Streptozotocin directly impairs cardiac contractile function in isolated ventricular myocytes via a p38 map kinase-dependent oxidative stress mechanism

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Abstract

Streptozotocin (STZ) has long been used to induce experimental diabetes mellitus to study diabetic complications such as diabetic cardiomyopathy. However, direct impact of STZ on cardiac function is unknown. This study was designed to evaluate the cardiac contractile effect of STZ in isolated adult rat ventricular myocytes. Contractile properties were assessed with an IonOptix MyoCam system including peak shortening (PS), time-to-PS (TPS), time-to-90% relengthening (TR90), and maximal velocities of shortening/relengthening (dL/dt). Intracellular Ca2+ handling was evaluated with the fluorescent dye fura-2. Acute exposure of STZ (10^-9–10^-5 M) depressed PS, prolonged TR90, and decreased electrically stimulated intracellular Ca2+ rise in a concentration-dependent manner. TPS, dL/dt, resting intracellular Ca2+ level, and intracellular Ca2+ clearing rate were unaffected. The STZ-induced mechanical alterations were alleviated by the antioxidant vitamin C (100 μM) and the p38 MAP kinase inhibitor SB203580 (1 μM). 2',7'-Dichlorofluorescein diacetate staining revealed enhanced production of reactive oxygen species following STZ treatment, which was prevented by either vitamin C or SB203580. Collectively, our data provided convincing evidence that the tool drug for experimental diabetes STZ may itself cause deleterious cardiac contractile dysfunction via an oxidative stress and p38 MAP kinase-dependent mechanism. Thus, caution should be taken when assessing diabetic heart complications using STZ-induced diabetic models.

Keywords: Diabetes; Myocytes; Streptozotocin; Oxidative stress; P38 MAP kinase

Streptozotocin (STZ) has long been the drug of choice to induce experimental diabetes mellitus due to its specific toxicity on pancreatic β-cells. Within days of STZ injection, animals develop severe hyperglycemia and diuresis coupled with weight loss and decreased motor activity [1]. One of the major complications in diabetes including STZ-induced experimental diabetes is diabetic cardiomyopathy, which develops independent of any macro-/micro-vascular diseases and is characterized by both systolic and diastolic dysfunctions [2]. The main mechanical defects of diabetic cardiomyopathy encompass reduced contractility, prolonged relaxation, and decreased compliance associated with impaired function of myosin Mg2+-ATPase, Ca2+-regulatory proteins, and mitochondria [3]. Both hyperglycemia and oxidative stress resulting from STZ administration have been speculated to play a role in the onset of cardiac dysfunctions [4,5]. Nevertheless, assessment of diabetic cardiomyopathy using STZ-induced experimental diabetic models has been conducted in the past decades under the assumption that STZ has little cardiac toxic effects. To elucidate the direct effect of STZ on cardiac mechanical function, cardiac contraction, intracellular Ca2+ transients, and generation of reactive oxygen species were evaluated in isolated ventricular myocytes following acute STZ administration. Since oxidative
stress and the stress-activated signaling pathway of p38 mitogen-activated protein (MAP) kinase have been implicated in the development of diabetic cardiac damage \[6,7\], the effect of STZ on cardiac mechanical function, intracellular Ca\(^{2+}\) transients, and reactive oxygen species generation was also examined in the presence of the antioxidant vitamin C or the p38 MAP kinase inhibitor SB203580.

**Materials and methods**

*Isolation of left ventricular myocytes.* The experimental procedures were approved by the Animal Investigation Committee of the University of North Dakota (Grand Forks, ND, USA). Adult male Sprague-Dawley rats (200–250 g) were purchased from Harlan Bioproducts (Indianapolis, IN, USA). Briefly, hearts were rapidly removed and perfused (at 37°C) with oxygenated (5% CO\(_2\)–95% O\(_2\)) Krebs-Henseleit bicarbonate (KHB) buffer (mM: NaCl 118, KCl 4.7, CaCl\(_2\) 1.25, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25, Na\(_2\)HPO\(_4\) 1.2, glucose 11.1, pH 7.4). Hearts were subsequently perfused with a nominally Ca\(^{2+}\)-free KHB buffer for 2–3 min followed by a 20 min perfusion with Ca\(^{2+}\)-free KHB containing 223 U/ml type II collagenase (Worthington Biochemical, Freehold, NJ, USA) and 0.1 mg/ml hyaluronidase (Sigma Chemical, St. Louis, MO, USA). After perfusion, the left ventricle was removed, minced, and further digested with trypsin (Sigma) before being filtered through a nylon mesh (300 µm) and collected by centrifugation. Cells were initially washed with Ca\(^{2+}\)-free KHB buffer to remove remnant enzyme and extracellular Ca\(^{2+}\) was added incrementally back to 1.25 mM \[2\].

*Measurement of myocyte shortening/relengthening and intracellular Ca\(^{2+}\) transients.* Mechanical and intracellular Ca\(^{2+}\) properties of ventricular myocytes were assessed using an IonOptix soft-edge system (IonOptix, Milton, MA, USA) \[2\]. Mechanical properties assessed including peak shortening (PS), time-to-PS (TPS), and maximal velocities of shortening/relengthening (±dL/dt), of intracellular Ca\(^{2+}\) fluorescence was recorded with a dual-excitation single-emission photomultiplier system (IonOptix) in myocytes loaded with fura-2 (0.5 µM). Intracellular Ca\(^{2+}\) properties analyzed included resting fura-2 fluorescence intensity (FFI), fluorescence decay rate, and electrically stimulated rise in intracellular Ca\(^{2+}\) (AFFI). All recordings were conducted while the ventricular myocytes were paced at 0.5 Hz.

*Experimental protocol.* Left ventricular myocytes (either fura-2-loaded or unloaded) were allowed to contract at a stimulation frequency of 0.5 Hz for 3 min to ensure steady state (myocytes with rundown >10% were not studied further) before exposing to STZ (10^{-9}–10^{-3} M) for 5 min. Since majority of the myocytes failed to survive or stopped twitching after exposing to 10^{-4} M STZ, 10^{-5} M was presented as the highest concentration in the results. Cells were then washed with normal contractile buffer for 3 min. In some studies, the antioxidant vitamin C (100 µM) or the p38 MAP kinase inhibitor SB203580 (10 µM) was pre-incubated with the myocytes for 5 min prior to STZ application and remained present during STZ treatment. Sodium citrate, a solvent often used for intravenous injection to deliver STZ, was used as a vehicle control for STZ.

*Measurement of intracellular reactive oxygen species.* Production of intracellular reactive oxygen species (ROS) was evaluated by analyzing changes in fluorescence intensity resulting from oxidation of the intracellular fluorophore 5,6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate (CM-H\(_2\)DCFDA, Molecular Probes, Eugene, OR, USA) \[8\]. In brief, ventricular myocytes were loaded with H\(_2\)DCFDA (1 µM) at 37°C for 30 min and the fluorescence intensity was measured with a fluorescent micro-plate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. H\(_2\)DCFDA-untreated cells had no fluorescence and were used as background fluorescence. The final fluorescence intensity was normalized to respective protein content in myocytes used for each assay and was normalized to the fluorescent intensity of myocyte group without treatment of STZ, vitamin C or SB203580.

*Data analysis.* A total of 14 rats were used for cell isolation and the myocytes shown in each figure were evenly distributed from these animals. Data were shown as means±SEM. Statistical significance (p<0.05) for each variable was estimated by two-way analysis of variance (ANOVA) with a Dunnett’s test as post hoc analysis.

**Results**

*Acute STZ treatment induced cardiomyocyte dysfunction: prevention by vitamin C*

The average resting cell length for ventricular myocytes used in this portion of study was 112.1±3.5 µm \((n=46\) cells). Vehicle for STZ administration (sodium citrate) had no observable effect on cell phenotype. Acute STZ administration (5 min) elicited a concentration-dependent \((10^{-9}–10^{-3} M)\) inhibition in peak shortening amplitude when normalized to the respective control value \((5.54±0.42\%\) of resting cell length). While STZ \((10^{-9}–10^{-5} M)\) failed to produce any notable effect on maximal velocities of shortening/relengthening \((±dL/dt)\) or time-to-peak shortening (TPS), it significantly prolonged time-to-90% relengthening \((TR_{90})\) with a threshold between 10^{-9} and 10^{-8} M, an effect reminiscent of cardiac myocytes isolated from in vivo diabetic animals \[2\]. A large portion of the ventricular myocytes (>70%) either died or ceased to twitch at 10^{-4} M STZ concentration, thus mechanical data obtained at 10^{-4} M STZ were not analyzed. Pretreatment of the cardiac myocytes with antioxidant vitamin C (100 µM) effectively prevented STZ-induced depression in PS and prolongation in TR_{90} while it did not affect the response of STZ on other mechanical indices tested. Vitamin C treatment itself did not exert any significant effect on PS, ±dL/dt, TPS, and TR_{90} (Fig. 1).

*Effect of acute STZ treatment on intracellular Ca\(^{2+}\) transients*

Effect of acute STZ administration (5 min) on intracellular Ca\(^{2+}\) transient properties was examined using fura-2/AM fluorescent dye. The time course of intracellular Ca\(^{2+}\) fluorescence signal decay was described by a single exponential equation and the decay constant was used as an indicator of the rate of intracellular Ca\(^{2+}\) clearing \[2\]. The vehicle for STZ (sodium citrate) had no effect on any parameters of fura-2 fluorescence (data not shown). Electrically stimulated increase of intracellular...
Ca\(^{2+}\) (or increase in fura-2 fluorescence intensity, ΔFFI) was significantly depressed only at the highest STZ concentration (10\(^{-5}\) M). Neither baseline FFI nor intracellular Ca\(^{2+}\) fluorescence decay rate was affected by 5 min STZ treatment within the concentration range tested (10\(^{-9}\)–10\(^{-5}\) M) (Fig. 2).

**Involvement of p38 MAP kinase in STZ-induced cardiomyocyte mechanical dysfunction**

Activation of the stress signaling molecule p38 MAP kinase has been shown to be involved in oxidative stress-induced cell injury, including diabetic hyperglycemic conditions [9,10]. To determine whether p38 MAP kinase was involved in STZ-induced cardiac contractile dysfunction, the effect of STZ on contractile function was re-examined by preincubating the myocytes with the selective p38 MAP kinase inhibitor SB203580 (1 μM). The average resting cell length for ventricular myocytes used in this portion of study was 110.1 ± 2.9 μm (n = 47 cells). Consistent with previous report [11], SB203580 itself did not affect the mechanical indices in cardiac myocytes. However, the STZ-induced decrease in PS and prolongation in TR\(_{90}\) were ablated by SB203580. SB203580 did not change the profile in STZ response for ± dL/dt and TPS. These results suggested that STZ-associated deleterious cardiac actions may be mediated, at least in part, through a p38 MAP kinase-dependent mechanism (see Fig. 3).

**Effect of STZ on ROS generation in ventricular myocytes**

STZ has been shown to induce oxidative stress [12], which is consistent with the role of oxidative stress in the progression and pathogenesis of diabetic cardiomyopathy [6,13]. Intracellular fluorescence dye DCF was used in order to assess the intracellular ROS generation in ventricular myocyte following STZ treatment. Myocytes treated with STZ (10\(^{-5}\) M) for 5 min displayed significantly enhanced fluorescence intensity, which was significantly attenuated by pretreatment with either vitamin C (100 μM) or the p38 MAP kinase inhibitor SB203580 (1 μM) (Fig. 4). These data suggest that oxidative stress via a p38
MAP kinase signaling-dependent mechanism is likely to play a critical role in STZ-induced cardiomyocyte dysfunction.

Discussion

Our study reported for the first time convincing evidence that acute STZ administration may directly impair cardiomyocyte contractile function independent of pancreatic toxicity and hyperglycemia. The working dogma for cardiovascular complications in STZ-induced diabetes has essentially been based on the secondary hyperglycemic effect of STZ following destruction of β-cells of the pancreas [14]. Hyperglycemia in turn promotes oxidative stress and impairs intracellular Ca²⁺ homeostasis, leading to diabetic heart dysfunction. The present study showed depressed PS and prolonged TR₉₀ (the hallmark phenotype of cellular diabetic cardiomyopathy) in myocytes acutely treated with STZ (in the absence of hyperglycemia). The observation that STZ-induced heart dysfunction is preventable by pre-treatment with either the antioxidant vitamin C or the p38 MAP kinase inhibitor SB203580 suggests that STZ-induced heart dysfunction is likely mediated through a p38 MAP kinase-dependent oxidative stress pathway.

The involvement of p38 MAP kinase-associated ROS production in response to STZ treatment in ventricular myocytes has been substantiated by our experimental evidence that STZ-induced ROS generation and myocyte dysfunction may be ablated by the antioxidant vitamin C. It is well known that the cardiac oxidant balance is impaired in a number of pathological states including diabetes and heart failure, which can be prevented by antioxidants [15–17]. p38 MAP kinase participates in the cellular responses to various assaults such as UV light, osmotic stress, and mechanical or chemical stress, resulting in cellular damage including cardiac contractile dysfunction [10]. Activation of p38 MAP kinase directly leads to a negative inotropic effect in cardiac myocytes [18] and inhibition of which may improve contractile function in ischemia/reperfusion-injured heart [19,20]. These findings have implicated a novel mechanism for stress-activated signals in the regulation of cardiac contractility. Interestingly, the negative inotropic effect of p38 MAP kinase is believed to be mediated by a reduction in responsiveness of the myofilaments to Ca²⁺ [18], consistent with our present findings in the discrepant
response between STZ-induced inhibition of myocyte shortening and intracellular Ca\(^{2+}\) transients. Our study observed a much more sensitive responsiveness in STZ-induced alteration in PS and TR\(_{90}\) whereas little or no response in DFFI and intracellular Ca\(^{2+}\) transient decay rate. Although diabetes and glucose have been shown to directly trigger the activation of p38 MAP kinase [21], whether p38 MAP kinase plays a role in the pathogenesis of diabetic cardiomyopathy has not been elucidated. Vitamin C has been shown to scavenge a wide variety of free radicals, thus offering cardiac protection against ROS. It is a water-soluble antioxidant and plays an indirect role in terminating lipid peroxidation and oxidative stress.

In conclusion, our study demonstrates that the pancreatic β-cell toxin STZ directly alters cardiac contractile function at the ventricular myocyte level, possibly through a p38 MAP kinase-dependent oxidative stress mechanism. These findings not only reveal novel interactions among STZ, p38 MAP kinase, and cardiac mechanical function but also provide warning for experimental application of STZ in the study of diabetic heart complications. Caution has to be taken when assessing cardiac function using STZ-induced experimental diabetes since STZ may produce hyperglycemia/diabetes-independent cardiac mechanical effects.
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References