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STAT3 and Nrf2 pathways modulate the protective effect of verapamil on lung injury of diabetic rats

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Objective. We aimed to assess the protective role of verapamil, L-type calcium channel blockers, against early lung damage in diabetic rats. Lung injury has recently been recognized as a consequent complication of diabetes mellitus. Hyperglycemia induces inflammatory changes in lung tissue early in the disease.

Methods. Twenty four adult male rats were grouped into control, diabetic, diabetic treated with verapamil, and verapamil control. Streptozotocin (STZ) was used to induce diabetes. Oxidative parameters and antioxidative mechanisms were assessed in lung homogenate. Tumor necrosis factor alpha (TNFα) protein was measured as a pro-inflammatory mediator. Signal transducer and activator of transcription 3 (STAT3) gene expression and nuclear erythroid factor 2 (Nrf2) immunoexpression were screened.

Results. The lung showed oxidative damage and inflammatory infiltration in STZ diabetic rats early at 2 weeks. The parameters significantly improved in lung tissue treated with verapamil. Histopathology of the lung tissue confirmed the results. Inhibition of STAT3/TNFα pathway was involved in the protection offered by verapamil. Activation of Nrf2 together with an increasing antioxidant capacity of diabetic lung significantly ameliorates the injury induced by diabetes.

Conclusions. Verapamil afforded protection in diabetic lung injury. The protection was mediated by the anti-inflammatory and antioxidant effects of verapamil.

Key words: streptozotocin, verapamil, lung, STAT3, TNFα, Nrf2

Diabetes mellitus (DM) is a global endocrine disease with various metabolic disturbances. About 422 million people suffer from diabetes mellitus, and the number is expected to increase (WHO 2016). DM is well-known for its serious complications, which affect almost all organs. Previous studies were focused on diabetic complications that affect kidney, retina, nerves, and heart (Kedziora-Kornatowska et al. 2002; Chawla et al. 2016). Lung, as an organ, has been recently recognized to be affected by diabetes (Pitocco et al. 2012). Hyperglycemia associated with DM is firmly linked to the progression of its complications (Chawla et al. 2016). Chronic hyperglycemia overproduces reactive oxygen species (ROS) and incapacitates the antioxidative cell defenses (Asmat et al. 2016). Together with the elaboration of various inflammatory mediators as tumor necrosis factor alpha (TNFα), the multi-organ injury is initiated (de Rekeneire et al. 2006).

Calcium channel blockers are sets of well-known, well-tolerated drugs that selectively block voltage calcium channels of L-type. They are the most broadly prescribed drugs for the treatment of cardiovascular diseases (Godfraind 2017). However, other beneficial

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roles recently emerged from this class in different body organs (Striessnig et al. 2015). Among calcium channel blockers, verapamil has been demonstrated with its valued antioxidant and anti-inflammatory effects (Kedziora-Kornatowska et al. 2002; Das et al. 2009). Verapamil exhibits an advantage in diabetic complications in heart and kidney (Tanaka et al. 1991; Tabur et al. 2015). However, the role of verapamil in lung injury has not been yet elucidated. Verapamil, in addition, displayed a pioneering effect on enhancing the survival of pancreatic beta cells of mice (Xu et al. 2012). It has been shown an improvement in the serum glucose levels of diabetic patients (Khodneva et al. 2016). These data encourage to investigate the role of verapamil in diabetic lung injury at an early stage of oxidative damage and explore the possible mechanisms underlying the developing results.

Materials and methods

Drugs and kits. The powders of verapamil as well as streptozotocin (STZ) were bought from Sigma-Aldrich Co., (St. Louis, MO, USA). Enzyme linked immunoassay (ELISA) kit for tumor necrosis factor alpha (TNFα) was getting from (Glory Science Co., Ltd). Rabbit Polyclonal antibody for nuclear erythroid factor 2 (Nrf2) was obtained from Lab Vision Corp. (USA). Other chemicals were obtained from commercial sources.

Animals. The study used 24 male albino rats weighing 180–200 g that were brought from the National Research Center, El-Giza, Egypt. Under standard conditions, animal were left one week for accommodation. The study was conducted according to the ethical standards approved by the faculty board committee of the faculty of medicine, Minia University, Egypt (REC1/2013/Pharm/1).

Induction of diabetes. Rats were injected at 8 am by STZ i.p at a dose of 50 mg/kg (Taghizadeh et al. 2018). STZ in 0.01 mM citrate buffer (pH 4.95) was prepared and injected within 10 min. STZ administered rats were given 20% sucrose in water within the first 48. Serum glucose levels were checked after 72 h by using a glucose meter (ACCU-CHEK®, Roche Diagnostics, Germany). Blood glucose above 250 mg/dl was considered diabetic and animals were involved in the study. Animals grouped as follows (6/group): control group received vehicle; verapamil group received verapamil (40 mg/kg) (Balakumaran et al. 1996); diabetic group was left untreated; diabetic-verapamil group received verapamil (40 mg/kg). Verapamil was suspended in 1% aqueous solution of carboxymethyl cellulose (vehicle) and administered orally by intragastric tube for 2 weeks after establishment of diabetes. Then, animals were sacrificed and parts of the lung were immersed in formalin. The lungs kept in –80 °C for various biochemical analyses, or processed for RNA extraction.

Serum and tissue measurement. At the end of the experiment, blood glucose levels were measured. Part of lung tissue was dried by filter paper and weighted then 1:10 w/v potassium phosphate buffer (prepared by dissolving 8.01 g NaCl, 0.20 g KCl, 1.78 g $Na_2HPO_4 \times 2H_2O$ and 0.27 g KH_2PO_4 in 1 liter of distilled water at pH 7.4) was added, mixed, then homogenized using Homogenizer (Tri-R Stir-R Homogenizer, Tri-R Instruments, Inc., Rockville Centre, NY). The homogenate was centrifuged at 10 000 rpm at 4 °C in a cooling centrifuge for 15 min and the supernatant was separated in epindorff.

Malondialdehyde, a lipid peroxidation product, was assessed by reacting with thiobarbituric acid. The colored supernatants were measured at 535 nm and calculated by the standard curve of 1, 1, 3, 3-tetramethoxy propane (Buege and Aust 1978). Antioxidant status was assessed in tissue homogenate by measuring the amount of reduced glutathione (GSH) and activity superoxide dismutase (SOD). GSH in samples reacted with 5,5-dithio-bis-2-nitrobenzoic acid to produce pale yellow color that measured at 412 nm by a spectrophotometer (Moron et al. 1979). SOD has a property to inhibit pyrogallol autoxidation. The activity was estimated colormetrically at 420 nm (Marklund and Marklund 1974).

Enzyme linked immunosorbent assay of TNFα. Tissue homogenate was used to assess TNFα protein expression. Instructions of the kit were strictly followed.

Real-time reverse transcription polymerase chain reaction (RT PCR). For signal transducer and activator of transcription 3 (STAT3) quantification, lung specimen was homogenized and RNA was extracted by ribozol reagent (Amresco, Solon, USA). cDNAs were produced by cDNA Synthesis kit from Fermentas (Life Sciences). They were reversely transcribed. RT PCR was done with 50 ng cDNA for each reaction consuming 25 µl of SYBR Green QPCR Mix (Solis BioDyne) encompassing 20 µM of the specific primers system in a relatively quantification to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene.

The sets of primers are: STAT3 forward, 5'-TGGAAGAGGCGGCAGCAGATAGC-3', and STAT3-reverse, 5'-CACGGCCCCCATTCCCACAT-3'. GAPDH sense primers: 5'-GTCGGTGTGAACGGATTTG-3' and antisense 5'-CTTGCCGTGGGTAGAGTCAT-3'.

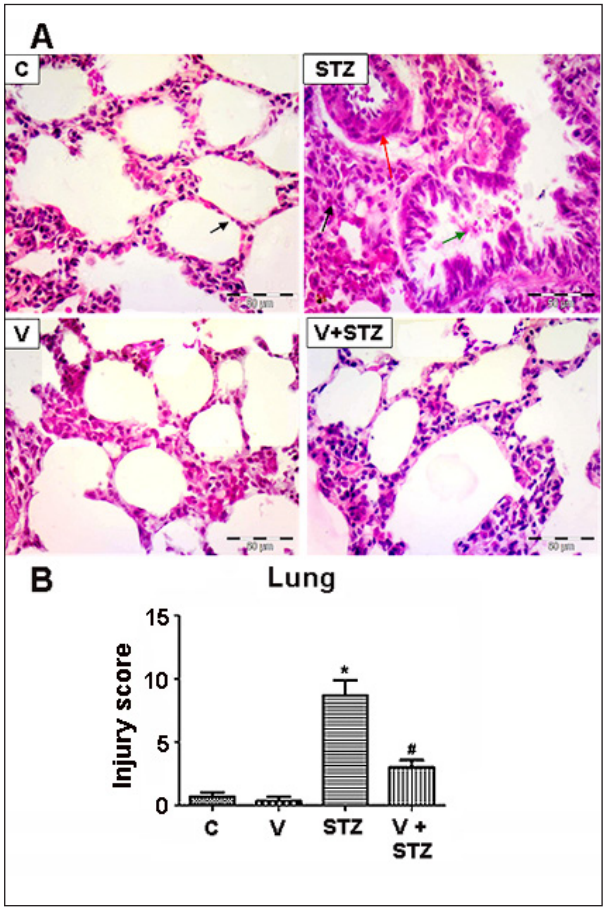

Figure 1. (A) A photomicrograph of lung tissue at power magnification ×400. Control rats (C) as (V) verapamil control showed normal open alveoli and thin interalveolar septum (black arrow). (STZ) streptozotocin diabetic rat showed thick congested inflamed interalveolar septum (black arrow), a thick walled blood vessel (red arrow) and cells inside bronchiolar lumen (green arrow). (V+STZ) verapamil treated rat showed marked improvement of the pathology. **(B)** Lung injury scoring. *# significantly different ($p<0.05$) from control rats and STZ-diabetic rats, respectively.

Control samples were set at a value of 1 and the expression for each gene was mounted relative to controls according to VanGuilder et al. (2008).

Histological tissue investigation. Fixed lung specimens were embedded into paraffin. Histological sections were stained with hematoxylin and eosin, while others charged slides were prepared for immunostaining according to the manufactured kit. Using light microscopy, the tissue sections were analyzed by histologist blinded to the experimental groups. The evaluation of lung injury was assessed by Mikawa score according to four parameters: alveolar congestion, hemorrhage, neutrophils infiltration, and alveolar wall thickness (Mikawa et al. 2003). Nrf2 immunoexpression was scored by Palanisamy et al. (2011).

Statistical analysis. The data of this study are articulated as the mean ± SEM and statistical significance was calculated with an ANOVA then Tukey's t-test. A value of $p<0.05$ was considered statistically significant.

Results

Fasting blood glucose level and body weight. At the end of two weeks, blood glucose levels showed an increase and body weight showed a decrease in diabetic rats as compared to control ones. Treatment with verapamil significantly decreased the glucose levels as well as prevented the significant decrease in the body weight (Table 1).

Oxidative parameters in lung tissue. In diabetic rats, MDA showed a significant increase, while both GSH concentrations and SOD activities exhibited a drop in their levels compared to control rats. Verapamil administration almost significantly normalized these measurements (Table 1).

Table 1
Effect of verapamil on body weight, serum glucose level, reduced glutathione, malondialdehyde, and superoxide dismutase in lung tissue of diabetic rats.

Group	Body Weight (g)	Serum Glucose (mg/dl)	Lung GSH (nmol/g tissue)	Lung MDA (nmol/g tissue)	Lung SOD (U/g tissue)
Conrol	213.3±21.07	93.0±9.07	583.2±26.78	76.50±1.88	928.8±50.42
Verapamil	197.3±4.33	91.3±5.93	509.7±108.5	72.79±4.33	1001±86.57
STZ	106.3±7.79*	372.3±6.64*	165.3±26.97*	240.2±8.90*	355.0±50.51*
STZ+verapamil	182.3±1.45#	191.0±10.97#	404.6±44.80#	83.88±0.46#	830.3±197.3#

Abbreviation: STZ – streptozotocin; GSH – glutathione; MDA – malondialdehyde; SOD – superoxide dismutase.
All parameters are expressed as means ± S.E.M. (n=6). *$p<0.05$ vs. normal control group; #$p<0.05$ vs. STZ group.

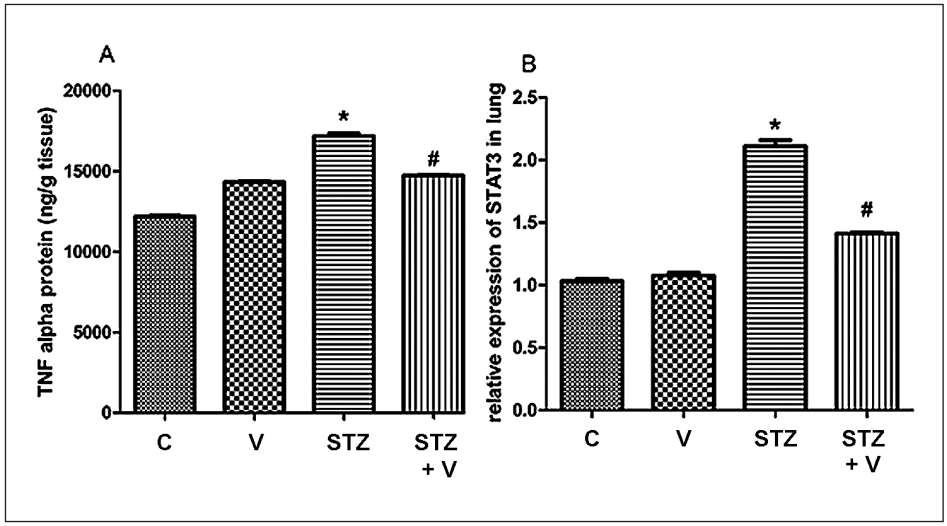

Figure 2. (A) Protein expression of TNFα in lung tissue. **(B)** Real time PCR for quantification of mRNA of STAT3 in lung tissue.*# significantly different ($p<0.05$) from control rats and STZ-diabetic rats, respectively. C – control; V – verapamil; STZ – streptozotocin.

TNFα expression and the relative expression of STAT3 in lung tissue. The high protein expression of TNFα was observed in diabetic rat lung. Significant improvement of TNFα expression by verapamil in STZ-treated rats is shown in Figure 2A. STAT3 was highly expressed in lung of diabetic rats in contrast to control rats. Verapamil significantly decreased STAT3 expression in the treated rats (Figure 2B).

Histology of lung tissue and expression of Nrf2. Lung tissue of diabetic rats showed significant cellular infiltration in the alveolar wall with marked congestion and hemorrhage. Verapamil treated rats displayed improvement in all the findings (Figure 1). Nrf2 immunostaining was evident in control and verapamil rats. Injection of STZ into rat displayed a marked decrease in Nrf2 expression as compared to controls. Verapamil treated rats expressed almost normal immunostaining for Nrf2 (Figure 3).

Discussion

Verapamil, the L-type calcium channel blocker, has protective effects on various organs in diabetic models. However, the present study offers an additional protective role for verapamil in the diabetic lung injury in rat.

Diabetes, with its catastrophic complications, embraces all body organs. In the present study, diabetes was found to affect lung tissues early by a prominent inflammatory cellular infiltration and a massive congestion. In line with the histopathology and others, lipid peroxidation and oxidative parameters in lung

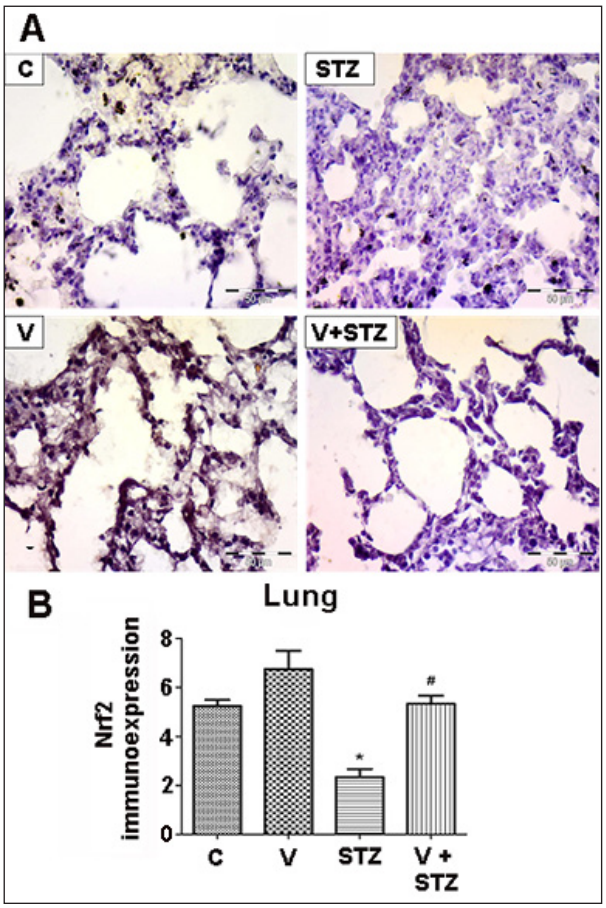

Figure 3. (A) A photomicrograph of lung Nrf2 expression. **(B)** Scoring of Nrf2 expression in lung tissue. *# significantly different ($p<0.05$) from control rats and STZ-diabetic rats, respectively.

tissue were disturbed significantly (Hu et al. 2014; Zhang et al. 2015). Verapamil successfully normalized the blood glucose and overcame the marbles body wasting in diabetic rats. This effect on serum glucose was strengthened by several studies performed on animals as well as humans (Xu et al. 2012; Poudel and Kafle 2017). These studies linked the hypoglycemic effect of verapamil with its ability to damping oxidative stress in the pancreatic beta cells. Likewise, our study showed an obvious antioxidant effect of verapamil both by inhibiting peroxidation of lipids and enhancing endogenous oxidative defenses as glutathione and antioxidant dismutase.

Diabetes-induced cellular inflammation has been discussed in depth in many previous studies. For example, cytokine, most importantly TNFα, was secreted abundantly in the insulin resistance state (Hotamisligil et al. 1995). Relation of TNFα with the occurrence of diabetic complication has also been confirmed by Kalantarinia et al. (2003). The role of TNFα in establishing the diabetic lung injury was prominent in the present study, in harmony with Zheng et al. (2017). The verapamil-induced decrease in the TNFα protein level has been explained well by Brown et al. (2004). Brown et al. (2004) have shown a unique anti-inflammatory effect of verapamil on its decline of calcium current into alveolar macrophages and subsequently TNFα production. In addition, a body of evidence supported the fact that TNFα mediates its inflammatory action through STAT3 signaling activation. TNFα tyrosine phosphorylation and activation of its receptor provides docking sites for STATs. STATs once phosphorylated, they are released from the receptor and translocated into the nucleus where they activate transcription of specific genes relevant for the inflammatory responses (Miscia et al. 2002). Moreover, STAT3 activation has been implicated in cytokine-induced insulin resistance (Mashili et al. 2013) and diabetic organ damage. The findings of the present study revealed upregulation of STAT3 mRNA in diabetic rats and its downregulation by verapamil treatment. Our results were supported by a pioneer study demonstrating that the STAT3 gene acts as a diagnostic and prognostic biomarker for lung inflammation and cancer. The study has reported upregulated STAT3 mRNA during the inflammatory lung insult in mice and human (Qu et al. 2009). We suggested here that the drop in STAT3 by verapamil treatment contributes to the attenuation of inflammation in the diabetes lung injury. The anti-inflammatory effect of verapamil also based on STAT3 inhibition in other models of neurotoxicity (Hashioka et al. 2012). In addition, STAT3 inhibition has been proven to be beneficial in the diabetic (Said et al. 2018) and non-diabetic lung injuries (Zhao et al. 2016).

Nrf2, the signaling cellular defense antioxidative damage, displays a crucial role in the glucose metabolism, possibly by increasing the cellular glucose uptake (Heiss et al. 2013). Verapamil treatment was capable of increasing the cytoprotective Nrf2 after marked attenuation by diabetes. A diabetes-induced decrease of the Nrf2 signalling pathway was a hallmark in the progress of oxidative damage and insulin resistance as shown by Tan et al. (2011). In the present study, that restoration of Nrf2 protein expression by verapamil was accompanied by an improvement in both antioxidant defenses and serum glucose levels.

In conclusion, the present study shows a new challenge for verapamil in the protection of the diabetic lung injury. The emerging anti-inflammatory, antioxidant, and hypoglycemic effects were mediated by STAT3 inhibition and Nrf2 activation.

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