Gene Deletion of the Kinin Receptor B1 Attenuates Cardiac Inflammation and Fibrosis During the Development of Experimental Diabetic Cardiomyopathy

Dirk Westermann,[1] Thomas Walther,[1] Konstantinos Savvatis,[1] Felcicitas Escher,[1] Meike Sobirey,[1] Alexander Riad,[1] Michael Bader,[2] Heinz-Peter Schultheiss,[1] and Carsten Tschöpe[1]

OBJECTIVE—Diabetic cardiomyopathy is associated with increased mortality in patients with diabetes. The underlying pathology of this disease is still under discussion. We studied the role of the kinin B1 receptor on the development of experimental diabetic cardiomyopathy.

RESEARCH DESIGN AND METHODS—We utilized B1 receptor knockout mice and investigated cardiac inflammation, fibrosis, and oxidative stress after induction of streptozotocin (STZ)-induced diabetes. Furthermore, the left ventricular function was measured by pressure-volume loops after 8 weeks of diabetes.

RESULTS—B1 receptor knockout mice showed an attenuation of diabetic cardiomyopathy with improved systolic and diastolic function in comparison with diabetic control mice. This was associated with a decreased activation state of the mitogen-activated protein kinase p38, less oxidative stress, as well as normalized cardiac inflammation, shown by fewer invading cells and no increase in matrix metalloproteinase-9 as well as the chemokine CXCL-5. Furthermore, the profibrotic connective tissue growth factor was normalized, leading to a reduction in cardiac fibrosis despite severe hyperglycemia in mice lacking the B1 receptor.

CONCLUSIONS—These findings suggest that the B1 receptor is detrimental in diabetic cardiomyopathy in that it mediates inflammatory and fibrotic processes. These insights might have useful implications on future studies utilizing B1 receptor antagonists for treatment of human diabetic cardiomyopathy. ***Diabetes*** **58:1373–1381, 2009**

Diabetic cardiomyopathy, as it occurs in patients with diabetes, carries a substantial risk concerning the subsequent development of heart failure and increased mortality (1). Different pathophysiological stimuli are involved in its development and mediate tissue injury leading to left ventricular systolic and diastolic dysfunction. Accumulation of cardiac fibrosis with distinct changes in the regulation of the extracellular matrix (2,3), excessive generation of reactive oxygen species (4), and cardiac inflammation (5,6), characterized by increased levels of proinflammatory cytokines and transendothelial migration of immunocompetent cells, plays a role in the manifestation of diabetic cardiomyopathy. Experimental stimulation of the local tissue kallikrein-kinin system has been shown to be beneficial in different forms of cardiomyopathies (7–11). Most of these effects are attributed to the kinin B2 receptor (B2R), while the role of the kinin B1 receptor (B1R) in cardiac failure is still under discussion. In contrast to the B2R, which is constitutively expressed in the cardiac tissue, the B1R is expressed at very low levels under basal conditions. Nevertheless, it is highly inducible under pathological conditions by pathological mediators such as bacterial lipopolysaccharide (12), cytokines (13), and ischemia but also by hyperglycemia (14), as can be shown in different animal models of cardiomyopathy. Also, in endomyocardial biopsies of patients with end-stage heart failure, this upregulation could be demonstrated and correlated with increased expression of proinflammatory cytokines in those patients (15). Whether B1R upregulation is cardioprotective, parallel to that of the B2R (16,17), or is cardiotoxic (13,18,19) remains debated. To further clarify the role of the B1R in the pathogenesis of diabetic cardiomyopathy, we investigated the left ventricular function in an animal model of streptozotocin (STZ)-induced type 1 diabetes using B1R knockout mice. Furthermore, changes in the left ventricular remodeling, inflammation, and oxidative stress were analyzed.

RESEARCH DESIGN AND METHODS

Twenty-five B1R knockout mice ($B1R^{-/-}$) on a C57/BL6 genetic background and 25 littermates ($B1R^{+/+}$) aged 2 months were obtained from the Max-Delbrück Center for Molecular Medicine (Berlin-Buch, Germany) (13). Diabetes was induced by injection of STZ (50 mg/kg i.p. for 5 days) in 15 $B1R^{-/-}$ ($B1R^{-/-}$-STZ) and 15 C57/BL6 mice ($B1R^{+/+}$-STZ), while the others served as nondiabetic controls ($B1R^{-/-}$ and control). Hyperglycemia (glucose >22 mmol/l) was confirmed 7 days later using a reflectance meter (Acutrend; Boehringer, Mannheim, Germany), as well as at the end of the study (glucose >30 mmol/l). The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH publ. no. 85-23, revised 1985).

Surgical procedures and hemodynamic measurements. Eight weeks after induction of diabetes, left ventricular function was analyzed using pressure-volume loops. The animals were anesthetized (125 mg/g i.p. thiopental), intubated, and artificially ventilated. As described recently (20), a 1.2-F microconductance pressure catheter (SciSense, Ontario, Canada) was positioned in the left ventricle for registration of left ventricular pressure-volume loops in a closed-chest model. Indexes of cardiac function were derived from pressure-volume data obtained both at steady state and during transient preload reduction by occlusion. Systolic function was quantified by left ventricual end systolic pressure (mmHg), by dP/dt_{max} (mmHg/s), and by ejection fraction (%). Global cardiac function was quantified by the end systolic volume (μl), end diastolic volume (μl), stroke volume (μl), cardiac

From the [1]Department of Cardiology and Pneumology, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany; and the [2]Max-Delbrück Center for Molecular Medicine, Berlin-Buch, Germany.
Corresponding author: Carsten Tschöpe, carsten.tschoepe@charite.de.
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TABLE 1

	$B1R^{+/+}$	$B1R^{-/-}$	$B1R^{+/+}$-STZ	$B1R^{-/-}$-STZ
Body weight (g)	27 ± 1	28 ± 1	17 ± 1*	17 ± 1*
Glucose levels (mmol/l)	6 ± 0.2	6 ± 0.2	31 ± 2*	32 ± 2*
Hemodynamic function				
Heart rate (beats/min)	465 ± 22	486 ± 32	302 ± 27*	389 ± 35
End diastolic volume (μl)	53 ± 2	48 ± 4	49 ± 4	47 ± 3
End systolic volume (μl)	18 ± 4	15 ± 3	28 ± 5	23 ± 5
Stroke volume (μl)	35 ± 6	31 ± 4	19 ± 6*	24 ± 5
Cardiac output (ml/min)	16.1 ± 1	15.8 ± 1	6.3 ± 0.6*	9.3 ± 1†
Cardiac output/body weight ($ml \cdot min^{-1} \cdot g^{-1}$)	0.61 ± 0.02	0.57 ± 0.04	0.41 ± 0.04*	0.56 ± 0.03
Left ventricular systolic pressure (mmHg)	98 ± 4	103 ± 5	72 ± 5*	86 ± 6†
dP/dt_{max} (mmHg/s)	6,658 ± 346	6,858 ± 256	3,215 ± 201*	5,214 ± 286†
Ejection fraction (%)	66 ± 4	64 ± 5	38 ± 7*	51 ± 4†
Left ventricular diastolic pressure (mmHg)	2.5 ± 1	2.8 ± 1	9.4 ± 2*	4.4 ± 2†
dP/dt_{min} (mmHg/s)	−5,896 ± 301	−5,485 ± 285	−2,248 ± 247*	−4,257 ± 244*†
Left ventricular stiffness (ml^{-1})	0.027 ± 0.002	0.034 ± 0.002	0.127 ± 0.001*	0.047 ± 0.004*†
Mean blood pressure (mmHg)	95 ± 4	98 ± 5	68 ± 7*	88 ± 6*†

Data are means ± SE. Hemodynamic function of control mice ($B1R^{+/+}$) or mice lacking the B1R ($B1R^{-/-}$) under basal conditions and 8 weeks after induction of STZ-induced diabetes. *$P < 0.05$ vs. nondiabetic controls; †$P < 0.05$ vs. $B1R^{+/+}$-STZ. dP/dt_{max}, contractility; dP/dt_{min}, relaxation.

output (μl/min), the ratio of cardiac output to body weight ($ml \cdot min^{-1} \cdot g^{-1}$), and heart rate (beats/min). Diastolic function was measured by left ventricular end diastolic pressure (mmHg), left ventricular pressure fall (dP/dt_{min}) (mmHg/s), and diastolic stiffness. Diastolic stiffness was calculated from the end diastolic pressure-volume relationship [$EDP = C \cdot \exp(b - Ved)$] where b is for stiffness (21). Moreover, mean arterial blood pressure was analyzed from measurements in the arteria carotis (22). Cardiac tissue was harvested and snap frozen for later measurements. All following measurements were performed in 10 animals per group.

Histological measurements. Immunohistochemistry was carried out using primary antibodies for collagen type 1 and type 3, the connective tissue growth factor (CTGF), the matrix-metalloproteinase-9 (MMP-9), and $CD3^+$, $CD11b^+$, $CD45^+$, and $CD68^+$ cells as well as nitrotyrosine and myeloperoxidase (MPO) (all from Serotec, Munich, Germany) followed by the DAKO

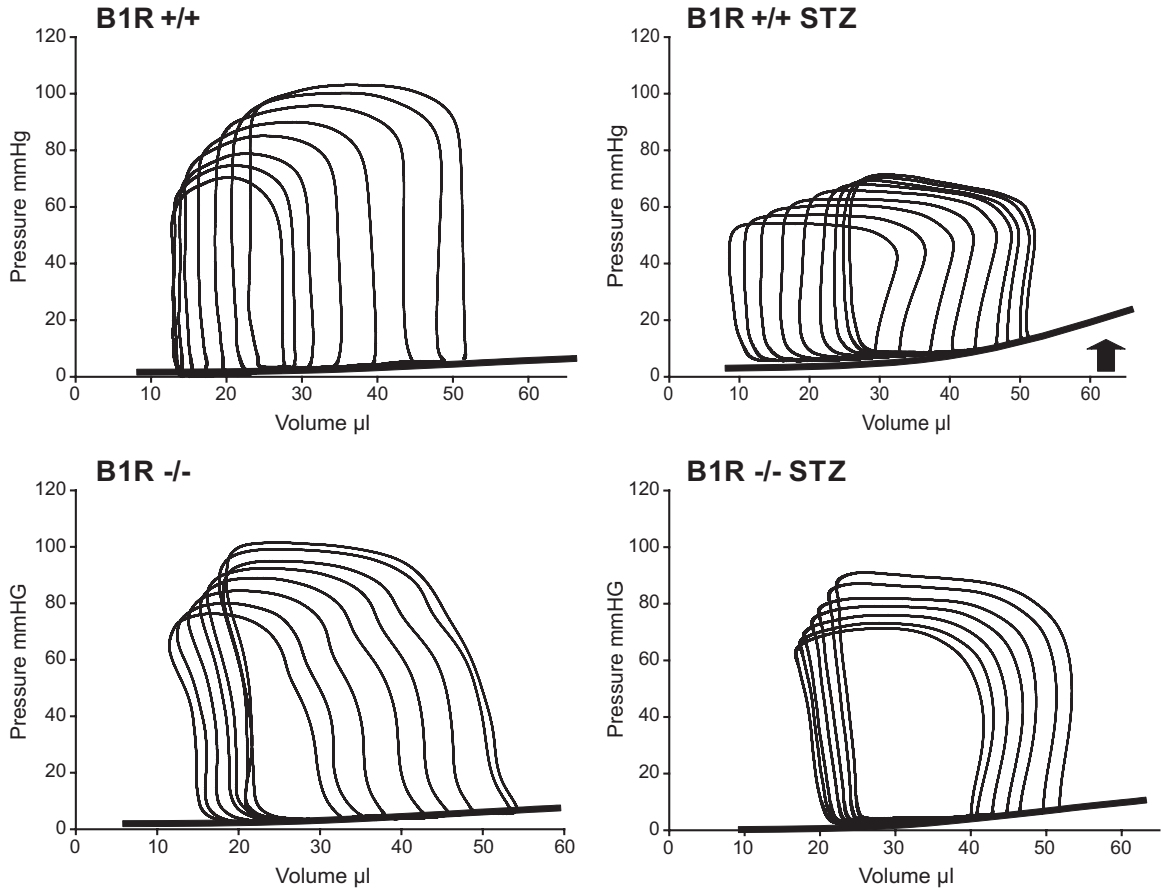

FIG. 1. Representative pressure-volume loops during a preload reduction of control mice ($B1R^{+/+}$) or mice lacking the B1R ($B1R^{-/-}$) under basal conditions and 8 weeks after induction of STZ-induced diabetes. The thick black line at the bottom indicates the left ventricular stiffness, which is increased in $B1R^{+/+}$-STZ (indicated by the thick black arrow).

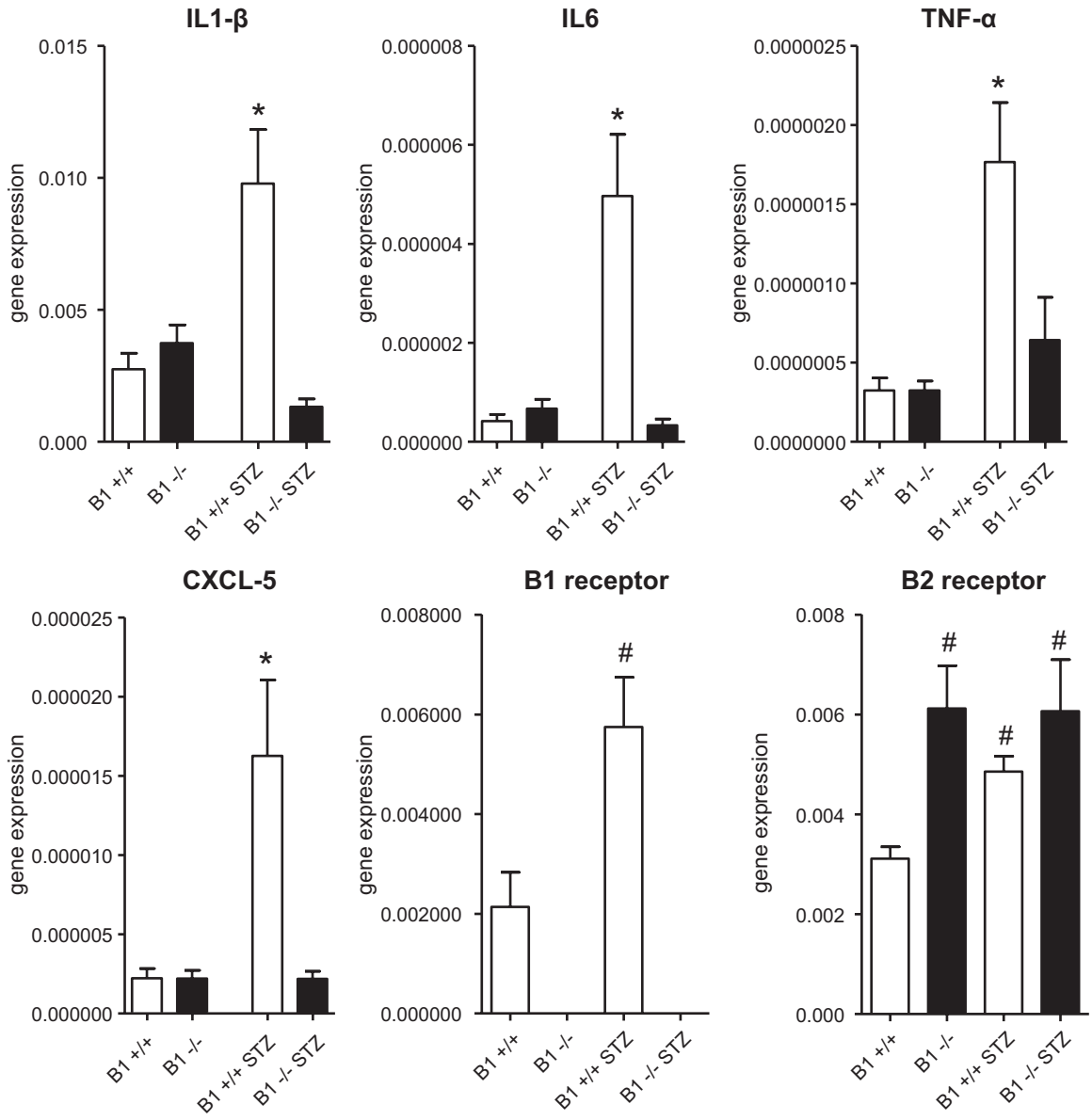

FIG. 2. mRNA levels of cardiac cytokines in control mice ($B1R^{+/+}$) or mice lacking the B1R ($B1R^{-/-}$) under basal conditions and 8 weeks after induction of STZ-induced diabetes with increased levels of IL-1β, IL-6, and TNF-α in STZ measured by real-time RT-PCR. Moreover, the figure shows mRNA levels of the chemokine CXCL-5 levels as well as mRNA levels of the B1R and B2R. *$P < 0.05$ vs. $B1R^{+/+}$ and $B1R^{-/-}$ STZ. #$P < 0.05$ vs. $B1R^{+/+}$.

Envision horseradish peroxidase technique (DAKO, Glostrup, Denmark). Histological costainings were performed using prinamry antibodies for CD68, tumor necrosis factor (TNF)-α (R&D Systems, Wiesbaden, Germany), sarcomeric actin, and TAB-1 (Cell Signaling Technology, Danvers, MA).

Real-time RT-PCR. Real-time RT-PCR (ABI Prism 7900 HT Sequence Detection System software, version 2.2.2.; Perkin Elmer) was carried out as previously described (23) using primers for B1R and B2R, the cytokine interleukin (IL)-1β, TNF-α, and IL-6, as well as for the chemokine CXCL-5. 18S was used as a housekeeping gene.

Western blot for evaluation of total p38 mitogen-activated protein kinase and p38 phosphorylation. Total p38 mitogen-activated protein kinase (MAPK) and phosphorylated p38 MAPK were detected with each specific antibody. Moreover, TAB-1 (all from Cell Signaling Technology) was detected. The blots were visualized with a chemiluminescene system (Amersham Bioscience, Buckinghamshire, U.K.). Quantitative analysis of the intensity of the bands was performed with NIH Image 1.63 software (National Institutes of Health, Bethesda, MD).

Statistical analysis. All data are expressed as means ± SE. Statistical significance between multiple groups was determined using ANOVA and post hoc analysis with a Bonferroni test. Values of $P < 0.05$ were considered significant.

RESULTS

Eight weeks after induction of STZ-induced diabetes, glucose levels were found to be highly increased in $B1R^{-/-}$-STZ and $B1R^{+/+}$-STZ mice but did not differ between both diabetic groups. Body weight decreased in both groups when compared with controls (Table 1).

Hemodynamic data. Lack of B1R had no effect on cardiac function under normoglycemic conditions. The heart rate was significantly decreased in $B1R^{+/+}$-STZ mice when compared with controls, due to the known effect of diabetic cardiac autonomy (24), while $B1R^{-/-}$-STZ mice were not statistically different from their controls. No ventricular dilatation was demonstrated in either STZ group when compared with their controls, while stroke volume was smaller in STZ, which contributed to impaired cardiac output in comparison with the controls. This decline of cardiac output could be partly prevented by B1R

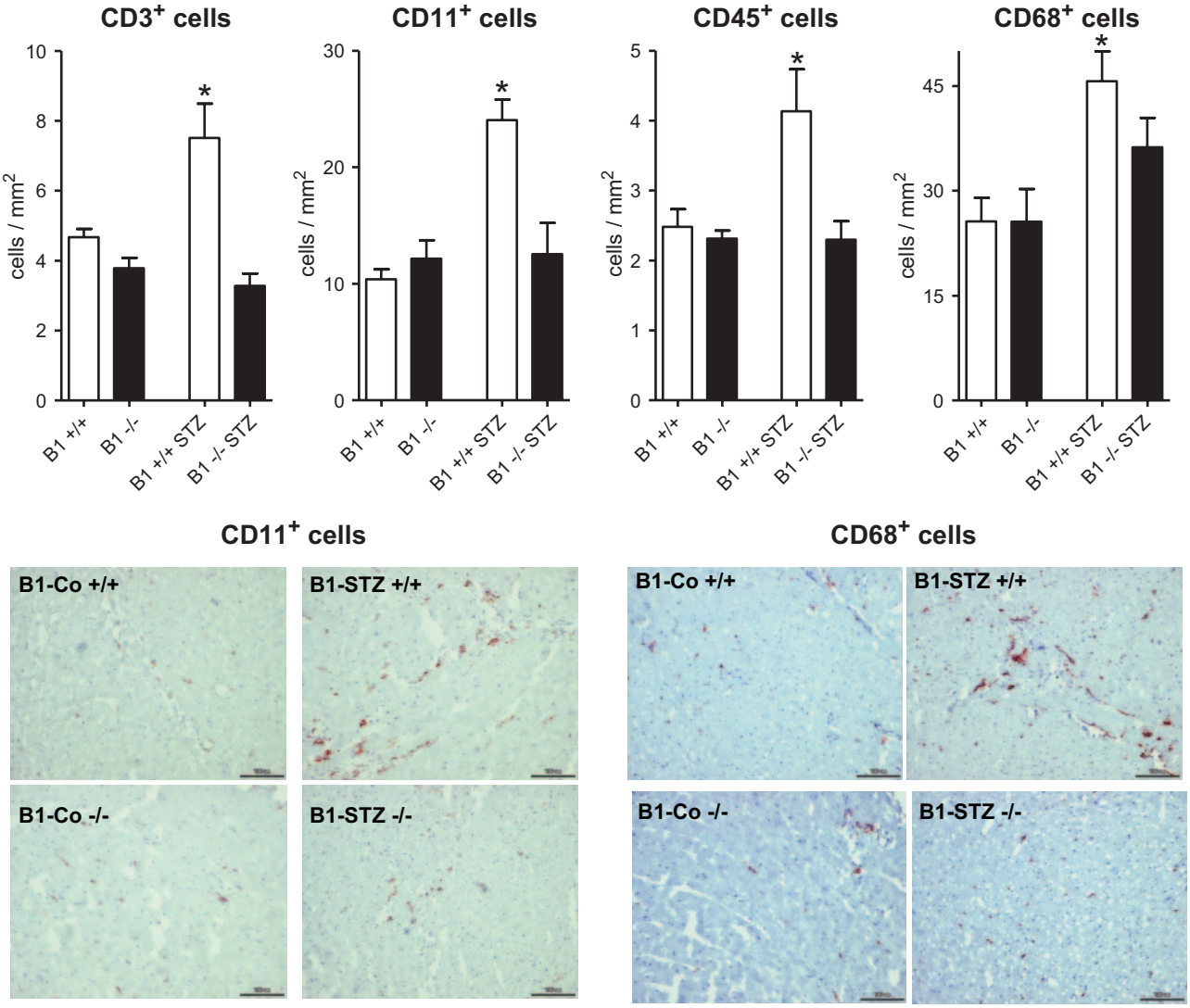

FIG. 3. Increased inflammatory cells ($CD3^+$, $CD11^+$, $CD45^+$, and $CD68^+$) in the cardiac tissue of $B1R^{+/+}$-STZ with representative pictures of all groups for $CD11^+$ and $CD68^+$ cells in control mice ($B1R^{+/+}$) or mice lacking the B1R ($B1R^{-/-}$) under basal conditions and 8 weeks after induction of STZ-induced diabetes. *$P < 0.05$ vs. $B1R^{+/+}$ and $B1R^{-/-}$ STZ. (A high-quality digital representation of this figure is available in the online issue.)

knockout but was still impaired when $B1R^{-/-}$-STZ mice were compared with controls. The systolic and diastolic parameters end systolic pressure, dP/dt_{max}, and ejection fraction were significantly decreased; the end diastolic pressure as well as diastolic stiffness was increased when STZ was compared with the control group. However, the impairment in these parameters was much less pronounced in $B1R^{-/-}$-STZ mice (Table 1 and Fig. 1).

Cardiac inflammation and oxidative stress. In the myocardium of the diabetic mice, the mRNA abundance of the proinflammatory cytokines IL-1β, IL-6, and TNF-α as well as the chemokine CXCL-5 were significantly increased by $B1R^{+/+}$-STZ compared with controls (Fig. 2). This was associated with increased numbers of $CD3^+$, $CD11b^+$, $CD45^+$, and $CD68^+$ cells (Fig. 3) and the protein abundance of MMP-9 (Fig. 4). This upregulation was prevented by $B1R^{-/-}$-STZ, resulting in normalized levels compared with controls. Moreover, a major part of TNF-α is produced by inflammatory cells (CD68) (Fig. 5). The abundance of nitrotyrosine and myeloperoxidase was increased in the cardiac tissue of the STZ group, as an indicator of increased oxidative stress (Fig. 4). The lack of the B1R reduced this increased expression of nitrotyrosine and myeloperoxidase in comparison with STZ, despite severe hyperglycemia when $B1R^{-/-}$-STZ was compared with $B1R^{+/+}$-STZ (Fig. 4).

Protein levels of endothelial nitric oxide synthase levels (eNOS) were downregulated in both diabetic groups. Nevertheless, the mRNA content was only downregulated significantly in the the $B1R^{+/+}$-STZ but not in the $B1R^{-/-}$-STZ mice (Fig. 4). Furthermore, the phosphorylation state of the MAPK p38, known to contribute to tissue inflammation, was increased in STZ mice compared with controls, again an effect that was reduced in $B1R^{-/-}$-STZ mice when compared with controls. Moreover, TAB-1 protein was significantly inreased in diabetic wild-type compared with $B1R^{-/-}$-STZ mice (Fig. 6).

Kinin receptor regulation. The B1R mRNA was increased by STZ-induced diabetes in wild-type mice. The B2R mRNA was also increased due to diabetic condition in wild-type mice. In $B1R^{-/-}$ animals, the B2R expression was higher compared with wild-type mice under basal conditions. Interestingly, there was no further B2R mRNA upregulation due to diabetic conditions as seen in wild-type mice (Fig. 2).

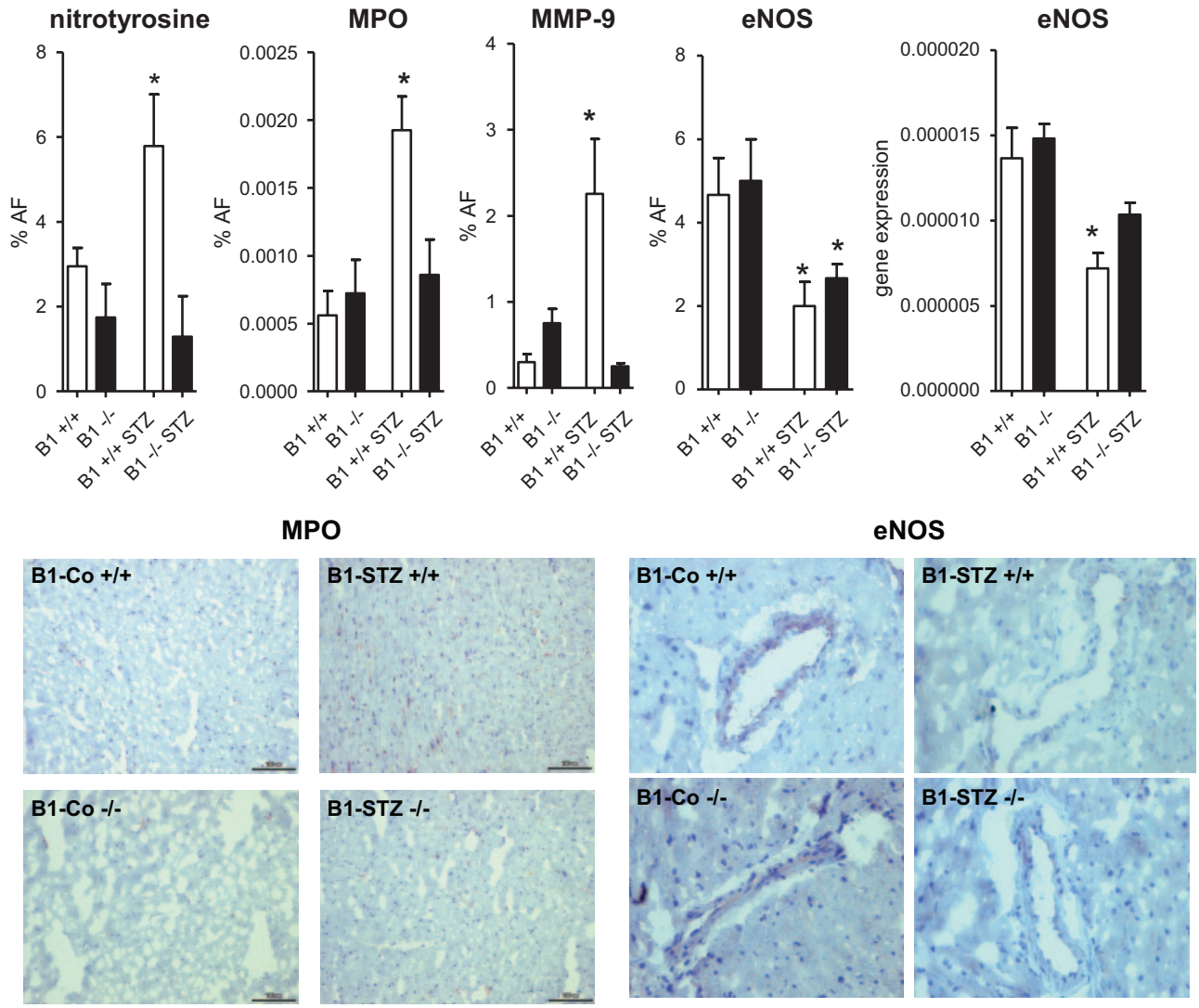

FIG. 4. Increased protein levels of oxidative stress (nitrotyrosin and MPO) as well as the MMP-9 in the cardiac tissue of B1R$^{+/+}$-STZ. Moreover, the figure shows protein levels and mRNA levels of eNOS and representative pictures of all groups for MPO and eNOS. *$P < 0.05$ vs. B1R$^{+/+}$. (A high-quality digital representation of this figure is available in the online issue.)

Cardiac fibrosis. CTGF was highly increased in diabetic animals. This increase in CTGF was accompanied by increased levels of collagen type 1 and 3, as an indicator of cardiac fibrosis. In contrast, CTGF was normalized in B1R$^{-/-}$-STZ, which translated into normalized collagen type 1 and type 3 levels as well, when compared with the controls (Fig. 7).

DISCUSSION

The salient finding of this study is that B1R gene deletion attenuates cardiac systolic and diastolic dysfunction in experimental diabetic cardiomyopathy. Diabetic cardiomyopathy is characterized by an increase in the phosphorylation state of the MAPK p38, which was associated with augmented cardiac inflammation, cardiac fibrosis, and oxidative stress in cardiac tissue. These changes were normalized in mice lacking the B1R, despite the occurrence of comparable severe hyperglycemia.

Experimental stimulation of the kallikrein-kinin system by gene transfer (25,26) and/or by the use of transgenic kallikrein overexpressing animals (10) attenuates diabetic cardiomyopathy. This is in agreement with other studies showing potent cardioprotective effects of the kallikrein-kinin system in animal models of ischemic (27–29), pressure overload (8), and septic (12) and hypertensive (9) cardiomyopathy. We, and others, using the STZ model of diabetes, were able to show that these cardiobeneficial effects of kallikrein-kinin mediate anti-inflammatory and antifibrotic effects and, furthermore, reduce oxidative stress (10,30,31) as well as improve glucose utilization and lipid metabolism (26,32). Both receptors of the system, the B1R and the B2R, are upregulated in the diabetic heart (14). The cardioprotective effects are mediated mainly by the B2R, since pharmacological inhibition of the B2R was seen to abolish these cardioprotective effects (31). The relationship between B1R and the development of heart failure is still under investigation. Recently, it was shown that the B1R may yield similar effects when compared with the B2R in an animal model of myocardial infarction (17). Nonetheless, other researchers have shown that a lack of the B1R reduced infarct size in ischemia reperfusion injury (19,33), a finding that indeed may imply an opposite function compared with that of the B2R. While there is good evidence that the B1R plays a detrimental role in autonomic diabetic nociception (34), obstructive nephropathy (35), and stroke (36) by modulating inflammatory

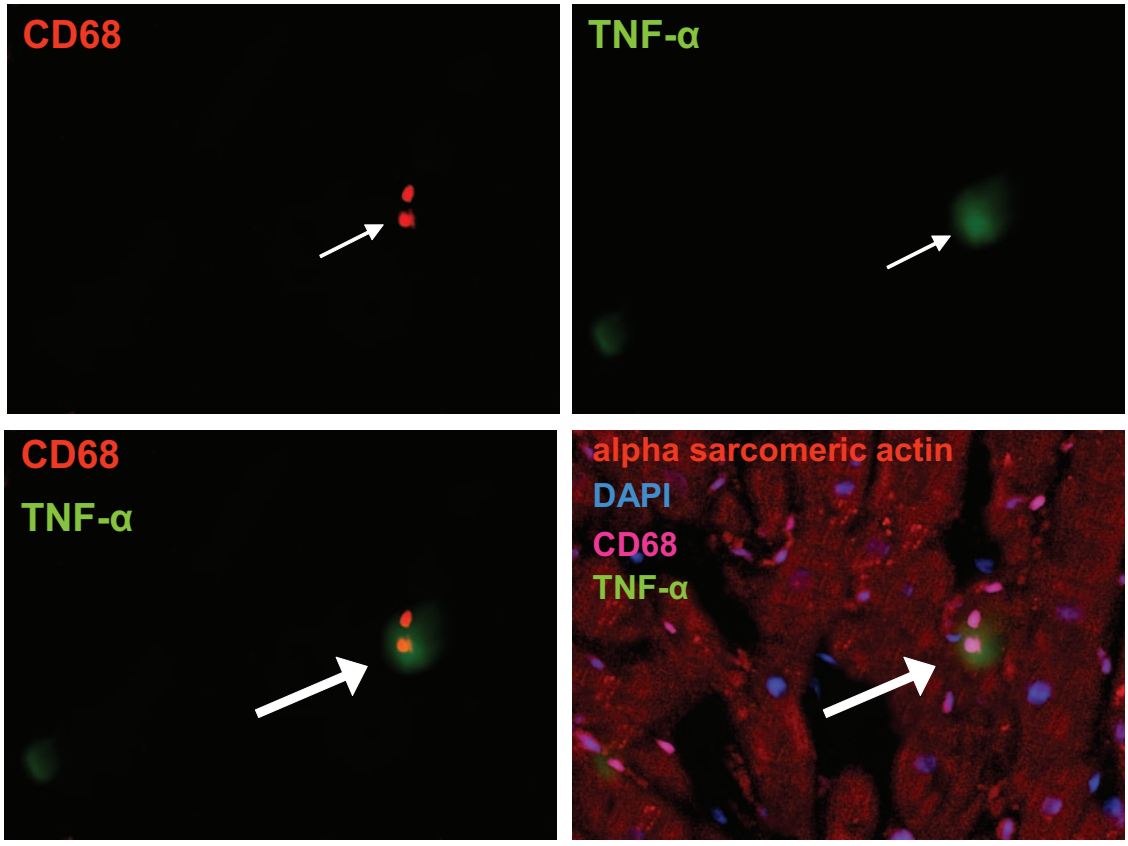

FIG. 5. Immunofluorescent stainings of cardiac tissue of a diabetic wild-type mouse with stainings for CD68, TNF-α, as well α-sacromeric actin and DAPI (for cell nuclei) showing that TNF-α is secreted by inflammatory CD68$^+$ cells. (A high-quality digital representation of this figure is available in the online issue.)

processes and increasing inflammation, its role in the development of diabetic cardiomyopathy has not yet been directly investigated.

We demonstrated recently that diabetic cardiomyopathy is associated with increased cardiac inflammation (2,5,10). These inflammatory processes were associated with increased oxidative stress and cardiac fibrosis, all contributing to systolic and diastolic dysfunction under diabetic conditions. Since the B1R is known to be upregulated by IL-1β (37) and hyperglycemia (14,38) and mediate tissue

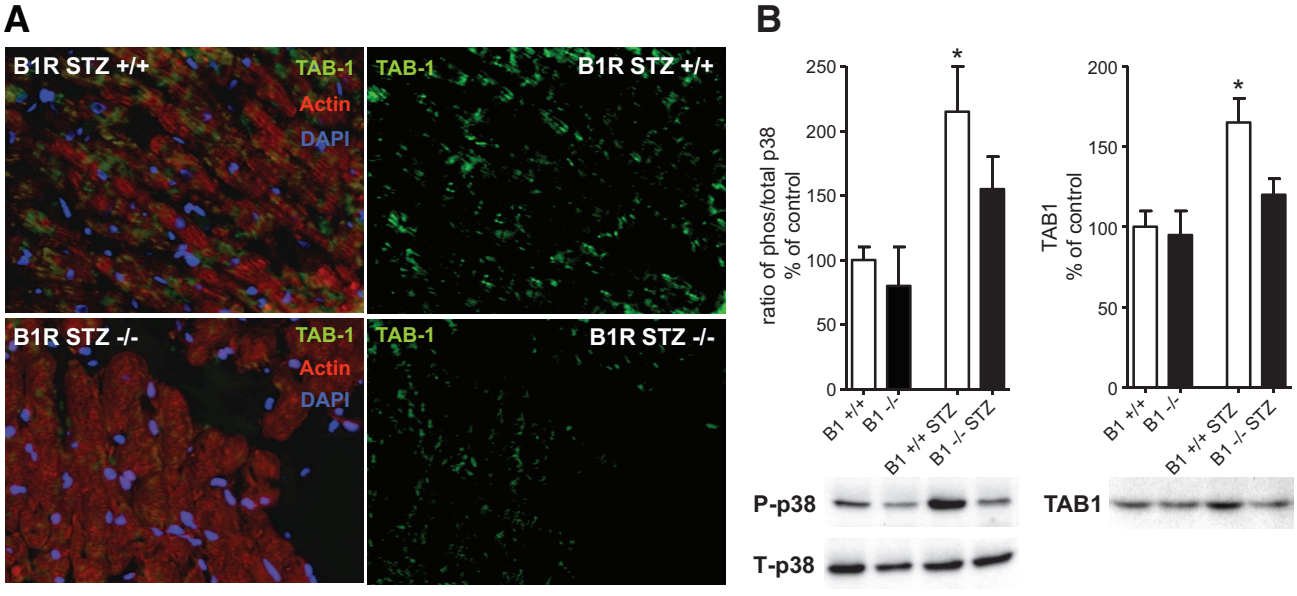

FIG. 6. *A*: Immunofluorescent stainings of cardiac tissue of B1R$^{+/+}$-STZ and B1R$^{-/-}$-STZ showing protein levels of TAK-1 binding protein (TAB-1) and α-sacromeric actin as well as DAPI (for cell nuclei). This demonstrates a reduced protein content of TAB-1 in B1R$^{-/-}$-STZ. *B*: Quantification of protein levels of the MAPK p38 and its phosphorylated form as well as TAB-1 showing a normalization of the p38 activation and the TAB-1 protein content in B1R$^{-/-}$-STZ compared with B1R$^{+/+}$-STZ. *$P < 0.05$ vs. B1R$^{-/-}$-STZ. (A high-quality digital representation of this figure is available in the online issue.)

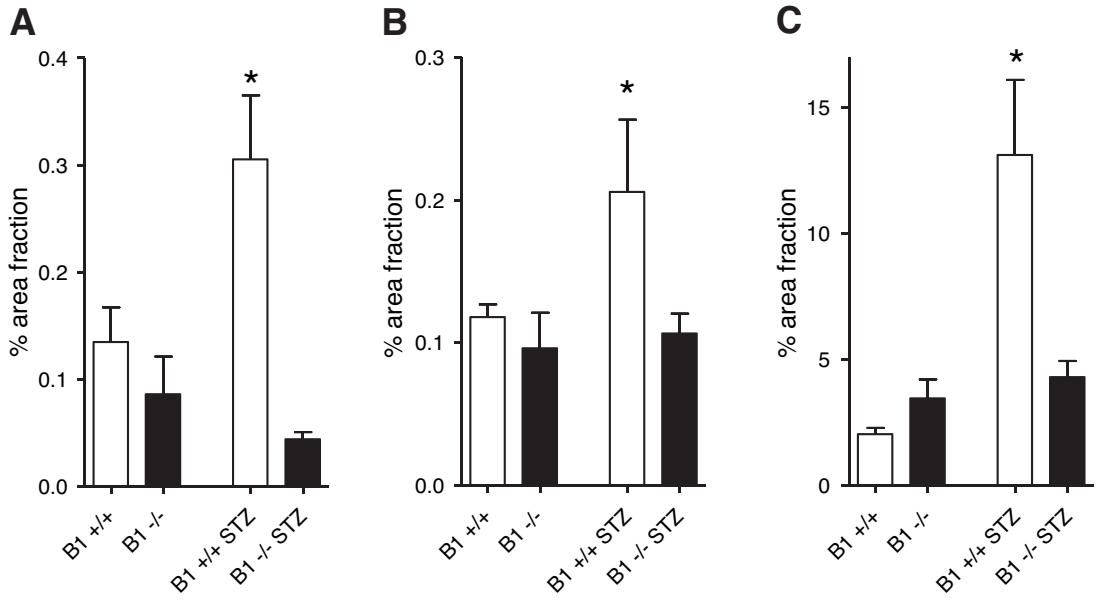

FIG. 7. Increased levels of CTGF (*A*) and collagen type 1 (*B*) and 3 (*C*) in cardiac tissue of B1R$^{+/+}$-STZ compared with nondiabetic controls. B1R$^{-/-}$-STZ have normalized cardiac fibrosis as well as normalized levels of CTGF. *$P < 0.05$ vs. B1R$^{+/+}$- and B1R$^{-/-}$-STZ.

inflammation by increasing invading cells and proinflammatory cytokines in airway diseases (39), we investigated its role during the development of diabetic cardiomyopathy. On one hand, in nondiabetic mice with gene deletion of the B1R, the cardiac function remained unchanged when this was compared with control mice in the current study. That is a finding that is in agreement with others (17,40–42). On the other hand, attenuated cardiac dysfunction despite severe hyperglycemia indicates a detrimental role of the B1R in diabetic cardiomyopathy when systolic and diastolic function of B1R$^{-/-}$-STZ were compared with STZ.

One intracellular pathway of the B1R was shown to be dependent on the MAPK p38 (43). The current study shows an activation of the p38 pathway in the STZ group, which was normalized in the B1R$^{-/-}$-STZ group. P38 phosphorylation can be induced by TAK-1 binding protein (44). In line with these findings, increased protein content of TAB-1 in diabetic wild-type mice compared with B1R$^{-/-}$-STZ mice could be documented here. Since p38 activation plays a role in diabetic cardiomyopathy by inducing cardiac inflammation (45), we documented increased cardiac levels of cytokines in the STZ group, namely of Il-1β, IL-6, and TNF-α, known to cause myocardial dysfunction and mediate leukocyte infiltration during tissue inflammation (46). This cytokine induction was decreased in diabetic mice lacking the B1R.

Moreover, the number of invading immunocompetent cells was increased in the STZ group, thus yielding another marker of cardiac inflammation. These increased numbers were normalized in mice lacking the B1R. This is important, since those invading cells are one major source of cytokine production within the cardiac tissue (e.g., as shown here by the colocalization of TNF-α with CD68^{+} cells). Furthermore, we show that the protein levels of MMP-9 were also increased in STZ and decreased in diabetic mice with gene deletion of the B1R. Recent findings (47) suggest that especially MMP-9 modulates the transendothelial migration of leukocytes from the vessel to the tissue, where an inflammatory progress is ongoing. This can be explained by the fact that MMP-9 does not solely cleave gelatin but indeed processes and activates many chemokines and cytokines and thereby directly modulates inflammation. In line with these effects, it was shown in a mouse model of hepatitis that MMP-9 knockout mice were protected against invading leukocytes undergoing transendothelial migration (48). Only recently could it be shown that induction of MMP-9 expression is triggered by bradykinin, using cell culture rat astrocytes (49). Although those authors showed that B2R antagonism inhibited this increase (49), our data also suggest that the B1R plays a role in MMP-9 expression in cardiac tissue under diabetic conditions. Moreover, recently, the B1R was shown to be essential for IL-1β–driven cell recruitment of immunocompetent cells by inducing the chemokine CXCL-5 in endothelial cells (50). This recruitment of CXCL-5, known to be one important player in leukocyte recruitment to sites of tissue inflammation, was abolished in mice without the B1R or when a pharmacological B1R antagonist was applied (50). In line with these data, we show here that the chemokine CXCL-5 was increased under diabetic conditions. This effect was completely normalized in mice lacking the B1R despite severe hyperglycemia. The normalized levels of CXCL-5 and MMP-9 result in reduced migration of inflammatory cells into the cardiac tissue. Therefore, inflammation leading to cardiac damage due to invasion of these cells was attenuated in diabetic B1R$^{-/-}$-STZ mice compared with diabetic controls with increased levels of CXCL-5 and MMP-9.

Furthermore, it was shown that the B1R, but not the B2R, increases the mRNA abundance of the profibrotic CTGF and thereby increases collagen mRNA and protein production in human fibroblasts, an effect that could be blocked by a B1R antagonist (51). Following these findings, we show increased levels of CTGF in the cardiac tissue of STZ mice, leading to increased collagen accumulation, which is known to be a hallmark in the development of diabetic cardiomyopathy leading to increased cardiac stiffness contributing to diastolic and systolic failure. Together with those findings, this mechanism could not be observed in mice with B1R gene

deletion, thus showing no increase in CTGF or collagen accumulation.

Much evidence has indicated that oxidative stress plays an important role in the failing diabetic heart (52). This can be attenuated by the kallikrein-kinin system (8,10). Consistently, we show increased nitrotyrosine and myeloperoxidase protein levels (expressed from inflammatory cells) in the cardiac tissue of the STZ group. Together with reduced inflammatory cells, nitrotyrosine and myeloperoxidase were reduced in the $B1R^{-/-}$-STZ group. These data suggest that the B1R might play a role in the generation of oxidative stress, most probably due to the increased recruitment of inflammatory cells resulting in increased myeloperoxidase present in the cardiac tissue. Future studies have to investigate whether this effect is only mediated by the B1R or if changes in basal B2R level expression, as shown in this study, are also influencing generation of oxidative stress in the $B1R^{-/-}$-STZ group.

The protein content of eNOS, known to exert antioxidative effects, was similarly reduced in both diabetic groups. Interestingly, $B1R^{-/-}$-STZ showed no significant downregulation on mRNA levels. Despite these changes in mRNA content, which may be explained by posttranscriptional modification, these data suggest that the diabetes-induced downregulation of eNOS protein is not mainly regulated by the B1R knockout. Again, further studies have to reveal the impact of B2R regulation on these effects. In conclusion, this study demonstrates that a lack of the B1R attenuates the development of STZ-induced diabetic cardiomyopathy with a decrease of cardiac inflammation, fibrosis, and oxidative stress.

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No potential conflicts of interest relevant to this article were reported.

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