psychological, vasculogenic, neurologic, hormonal, or combined factors (Lue 2000). Diabetes is an important risk factor for ED. It was reported that more than 50% of diabetes patients display ED, which seriously affects the quality of life (Thorve et al. 2011). However, the underlying pathological mechanism of diabetes induced ED is not fully clarified.

The pathogenesis of ED in diabetes is multifactorial. The possible mechanisms for diabetic ED include hyperglycemia, impaired nitric oxide (NO) synthesis, cyclic guanosine monophosphate (cGMP) pathway dysfunction, increased levels of reactive free-radicals, up-regulation of the RhoA/Rho-kinase pathway, and neuropathic damage (Gur et al. 2014). The hyperglycemic conditions in diabetes may cause oxidative stress, which impairs the function of endothelium and nerve in the penile tissue and decreases the synthesis of endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) (Bivalacqua et al. 2003; Li et al. 2012). Recent studies reported that RhoA/Rho-kinase pathway is up-regulated in diabetic rats which leads to suppress eNOS and decreases the production of NO, which finally contributes to the pathogenesis of diabetic ED (Buyukafsar and Un 2003; Bivalacqua et al. 2004). Furthermore, an early study has shown that the activation of guanylyl cyclase is reduced in diabetic mice, which decreases the cGMP production and leads to erectile dysfunction (Seftel et al. 1997).

Multiple cellular systems, factors and signaling pathways are involved in penile erection. NO is widely regarded as a principle factor controlling normal erectile function (Burnett et al. 1992; Lue 2000). Recent studies indicated that A2B adenosine receptor (ADORA2B) signaling pathway is a very important signaling involved in nor-

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mal and abnormal erection. It is reported by our early work that endogenous adenosine through ADORA2B plays important role in the physiological process of penile erection in smooth muscle cells by two mechanisms: direct induction of cAMP production and indirect induction of cGMP production following the activation of eNOS in endothelial cells via the PI3K/AKT signaling cascade (Wen et al. 2011a, b). Additionally, excess adenosine lead to priapism in adenosine deaminase (ADA) deficient mice and sickle cell disease transgenic (Tg) mice, also via ADORA2B (Mi et al. 2008; Wen et al. 2010a, b). Moreover, Faria et al. (2006) reported that adenosine regulates smooth muscle tone of human corpus cavernosum through the activation of A2A and A2B adenosine receptors, which are located on smooth muscle fibers and on endothelial cells, respectively. In addition, endothelial dysfunction was due to loss of ADORA2B activity in penile vessels from men with vasculogenic ED (Faria et al. 2006). However, the pathological role of ADORA2B in diabetic ED has not been elucidated.

Based on previous studies, we hypothesize that diabetic ED may be attributed to impaired A2B adenosine signaling pathway. In this study, diabetic ED rats were used as animal model to determine the pathological role of ADORA2B in diabetic ED. More importantly, we sought to explore the therapeutic possibility for diabetic ED by targeting A2B adenosine receptor signaling.

Materials and Methods

Animal model

Seven weeks old male Sprague-Dawley rats were administered with a single intraperitoneal injection with Streptozocin (STZ, Sigma, Louis, MO, USA) at a dose of 60 mg/kg in citrate buffer (50 mM sodium citrate, pH 4.5) (De Young et al. 2004; Li et al. 2012). Blood glucose levels in samples collected from tail vein were measured 72 hours and every 2 weeks after the injection of STZ with a blood glucose Meter. The rats with the constant blood glucose level higher than 16.7 mmol/L were considered as diabetic models. After 12 weeks, evaluation of erectile function was performed for the diabetic rats. For one group of diabetic rats, we treated them with a single intravenous bolus of BAY 60-6583 through tail vein (60 µg/kg body weight, a specific ADORA2B agonist, Tocris Bioscience, Bristol, United Kingdom) 60 min before functional evaluation (Eckle et al. 2007; Grenz et al. 2008). Bay 60-6583 (2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide) is a compound with EC_{50} equal to 2.83 nM for murine A2B receptor. For the control groups of rats and diabetic rats without BAY 60-6583 treatment, an equal volume of vehicle solution (dimethylsulfoxide) was injected. All rats were maintained and housed with the approval of the Animal Care and Use Committee at Zhejiang University.

Immunohistochemical analysis

The penises of rats were isolated and pressure-infused with 4% paraformaldehyde in PBS and fixed overnight at 4°C. Fixed penises were rinsed in PBS, dehydrated through graded ethanol washes, and embedded in paraffin. Sections of 5 μ m were collected on slides for immunohistochemistry studies. To evaluate the expression of ADORA2B, slides were processed following the instructions for ABC

Elite staining reagent and incubated with a 1:50 dilution of polyclonal antibody to ADORA2B (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) for 2 h at room temperature following the previous protocol (Wen et al. 2010b).

Preparation of protein extracts and Western blot analysis

Supernatants of corpus cavernosal tissue homogenates were electrophoresed and then transferred to a nitrocellulose membrane. The membranes were blocked for 1 hour at room temperature with blotto-Tween (5% nonfat dry milk/0.1% Tween-20) and incubated with primary ADORA2B antibody (1:100 dilution). Bound antibody was detected with labeled anti-goat secondary antibody (1:20,000) (Santa Cruz Biotechnology, CA, USA). Protein bands were quantified by densitometry using BandScan 5.0 software.

Cavernous nerve stimulation and intracavernosal pressure (ICP) measurement

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (35 mg/kg). During the procedure a heating pad at 37°C was employed to keep the rats warm (Wen et al. 2010a, b, 2011a, b; Ning et al. 2012, 2014). The bladder and prostate were exposed via a midline suprapubic incision. Bilateral cavernosal nerve, located postero-lateral to the prostate, was identified and isolated. The shaft of the penis was freed of skin and fascia. Electric stimulation of the cavernous nerve was carried with a bipolar silver electrode, positioned by a micromanipulator and placed around the cavernous nerve. The electrode cable was attached to a stimulator and the electrical stimulation was delivered by a stimulator at 2.5, 5 or 7.5 voltage with 15 Hz frequency and pulse width of 1.2 ms for 1 minute to induce penile erection (Wen et al. 2010a, b, 2011a, b; Ning et al. 2012, 2014).

To monitor the ICP, the left corpus cavernosum was penetrated with a 25-gauge needle filled with heparinized (150 U/mL) solution, which was connected to PE-50 tubing. The PE-50 tubing was connected to a pressure transducer and an amplifier unit (BL-420S data acquisition and analysis system). The amplifier was connected to a data acquisition module. The ICP before and after electrical stimulation were recorded on a computer by BL-420S system record software (Wen et al. 2010a, b, 2011a, b; Ning et al. 2012, 2014).

ICP data were normalized by mean systemic arterial pressure (MAP) and the MAP was monitored simultaneously with ICP monitoring. The right carotid artery was dissected via a midline cervical incision under the microscope, and then PE-50 tubing was inserted into the carotid artery. The catheter was connected to a pressure transducer and an amplifier unit. The amplifier was connected to a data acquisition module, and the MAP was recorded simultaneously with ICP monitoring on a computer by BL-420S system record software (Wen et al. 2010 a, b, 2011a, b; Ning et al. 2012, 2014). To evaluate the erectile function, we calculated three parameters analyzed by the BL-420S system record software. The first one is the ratio of maximal ICP to MAP. The second one is the area under the ICP curve which means the ICP multiply stimulated time (mmHg*s), we defined the ICP multiply stimulated time as total ICP in this study. The third one is the slope at which ICP increased with time following the initial carvernosal nerve stimulation.

Quantitative RT-PCR analysis

Total RNA was isolated using TRIzol reagent. RNase-free DNase was used to eliminate genomic DNA contamination.

Transcript levels were quantified using real-time quantitative RT-PCR. Cyber green was used for analysis of ADORA2B using the following primers (forward 5'-TCTTCCTCGCCTGCTTCGT-3' and reverse 5'-GGAGTCAGTCCAATGCCAAA-3') under the conditions as described previously (Wen et al. 2010b). To determine the differences of mRNA levels in various groups of rats, the median in each triplicate was used to calculate relative mRNA concentrations (Δ Ct = Ct median mRNA – Ct median β -Actin). Expression fold change compared to control rats were calculated using 2- Δ Ct methods.

The measurement of cAMP in the penile tissue

For cAMP content in the penile tissue, frozen penile tissues were homogenized in 6% trichloroacetic acid (1 ml of trichloroacetic acid per 100 mg of tissue), centrifuged and extracted with water-saturated diethyl ether. Quantitative assays for cAMP were performed using a commercial enzyme immunoassay kit (Amersham Pharmacia, Pittsburgh, USA).

Statistic analysis

All data were expressed as the mean \pm SEM. Data analysis was performed by using GraphPad Prism 6.0 software. For the two group's analysis, Student's *t* tests were used to identify statistical significance. For the multiple groups's compare, we employed the oneway analysis of variance (ANOVA) followed by a Tukey's multiple comparisons test to determine the statistical difference. A value of P < 0.05 was considered as significant.

Results

Animal model of diabetic rats

In order to generate diabetic rats, seven-week old male Sprague-Dawley rats were administered with a single intraperitoneal injection of STZ. Blood glucose levels were measured 72 hours and every two weeks after the injection of STZ. The rats with the constant blood glucose level higher than 16.7 mmol/L were considered as diabetic models. In this study, the average blood glucose concentration 72 hours after STZ injection was 19.4 ± 2.3 mmol/L compared to 5.9 ± 0.8 mmol/L in age-matched control rats, which were treated with equal volume of vehicle solution. The average blood glucose level 12 weeks after STZ injection was 36.4 ± 5.9 mmol/L in diabetic rats compared to 5.6 ± 0.9 mmol/L in age-matched control rats. The blood glucose levels in diabetic rats were summarized in Table 1.

Evaluation of erectile function in diabetic rats

Diabetes is a risk factor of erectile dysfunction (Thorve et al. 2011), and the STZ-induced diabetic rat is a well-accepted animal model of diabetic erectile dysfunction (Li et al. 2012; Liu et al. 2015; Wang et al. 2015). To deter-

mine the erectile function of our diabetic rats, we used cavernous nerve stimulation to induce the penile erection and ICP increase was measured to quantify the erectile function, which is a widely used experimental strategy to determine the erectile function in animals (Wen et al. 2011a, b; Fandel et al. 2012; Qiu et al. 2012). The ICP data were normalized with MAP and presented as ratio of maximal ICP to MAP. The result showed that the erectile response was increased in a voltage dependent manner. The ratio of maximal ICP to MAP was increased following by gradually enhanced voltage of electrical stimulation in both diabetic rats and control rats. However, the average ratio of maximal ICP to MAP in diabetic rats was remarkably decreased compared with the age-matched control rats under different voltage of stimulation (Fig. 1A, D). Furthermore, the total ICP (ICP multiply stimulation time, area under curve) and the slope at which ICP increased with time following the initial carvernosal nerve stimulation were also decreased in diabetic rats compared to control rats (Fig. 1B-D). Taken together, the functional analysis demonstrated that the erectile function was significantly impaired in the diabetic rats, which provided us with qualified erectile dysfunction animal models for the further studies.

A2B adenosine receptor is widely expressed in the penile tissue of rats

Our previous studies have demonstrated that endogenous adenosine through ADORA2B directly induces cAMP production and indirectly induces cGMP production in smooth muscle cells, which plays an important role in normal penile erection (Wen et al. 2011a, b). A human study has demonstrated that vasculogenic ED is due to loss of ADORA2B activity in penile vessels (Faria et al. 2006). We hypothesize that the diabetes induced ED may be associated with impaired A2B adenosine receptor signaling in the penile tissue. To test this hypothesis, we firstly used immunohistochemistry to localize the expression of ADORA2B in the penile tissue. The results showed that the expression of ADORA2B was detected in the nerve bundle, smooth muscle, and endothelium in the penile tissue, which were the major cell types involved in the process of penile erection (Fig. 2).

The expression level of A2B adenosine receptor is decreased in diabetic rats

To explore whether the expression level of ADORA2B in penile tissue of diabetic rats is changed compared with control rats, Quantitative PCR was employed to determine

Table 1. Blood glucose concentration in diabetic fats and control fats.								
Interval after STZ injection	72 hours (mmol/L)	1 week (mmol/L)	2 weeks (mmol/L)	4 weeks (mmol/L)	6 weeks (mmol/L)	8 weeks (mmol/L)	10 weeks (mmol/L)	12 weeks (mmol/L)
Diabetic rats	19.4 ± 2.3	31.7 ± 5.3	33.6 ± 7.7	37.5 ± 5.9	38.7 ± 6.2	34.7 ± 9.6	38.4 ± 7.6	36.4 ± 5.9
Control rats	5.9 ± 0.8	6.1 ± 0.9	5.5 ± 0.6	5.5 ± 1.0	5.7 ± 0.5	5.9 ± 0.7	6.0 ± 0.6	5.6 ± 0.9

Table 1. Blood glucose concentration in diabetic rats and control rats.

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Fig. 1. Impaired erectile function in diabetic rats with different voltage of electrical stimulation.
(A) Maximal intracavernosal pressure (ICP) which was normalized by mean systemic arterial pressure (MAP). (B) Total ICP (ICP plus stimulation time, area under curve). (C) The rate at which ICP increased with time following the initial carvernosal nerve stimulation (Slope). Data are expressed as the mean ± SEM (n = 7-8). *P < 0.05 vs. control rats. (D) Representative recordings of ICP induced by cavernous nerve stimulation at 2.5 V for 1 minute in diabetic rats and control rats. DM means diabetes mellitus rats.



Fig. 2. A2B adenosine receptor is expressed in penile tissue of rats.(A, B) The expression of A2B adenosine receptor is detected in the nerve bundle (Arrow 1), smooth muscle (Arrow 2), and endothelium (Arrows 3, 4) in penile tissue. (C, D) Negative controls for the immunohistochemistry staining. All of panels showed the sections from control rats.

the ADORA2B mRNA level in penile tissue. To determine the differences of mRNA levels in various groups of rats, the median in each triplicate was used to calculate relative mRNA concentrations ($\Delta Ct = Ct$ median mRNA – Ct median β -Actin). Expression fold change compared to control rats were calculated using 2- $\Delta\Delta Ct$ methods. The result



Fig. 3. The expression level of A2B adenosine receptor is decreased in diabetic rats. (A, B) Western blot (A) and quantitative bands analysis (B) showed the expression of A2B adenosine receptor in penile tissue of diabetic rats is significant lower than control rats. Data are expressed as the mean ± SEM (n = 5-6). *P < 0.05 vs. control rats. (F) Real-time PCR analysis showed that the mRNA level of A2B adenosine receptor in penile tissue of diabetic rats is decreased compared to control rats. Data are expressed as the mean ± SEM (n = 6-8). *P < 0.05 vs. control rats. Expression fold change compared to control rats were calculated using 2-⊿⊿Ct methods.</p>

indicated that mRNA level of ADORA2B in penile tissues of diabetic rats was significantly decreased compared to control rats (Fig. 3C). Western blot assay combined with quantitative band analysis was used to further determine the protein level of ADORA2B in both diabetic rats and control rats, showing that the expression of ADORA2B protein level in the penile tissues of diabetic rats is significantly lower than control rats (Fig. 3A, B). Thus, we have demonstrated that ADORA2B expression level is significantly decreased in penile tissues of diabetic rats in both protein and mRNA levels, which suggests that impaired A2B adenosine signaling pathway may be a pathological mechanism of diabetic ED.

Treatment with BAY 60-6583 improves erectile function in diabetic ED rats

Next, to further confirm the pathological role of impaired A2B adenosine signaling pathway in diabetic ED and address the therapeutic possibility for diabetic ED by targeting A2B adenosine receptor, we treated some of diabetic rats and control rats with a single intravenous bolus of BAY 60-6583 (60 μ g/kg body weight) 60 min before functional evaluation, which is a potential therapeutic role of a specific ADORA2B agonist (De Young et al. 2004; Li et al. 2012). Recent studies have demonstrated selectivity and functional in vivo evidence of this compound for ADORA2B (Eckle et al. 2007; Grenz et al. 2008; Koscso et al. 2013). ADORA2B is coupled to stimulatory G protein subunit (G α s) to activate adenylyl cyclase and serves to increase cAMP level (Wen and Xia 2012). To confirm that BAY 60-6583 functions as an ADORA2B agonist in our model, we measured the cAMP level in penile tissues of both diabetic rats and control rats with and without BAY 60-6583 treatment. The results showed that with BAY 60-6583 treatment, the cAMP levels in penile tissues of both diabetic rats and control rats were increased compared to the rats without treatment. These data demonstrated that the BAY 60-6583 functioned as an ADORA2B agonist and activated the ADORA2B in our rat model (Fig. 4E). At 60

min after BAY 60-6583 treatment, cavernous nerve stimulation at 5 voltages for 1 minute was employed to induce erectile response and the change of ICP was monitored with a needle connected to a pressure transducer. The results showed that diabetic ED rats with BAY 60-6583 treatment displayed dramatically elevated maximal ICP to MAP ratio as well as total ICP (ICP multiply stimulation time, area under curve) which reflects the maintenance of penile erection, compared to diabetic ED rats without treatment (Fig. 4A-C). Furthermore, the slope at which ICP increased with time following the initial carvernosal nerve stimulation was also increased in diabetic ED rats with BAY 60-6583 treatment, indicating BAY 60-6583 treated diabetic ED rats exhibited heightened initiation of penile erection (Fig. 4D). However, the average ratio of maximal ICP to MAP and total ICP in control rats with BAY 60-6583 treatment were increased, but without statistical significance, compared the control rats without treatment (Fig. 4). The increases of maximal ICP, total ICP and slope indicated that BAY 60-6583 treatment significantly improved erectile function in diabetic ED rats and demonstrated that impaired A2B adenosine signaling is one of the pathological mechanisms of diabetic ED. In addition, enhancement of A2B adenosine signaling level was capable of improving erectile function in diabetic ED, suggesting the therapeutic possibility to treat the diabetic ED by activating ADORA2B.

Discussion

In this study, we demonstrated firstly that the expression of ADORA2B in penile tissue of diabetic rats is dramatically reduced in both mRNA and protein level and enhancement of A2B adenosine signaling by BAY 60-6583, a specific ADORA2B agonist, improves erectile function in diabetic ED rats. Overall, this preclinical study has revealed previously unrecognized novel therapeutic possibility of BAY 60-6583 as an effective and mechanismbased drug to treat diabetic ED and provide strong rationale for future clinical trials involving the application of BAY 60-6583 to treat diabetic ED patients.



(A) Representative recordings of intracavernous pressure (ICP) induced by cavernous nerve stimulation at 5 V for 1 minute in diabetic rats and control rats with or without BAY 60-6583 treatment. DM indicates diabetes mellitus rats.
(B) Maximal ICP was normalized by mean systemic arterial pressure (MAP). (C) Total ICP (ICP plus stimulation time, area under curve). (D) The rate at which ICP increased with time following the initial carvernosal nerve stimulation (Slope) for both diabetic ED rats and control rats with or without BAY 60-6583 treatment. Data are expressed as the mean ± SEM (n = 5-8). **P < 0.05 vs. diabetic ED rats and control rats and control rats without BAY 60-6583 treatment. (E) With BAY 60-6583 treatment, the cAMP levels in penile tissues of both diabetic rats and control rats were increased compared to the rats without treatment. Data are expressed as the mean ± SEM (n = 7). **P < 0.05 vs. control and diabetic rats without BAY 60-6583 treatment.

Adenosine is a key extracellular signaling molecule that elicits a multitude of effects on target cells by activating specific G protein-coupled receptors (Wen and Xia 2012; Antonioli et al. 2015). Four adenosine receptors, ADORA1, ADORA2A, ADORA2B, and ADORA3, have been identified. ADORA1 and ADORA3 couple to the inhibitory G protein subunit (G α i) to inhibit adenylyl cyclase, leading to the decrease of intracellular cAMP level. ADORA2A and ADORA2B are usually coupled to stimulatory G protein subunit (G α s) to activate adenylyl cyclase and serve to increase intracellular cAMP (Wen and Xia 2012). Recent studies have clarified the potential role of ADORA2B in the penile erection. It has been demonstrated that endogenous adenosine through A2B adenosine receptor play important role in the physiological process of penile erection (Wen et al. 2011a, b) and excess adenosine, also via ADORA2B, leads to priapism in ADA deficient mice and sickle cell disease Tg mice (Mi et al. 2008; Wen et al. 2010a, b). In additional, Faria et al. (2006) reported that vasculogenic erectile dysfunction is resistant to adenosine relaxation due to endothelial dysfunction caused by decreased activation of ADORA2B. These convincing studies lead us to hypothesize that impaired ADORA2B signaling pathway may be one of the potential pathogenesis of diabetic ED. The experiment results have shown that the expression level of ADORA2B is significantly reduced in penile tissues of diabetic rats, which confirmed our hypothesis

PDE5 inhibitors serve as the first-line treatment option for diabetic ED, however they fail for a certain percentage of patients due to the pathogenesis of ED in diabetes is not fully understood. In this study, we have demonstrated that BAY 60-6583 is capable of reversing the impaired erectile function in diabetic rats by elevating the ADORA2B activity and presents potential therapeutic role for diabetic ED. BAY 60-6583 is a novel specific ADORA2B agonist. Tobias Eckle et al. (2007) have reported that intra-arterial application of BAY 60-6583 generates significant attenuation of infarct size following by myocardial ischemia. Grenz et al. (2008) have demonstrated that intravenous administration of BAY 60-6583 resulted in renal protection from ischemia and generated a dramatic improvement of glomerular filtration rate. A recent study has shown that ADORA2B stimulation with BAY 60-6583 during resuscitation protects against trauma and hemorrhagic shockinduced lung injury (Koscso et al. 2013). Taken together, these studies have demonstrated the selectivity and therapeutic function in vivo of BAY 60-6583, which provides strong rationale for therapeutic possibility by targeting the ADORA2B.

Multiple factors and cellular signaling contribute to the pathogenesis of diabetic ED. Previous studies showed that glycation end-products is prevalent in penile tissue of diabetic animal, which generates free radicals or reactive oxygen species (ROS) to impair the relaxation of smooth muscle in corpus cavernosum (Cartledge et al. 2001). Chang et al. (2004) reported that significant down-regulation of PKG-1 expression associated with decreased PKG-1 activity were detected in the corpus cavernosum in diabetic rabbits, which is response for the pathogenesis of diabetic ED. Recent studies revealed that RhoA/Rho-kinase pathway is up-regulated in diabetic rats which leads to suppress eNOS and decreases the production of NO, which leads to diabetic ED (Buyukafsar and Un 2003; Bivalacqua et al. 2004). In this study, we have demonstrated that A2B adenosine signaling is impaired in diabetic rats, which might attenuate the activation of downstream signaling of ADORA2B. However, the molecular mechanism how ADORA2B mediated the pathogenesis of diabetic ED and the interaction between A2B adenosine signaling and wellknow factors and signaling pathways involved in diabetes ED are largely unknown, which requires for the future studies.

In our study, no significantly enhanced erectile response was observed in control group of rats after BAY 60-6583 treatment. Thus, full erection and saturated activation of signaling pathway involved in penile erection were already achieved in the control rats without treatment, which also support that BAY 60-6583 is safe without priapic-like effect. The decreased expression level of ADORA2B in penile tissues of diabetic ED rats might attenuate the activation of downstream signaling of ADORA2B involved in penile erection and lead to ED; thus, the activation of ADORA2B by BAY 60-6583 in diabetic rats is capable of up-regulating the downstream signaling and improving the erectile function. However, due to the decreased ADORA2B expression level in penile tissues of diabetic rats and some other pathogenesis involved in diabetic ED, BAY 60-6583 treatment could not fully correct the erectile dysfunction of diabetic rats (Fig. 4).

The STZ induced type 1 diabetic model has contributed to significant advancement in the study of ED (Gur et al. 2014). It is a well-accepted erectile dysfunction animal model (Li et al. 2012; Liu et al. 2015; Wang et al. 2015). The rats with the constant blood glucose level higher than 16.7 mmol/L were considered as a diabetic model. The slower initiation of erection and decreased ICP is detected in these rats. In this study, we used cavernous nerve stimulation to induce the penile erectile function. The results showed the erectile response in diabetic rats under different voltage of cavernous nerve stimulation is attenuated compared to control rats. These data indicated that the diabetic rats were reliable erectile dysfunction animal model.

In conclusion, we provide the in vivo evidence that BAY 60-6583 is an effective drug to improve erectile function in diabetic rats probably by activating ADORA2B. Thus, our preclinical studies have identified a previously unrecognized novel application of BAY 60-6583 as effective and mechanism-based drug to treat diabetic ED and set up a solid experimental foundation for future clinical trials in men.

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Conflict of Interest

The authors declare no conflict of interest.

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