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European Journal of Pharmacology 554 (2007) 40–45
www.elsevier.com/locate/ejphar

Chronic inhibition of p38MAPK improves cardiac and endothelial function in experimental diabetes mellitus

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Received 3 May 2006; received in revised form 19 August 2006; accepted 23 August 2006
Available online 14 September 2006

Abstract

To investigate the influence of p38 mitogen activated kinase (p38MAPK) on the development of diabetic cardiac and endothelial dysfunction, we assessed left ventricular and vascular function as well as inflammatory markers in diabetic rats after chronic pharmacological inhibition of p38MAPK. Diabetes mellitus was induced in rats by a single injection of streptozotocin. Rats were treated with the p38MAPK inhibitor SB 239063 (40 mg/kg/day, p.o.) or vehicle. 48 days after diabetes mellitus-induction, left ventricular function and vascular function were assessed *in vivo* by TIP-catheter and the autoperfused hindlimb, respectively. Cell adhesion molecules staining was quantified immunohistochemically in the heart and quadriceps muscle, respectively, as well as cardiac phosphorylation of p38MAPK by Western blot analysis. Treated and untreated diabetic groups displayed similar severe hyperglycemia. Left ventricular and endothelial function were impaired in the untreated diabetic group compared to controls (dp/dtmax: −40%, dp/dtmin: +49%, maximal vasodilatation: −57%; $P<0.05$) associated with significantly increased cardiac (3-fold) and peripheral cell adhesion molecules staining, respectively. Treatment of diabetic rats with SB 239063 led to a significant reduction of diabetes-induced enhancement of p38MAPK phosphorylation associated with improved left ventricular function (dp/dtmax: +39%, dp/dtmin: +47%; $P<0.05$) and peripheral endothelial function (maximal vasodilatation: +71%; $P<0.05$) under diabetic conditions. This was associated with reduced cardiac and peripheral inflammation indexed by reduced adhesion molecules content. Pharmacological inhibition of p38MAPK is sufficient to mitigate the development of diabetic cardiac and endothelial dysfunction despite of hyperglycemia. Our data suggest that the anti-inflammatory properties due to p38MAPK inhibition contribute to these beneficial cardiovascular effects.

Keywords: Diabetes mellitus; Endothelial dysfunction; Diabetic cardiomyopathy; p38MAPK; Inflammation; Adhesion molecule

1. Introduction

Activation of mitogen activated kinases (MAPKs) plays a key role in intracellular signalling and contributes to cardiovascular diseases. Among others such as c-Jun N-terminal kinase (JNK) or extracellular signal-regulated kinase (ERK 1/2), p38MAPK is known as a relevant regulator of pathophysiological gene activation leading to left ventricular and vascular dysfunction (Cain et al., 1999; Communal et al., 2000; Liang and Molkentin, 2003; Li et al., 2005; Chen et al., 2006). Thereby, phosphorylation of p38MAPK results in the activation of pro-inflammatory transcription factors such as nuclear factor κB (NF-κB) (Dai et al., 1995) inducing a variety of pathophysiologic mechanisms like apoptosis (Baines and Molkentin, 2005) or left ventricular remodelling (Takimoto et al., 2005). In humans, p38MAPK activation leads to enhanced expression of cytokines such as tumor necrosis factor α (TNF-α) resulting in impaired post-ischemic myocardial function (Cain et al., 1999). Expression of cellular adhesion molecules, which is necessary for adherence of neutrophils to endothelial cells, following cytokine activation is also regulated by p38MAPK (Pietersma et al., 1997) which might constitute a further mechanism of tissue damage. Several pathophysiological mechanisms present in diabetes mellitus including hyperglycemia, oxidative stress and enhanced

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doi:10.1016/j.ejphar.2006.08.065

angiotensin II have been implicated in pathophysiological gene activation due to upstream regulators such as p38MAPK (Natarajan et al., 1999). In cultured vascular cells increasing levels of glucose enhanced the activation of p38MAPK (Igarashi et al., 1999). Moreover, this study demonstrated a significant activation of p38MAPK in cultured vascular cells derived from diabetic rats (Igarashi et al., 1999) supporting a role of p38MAPK in diabetic complications. This may include endothelial and cardiac dysfunction, for which it was shown that inflammatory mechanisms are important (Dorenkamp et al., 2005; Tschope et al., 2005).

However, whereas the effects of pharmacological inhibition of p38MAPK have been demonstrated in myocardial ischemia and apoptosis as well as in left ventricular hypertrophy (Cain et al., 1999; Ma et al., 1999; Behr et al., 2001), the role of p38MAPK inhibition in diabetic individuals had not been studied *in vivo* so far. Therefore we investigated the significance of p38MAPK in the development of diabetes-induced complications by measuring left ventricular and endothelial function *in vivo* as well as cardiac and peripheral inflammation in the streptozotocin rat model of diabetes mellitus after chronic pharmacological inhibition of p38MAPK.

2. Methods

2.1. Animals and treatment

Experiments were conducted in 8 weeks old male Sprague Dawley (SD) rats (300–320 g, Charles River, Germany). Rats were housed under standard conditions (20 °C, 12-h light/dark cycle) and given free access to standard chow and tap water. The experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Diabetes mellitus was induced by a single injection of streptozotocin (70 mg/kg, i.p.) as previously described (Tschope et al., 2004). Only rats with blood glucose levels >16.5 mmol/l 5 days after streptozotocin injection were used in the study. Rats were randomly separated into 2 sets, one for the assessment of cardiac function and one for the assessment of vascular function. Every set contains 4 groups ($n=8$ per group): two non-diabetic and two diabetic groups. One diabetic group (STZp38I) of each set was treated with the p38MAPK inhibitor SB 239063 [*trans*-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyridimidin-4-yl)imidazole] (40 mg/kg/day, p.o.) for 43 days. SB 239063 is a second-generation p38MAPK inhibitor that has been shown to be extremely selective for p38MAPK (Barone et al., 2001). One non-diabetic group (SD-p38I) of each set was also treated with SB 239063 in the same way. The vehicle-treated non-diabetic (SD-Co) and diabetic group (STZ-Co) served as normoglycemic and diabetic controls using β-cyclodextrane solution (10%) as vehicle. 48 days after streptozotocin injection both vascular and left ventricular function were measured as well as cardiac and peripheral cell adhesion molecules content.

2.2. Assessment of left ventricular function in vivo

For *in vivo* hemodynamic measurement, the rats were anaesthetized (chloral hydrate, 400 mg/kg, i.p.), intubated and mechanically ventilated. Maximal rate of left ventricular pressure rise (dp/dtmax [mm Hg/s]) as an index of systolic function and the minimal rate of left ventricular pressure fall (dp/dtmin, [mm Hg/s]) as an index of diastolic function were recorded via a 2F Millar-Tip-catheter system in a closed chest model as previously described (Tschope et al., 2004). Briefly, after intubation the Tip-catheter was positioned in the left ventricular via the carotid artery. All measurements were performed during transient apnoe.

2.3. Assessment of vascular function in vivo

For *in vivo* assessment of vascular function, we used the autoperfused hindlimb model in the rat as described previously (Dorenkamp et al., 2005). Briefly, the rats were anaesthetized (chloral hydrate, 400 mg/kg, i.p.), intubated and mechanically ventilated. Under aseptic conditions, an extracorporal catheter system was placed between the left carotid artery and the right femoral artery using polyethylene catheters. An integrated roller peristaltic pump (Minipuls 3, Abimed, Langenfeld, Germany), which delivered blood from the left carotid artery performed a laminar flow through the femoral artery into the left hindlimb. Flow of the pump was set at 2.2 ml/min per kilogram body weight. After 20 min of perfusion, a pressure baseline was recorded. In order to induce endothelial-dependent vasodilation, three different volumes of Krebs–Henseleit-Solution (KHS) (80, 200 and 600 μl/kg) were administered into the femoral artery via a three-way-gain integrated in the catheter system. The area of pressure decrease (Integral [I in mm Hg s]) after KHS was analyzed as an index of the degree of vasodilatation.

2.4. Western blot analysis of phosphorylated and unphosphorylated cardiac p38MAPK

Western blot analyses were performed in heart muscle. 40 μg of protein for each sample was loaded. The reaction was carried out using primary antibodies raised against p38MAPK (Santa Cruz, USA). Activation of p38MAPK was examined using specific phosphorylated antibodies (Santa Cruz, USA) and β-Tubulin (Santa Cruz, USA) served as loading control which was not affected by diabetes mellitus or treatment with SB 239063 resulting in similar expression among all groups. Specific protein was detected by chemiluminescence reaction (Amersham, UK), followed by immunoblot density by dedicated software (TINA 2.0, Raytest).

2.5. Immunohistology and quantification of cardiac and peripheral ICAM-1 and VCAM-1

Tissue samples of quadriceps muscles were embedded in O. C.T. (optimal cutting temperature) compound (Tissue Tek®, Sakura Finetek, USA), frozen in liquid nitrogen and stored at −80 °C. Serial 5-μm-thick cryosections were placed on 10%

Table 1
Basal and hemodynamic characteristics

	SD (n=8)	STZ (n=8)	STZp38I (n=8)	SD-p38I (n=8)
Blood glucose (mmol/l)	4.4±0.3	>30.5	>30.5	4.2±0.4
Body weight (g)	350±10	255±5[a]	257±6[a]	345±12
dp/dtmax (mm Hg/s)	4373±111	2606±170[a]	3627±102[b]	4410±118
dp/dtmin (mm Hg/s)	−3901±99	−1999±81[a]	2931±102[b]	−3998±81

Values depict means±S.E.M.
[a] P<0.05 vs. SD (n=8 per group).
[b] P<0.05 vs. STZ and SD.

poly-L-lysine precoated slides and fixed in cold acetone. After blocking endogenous peroxidase activity, sections were incubated with an avidin/biotin blocking kit (Vector Laboratories Inc., Burlingame, USA). Staining was performed with the following primary antibodies at the given dilutions: mouse–anti-rat ICAM-1 (Serotec, Oxford, UK; 1:100) and mouse–anti-rat VCAM-1 (HISS Diagnostics, Freiburg, Germany; 1:50). These antibodies were detected using a biotinylated goat–anti-mouse IgG (Dianova, Hamburg, Germany; 1:400). Sections were incubated with peroxidase-conjugated streptavidin (Vectastatin ABC Elite, Vector Laboratories Inc, Burlingame, USA). Antigen–antibody complexes were visualized by 3-amino-9-ethylcarbazole (Merck, Darmstadt, Germany). The slides were counterstained (Mayer's hematoxylin), mounted in Kaiser's gelatine (both Merck, Darmstadt, Germany) and evaluated in a blinded fashion using the digital image analysis system Lucia G Version 3.52b (Nikon Deutschland GmbH, Düsseldorf, Germany) as described before (Dorenkamp et al., 2005).

2.6. Statistical analysis

Statistical analyses were performed using JMP Statistical Discovery Software Version 4.02 (SAS Institute, USA). All data are expressed as the mean±S.E.M. Statistical differences were assessed using the two-way analysis of variance (ANOVA) in conjunction with the post-hoc multiple comparison-corrected Student's t-test. Differences were considered statistically significant at a value of P<0.05.

3. Results

3.1. Basic parameters

Blood glucose levels were monitored in all of the studied animals. 48 days after streptozotocin administration blood glucose increased to a level greater than 30.5 mmol/l in all diabetic groups and the body weight was reduced compared to normoglycemic controls (Table 1). Heart weight decreased in both STZ-Co groups to a similar degree and significantly compared to the SD-Co rats (heart weight: STZP38I 0.82±0.03 and STZ 0.83±0.02 vs. SD-Co 1.36±0.05, mg, P<0.05). The calculated heart/body weight ratio showed no significant differences between the groups (STZP38 3.2±0.3, STZ 3.3±0.4, CO 3.8±0.3, g/kg, n.s.).

3.2. Protein expression of p38MAPK

To prove the effect of SB 239063 on p38MAPK activity we examined total p38MAPK and its activated phosphorylated p38MAPK (pp38MAPK) using Western blot analysis. Whereas the expression of total p38MAPK did not differ among the groups, pp38MAPK was significantly enhanced in STZ-Co hearts compared to SD-Co hearts (P<0.05). Chronic treatment with SB 239063 led to a decrease of pp38MAPK protein expression in streptozotocin rat hearts to basal level of non-diabetic rats (Fig. 1).

3.3. Left ventricular function

As shown in Table 1, left ventricular function between SD-Co rats and SD-p38I rats did not differ. Six weeks after streptozotocin injection, diabetic rats displayed impaired systolic and diastolic function. Dp/dtmax as an index of systolic function and dp/dtmin as an index for diastolic function were significantly decreased in the streptozotocin group (−40% and −49%) compared to normoglycemic controls (P<0.05; Table 1). After chronic p38MAPK inhibition, both systolic and diastolic parameter were improved compared to non-treated diabetic rats (dp/dtmax +39%, dp/dtmin +47%, P<0.05).

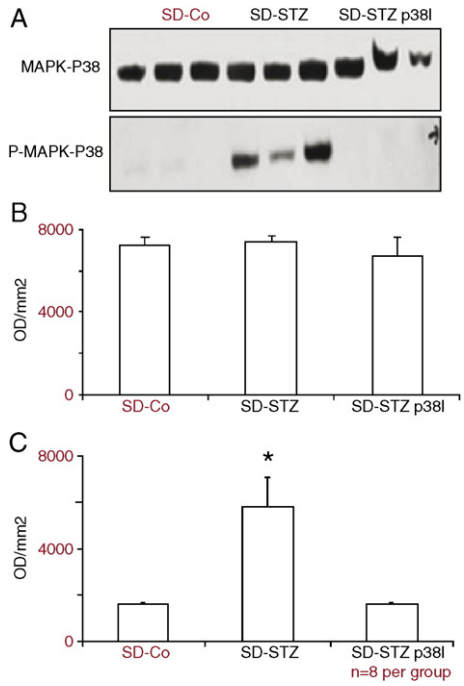

Fig. 1. p38MAPK phosphorylation in the heart muscle. Representative immunoblots of phosphorylated p38MAPK and total p38MAPK (A) were identified by Western blotting. The specific immunoblots were quantified by densitometry. The quantification of intensities of the total p38MAPK (B) and phosphorylated p38MAPK (C) were expressed below. Data obtained from quantitative densitometry were presented as mean±S.E.M (n=8 per group). *P<0.05 vs. SD-Co and SD-STZp38I. SD, Sprague Dawley; Co, control; STZ, streptozotocin; p38I, SB 239063.

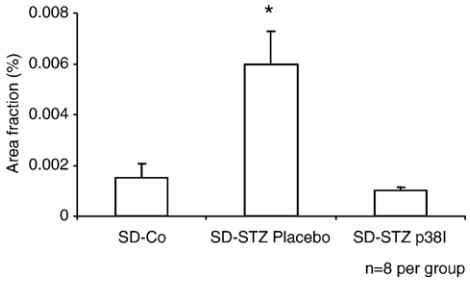

Fig. 2. VCAM-1 protein expression in heart muscle. Quantification of VCAM-1 expression in the heart. Data expressed as mean of area fraction (AF in %) ± S.E.M. *$P<0.05$ vs. SD-Co and SD-STZp38I. SD, Sprague Dawley; STZ, streptozotocin; p38I, SB 239063; $n=8$ per group.

3.4. Histochemical quantification of cardiac vascular cellular adhesion molecule 1 content

As shown in Fig. 2, the quantification of vascular cellular adhesion molecule 1 (VCAM-1) content by digital image analysis revealed significantly increased expression in STZ-Co heart muscle compared to SD-Co rats. The chronic inhibition of p38MAPK in diabetic rats was associated with a significant reduction of VCAM-1.

3.5. Endothelial function

Endothelial function between untreated SD-Co rats and SD-p38I rats did not differ significantly (KHS 80: 1219±191 vs. 1208±161; KHS 200: 2250±175 vs. 2278±210; KHS 600: 3740±221 vs. 3698±252; n.s.). As shown in Fig. 3, endothelial-dependent vasodilatation induced by shear stress in the STZ-Co group was significantly reduced at all doses of KHS (80, 200 and 600 µl/kg) compared to SD-Co group (−52%, −53% and −57%; $P<0.05$). Endothelial-dependent vasodilatatory response was significantly increased in STZp38I rats compared to STZ-Co rats at all doses of KHS (+102%, +93% and +71%; $P<0.05$).

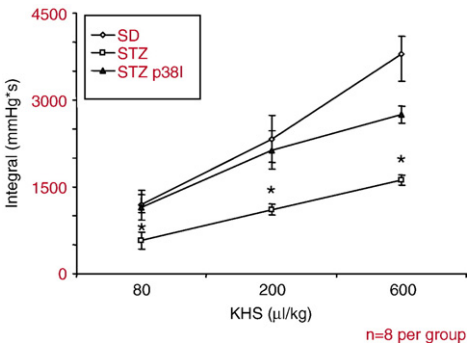

Fig. 3. Endothelial-dependent vasodilatation. Flow-mediated endothelium-dependent vasodilatation elicited by the administration of KHS ($n=8$ per group). Data were expressed as mean±S.E.M. and displayed as the integral of pressure decrease after administration of Krebs–Henseleit-Solution (mm Hg *s). Significantly different values vs. SD, STZ and STZp38I are marked by * (Student's t-test; $P<0.05$). SD, Sprague Dawley; STZ, streptozotocin; p38I, SB 239063.

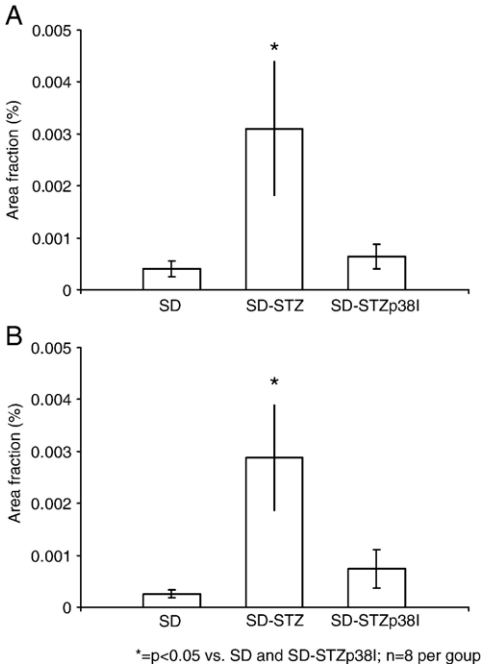

Fig. 4. ICAM-1 and VCAM-1 protein expression in quadriceps muscle. Quantification of ICAM-1 (A) and VCAM-1 (B) expression in quadriceps muscle. SD, Sprague Dawley; STZ, streptozotocin; p38I, SB 239063; $n=8$ per group.

3.6. Histochemical quantification of peripheral intercellular and vascular cellular adhesion molecule 1 content

To examine the peripheral pro-inflammatory aspects of the diabetic state, expression of the inflammatory mediators intercellular and vascular cellular adhesion molecule 1 (ICAM-1 and VCAM-1) was examined in the non-perfused quadriceps muscle at the end of the protocol (Fig. 4). STZ-Co rats demonstrated a marked increase in ICAM-1 protein expression compared to non-diabetic controls. Diabetic rats treated with p38MAPK inhibitor exhibited a low ICAM-1 immunoreactivity, similar to this of non-diabetic controls. VCAM-1 expression was also markedly increased under diabetic conditions. As shown for ICAM-1, p38MAPK inhibition did significantly reduce VCAM-1 protein expression.

4. Discussion

The salient finding of the present study is that a chronic p38MAPK inhibition reduces the progression of cardiac and endothelial dysfunction in severe streptozotocin-induced diabetes mellitus. We show that these beneficial effects are associated with a reduction in inflammatory response indicating an important role of p38MAPK in triggering inflammatory signal transduction pathways in streptozotocin-induced diabetes mellitus, without influencing hyperglycemia.

MAPKs including JNK, ERK and p38MAPK are up-regulated by oxidative stress leading to downstream activation of several transcription factors including activating transcription factor-2 (ATF-2), NF-κB and myocyte enhancer factor-2 (MEF-2) triggering transcriptional activation of inflammatory

response (Ono and Han, 2000) and apoptosis (Baines and Molkentin, 2005). Growing evidence for causality between p38MAPK activation and the development of cardiovascular diseases comes mostly from studies using specific p38MAPK inhibitors under several conditions of heart failure in animals and humans including cardiac ischemia and hypertension (Cain et al., 1999; Ma et al., 1999; Behr et al., 2001). In addition, p38MAPK activation has been found to be induced by hyperglycemia and by diabetes mellitus in an experimental study (Igarashi et al., 1999). It is known that hyperglycemia causes adverse effects on different cell lines including vascular cells possibly by a variety of mechanisms including the activation of advanced glycation end products, oxidant formation, abnormality of sorbitol and myoinositol metabolism and diacylglycerol-protein kinase C (PKC) activation (Igarashi et al., 1999). Further, it has been shown that PKC activates p38MAPK in cultured cells due to hyperglycemia and in aortic smooth muscle cells of streptozotocin-diabetic rats (Igarashi et al., 1999). However, the effects of p38MAPK inhibition on cardiac and endothelial function in diabetes mellitus were not investigated so far *in vivo*. Diabetes mellitus is a significant entity (Bell, 1995) leading to functional changes including left ventricular and endothelial function (Farhangkhoee et al., 2006). Several mechanisms contribute to this disease. A main mechanism for the development of diabetic cardiovascular complications constitutes the generation of oxidative stress in the heart leading to pro-inflammatory gene activation which are among others regulated by MAPKs. Here we show that the pharmacological inhibition of p38MAPK is sufficient to improve left ventricular and vascular function *in vivo*. Interestingly, p38MAPK inhibition did not alter blood glucose concentration.

Several other studies have demonstrated that vascular function is also abnormal in patients with diabetes mellitus (Johnstone et al., 1993) predicting strongly and independently adverse cardiovascular events and long-term outcome (Targonski et al., 2003; Widlansky et al., 2003). We found in agreement with previous studies an impaired endothelial function under experimental type 1 diabetic conditions (Angulo et al., 1998; Dorenkamp et al., 2005; Tschope et al., 2005) and show improved endothelial function after chronic inhibition of p38MAPK *in vivo*.

Because of the pivotal role of inflammation in the progression of diabetic complications, especially vascular damage, we analyzed the expression of cell adhesion molecules in peripheral quadriceps muscles reflecting the environment of the analyzed vessels. The expression of adhesion molecules on the surface of endothelial cells is an important pathophysiologic factor in endothelial dysfunction. It constitutes the basis for leukocyte transmigration into endothelial cells resulting in endothelial inflammation (Libby et al., 2002). Therefore the expression of cell adhesion molecules plays a key role in the development of vascular damage. p38MAPK inhibition in our study led to a suppression of cell adhesion molecules expression in the quadriceps muscle. This finding supports the protective role of p38MAPK inhibition in the reduction of the development of peripheral vascular damage via anti-inflammatory mechanisms.

There is growing evidence for the emerging role of cardiac microvasculopathy in the initiation and perpetuation of diabetic cardiomyopathy (Brutsaert, 2003), also as a sign paralleled by other secondary complications such as retinopathy and nephropathy (La Selva et al., 1993). It has been shown that most cardiovascular risk factors that cause alterations of peripheral vascular endothelial cells also affect cardiac endothelium including myocardial microvessels (Brutsaert, 2003). In consideration of the priority of microvasculopathy in diabetic cardiomyopathy, we estimated microvascular damage in the heart by analyzing cardiac cell adhesion molecules as inflammatory markers. We evaluated similar results regarding cell adhesion molecules expression in the heart compared to quadriceps muscle. We observed in agreement with previous findings (Fasching et al., 1996; Tschope et al., 2005) a marked cardiac up-regulation of cell adhesion molecules in diabetic rat hearts indicating not only peripheral but also myocardial damage. It has been suggested that enhanced CAM expression facilitate transmigration of leukocytes into myocardial tissue (Marfella et al., 2000). Moreover, a direct inverse correlation of left ventricular function and cardiac inflammation in experimental diabetic cardiomyopathy could be demonstrated (Tschope et al., 2005). Inhibition of p38MAPK was sufficient to reduce cardiac cell adhesion molecules expression indicating both anti-inflammatory and vasculoprotective effects in the diabetic heart.

In summary, we found improved cardiovascular function indexed by improved left ventricular and endothelial function after chronic pharmacological inhibition of p38MAPK associated with suppressed inflammatory response under diabetic conditions supporting a causal role for p38MAPK in the development of cardiovascular complications in diabetes mellitus. p38MAPK inhibition exerts these beneficial effects despite of severe hyperglycemia. Our data support the pharmacological inhibition of p38MAPK as a new promising therapeutic strategy regarding the prevention of diabetic complications. However, further studies have to prove whether these finding can be translated to human conditions.

Acknowledgement

This study was supported by a grant from the Deutsche Forschungsgesellschaft (DFG; GRK 865).

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