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Original Paper

Hyperketonemia (Acetoacetate) Upregulates NADPH Oxidase 4 and Elevates Oxidative Stress, ICAM-1, and Monocyte Adhesivity in Endothelial Cells

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Key Words
NOX4 • ROS • ICAM-1 • Acetoacetate, and type 1 diabetes

Abstract
Background/Aims: The incidence of developing microvascular dysfunction is significantly higher in type 1 diabetic (T1D) patients. Hyperketonemia (acetoacetate, β-hydroxybutyrate) is frequently found along with hyperglycemia in T1D. Whether hyperketonemia *per se* contributes to the excess oxidative stress and cellular injury observed in T1D is not known. ***Methods:*** HUVEC were treated with ketones in the presence or absence of high glucose for 24 h. NOX4 siRNA was used to specifically knockdown NOX4 expression in HUVEC. ***Results:*** Ketones alone or in combination with high glucose treatment cause a significant increase in oxidative stress, ICAM-1, and monocyte adhesivity to HUVEC. Using an antisense approach, we show that ketone induced increases in ROS, ICAM-1 expression, and monocyte adhesion in endothelial cells were prevented in NOX4 knockdown cells. ***Conclusion:*** This study reports that elevated levels of ketones upregulate NOX, contributing to increased oxidative stress, ICAM-1 levels, and cellular dysfunction. This provides a novel biochemical mechanism that elucidates the role of hyperketonemia in the excess cellular injury in T1D. New drugs targeting inhibition of NOX seems promising in preventing higher risk of complications associated with T1D.

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Introduction

Type 1 diabetic (T1D) patients have frequent episodes of ketosis and elevated blood levels of ketone bodies acetoacetate (AA) and 3-β-hydroxybutyrate (BHB), in addition to hyperglycemia. In cases of very severe insulin deficiency, the serum concentration of these ketone bodies can exceed 25 mM, compared with levels of <0.5 mM in normal individuals [1-3]. Ketonemia levels of 1-2 mM (1-2 μmol/mL) are frequently seen in T1D patients during routine check-up visits at the clinic [1, 4-6]. Newer data indicate that hyperketonemia is also being identified in type 2 diabetic (T2D) patients [7, 8]. T1D patients are at a significant risk of developing microvascular complications that include nephropathy, retinopathy, and neuropathy [9-11]. The role of ketones in contributing to the risk of development of cardiovascular disease (CVD) is not known. Nevertheless, there is some evidence that elevated ketone levels are associated with congestive heart failure and carotid atherosclerosis [12, 13]. On the other hand elevated blood ketones are associated with the worsening of hyperglycemia, and patients with ketosis onset T2D showed much more severe insulin resistance compared to nonketotic onset T2D patients [14, 15]. Several studies have also shown that diabetes associated ketoacidosis can cause cerebral edema, hemorrhagic stroke, or acute ischemia leading to intracerebral complications [16, 17]. Recent studies using MRI measures show altered brain structure and injury associated with adverse neurocognitive outcomes including impairment in neuronal function and viability in T1D children [18, 19]. Ketosis mediated cerebrovascular endothelial dysfunction has been shown to be associated with intracranial microvascular complications [20]. Studies have also shown that ketones can promote pro-inflammatory factors and elicit systemic inflammation [20, 21]

Intercellular adhesion molecule-1 (ICAM-1) is a cell surface glycoprotein expressed on cells and induction of ICAM-1 promotes monocyte/macrophage recruitment allowing the macrophages to adhere. The ICAM-1 level is considered a predictor of endothelial dysfunction, and is reported to be elevated in T1D patients [11, 22, 23]. Increased ICAM-1 expression was reported with ketones in human brain endothelial cells [24].

NADPH oxidase-derived ROS plays a physiological role in the regulation of endothelial function and the inflammation underlying vascular remodeling in diabetes. Various NADPH oxidase (NOX) isoforms have now been identified and characterized as having an important function in mediating oxidative stress and thereby regulating cellular functions. Several reports indicate that NOX contribute to oxidative stress in diabetes [25-27]. There is also evidence that the activation of these enzymes may be driven by various stimuli such as cytokines, growth factors, hyperglycemia, and lipids [28]. High glucose is known to activate NOX [29]. However, there is no study that has investigated the effect of ketones on the expression or the activity of NOX in HUVEC or any other cell type.

This study demonstrates for the first time that ketones upregulate NOX4 expression and NOX activity in HUVEC. Using an antisense approach we show that ketone induced increases in ROS, ICAM-1 expression, and monocyte adhesion in endothelial cells was prevented in NOX4 knockdown cells. This suggests that NOX4 upregulation mediates the effect of ketones on ROS, ICAM-1, and monocyte-endothelial adhesion. This provides a novel biochemical mechanism that elucidates the role of hyperketonemia in the increased oxidative stress that potentially mediates the excess cellular injury in T1D. This study suggests that cellular dysfunction associated with T1D might be preventable by targeting NOX.

Materials and Methods

Endothelial cells

Primary human umbilical vein endothelial cells (HUVEC): Cells were purchased from Lonza Walkersville Inc., Walkersville, MD. They were cultured in Endothelial Growth Medium-2 BulletKit from Lonza Walkersville Inc., Walkersville, MD and grown to confluence at 37 °C in a humidified atmosphere containing 5% CO_2. The culture was passaged according to standard procedures. For experiments, HUVEC were used within 24 h after reaching confluence, between passages 3 and 9.

Monocytes

Human THP-1 monocytes were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 100 µg/mL streptomycin, 7 mM glucose, 12 mM sodium carbonate, 12 mM HEPES, and 2 mM glutamine. The culture was grown and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were counted using the Trypan Blue method before all treatments. The number of cells was maintained at around one million per mL of media.

Treatment with AA, BHB, or HG

Stock solutions for AA, BHB, and HG were made in sterilized water. Cells were treated with ketones: acetoacetate (AA, 0-4 mM) or DL-β-hydroxybutyrate (BHB, 0-12 mM) and HG (25 mM) for 24 h. Previous studies have reported ketone concentrations as high as 25 mM in patients with uncontrolled diabetes [2]. Physiologically AA and BHB occur in either 1:2 or 1:3 ratios [2]. To recreate this ratio we have incorporated both AA (4 mM) and BHB (12 mM) together in cell treatments. Thus, the ketone concentrations used to mimic hyperketonemia in this cell culture study are reasonable. The glucose concentration of 25 mM was similar to that used in other studies [30]. Patient data suggest that glucose levels can sometimes become elevated to 30 mM [1]. Cell viability was determined in all treatments to rule out cell death.

siRNA knockdown in HUVEC

NOX4 siRNA (s224161) was purchased from Invitrogen (Eugene, OR). siRNA was diluted in serum free transfection medium (Santa Cruz) and plated into wells. An aliquot of 3 µL transfection reagent (lipofectamine Invitrogen) was added to each well of diluted siRNA and gently mixed before incubating for 20 min. Trysinized HUVEC were resuspended in the transfection media and added to the siRNA-lipofectamine complexes. The cell suspension added to the wells was calculated accordingly to give a final concentration of 200 nM siRNA. Cells were incubated for 4 h at 37 °C. Complete media with serum was then added to the cells and they were incubated at 37 °C. After 24 h fresh medium was added and the cells were treated with different reagents as per the experimental protocol. Confluent cells were used to perform ROS, Western blotting, quantitative PCR, or adhesion assays.

ROS assay

Cells were plated in a 96 well plate and grown to subconfluence. They were treated with ketones and HG. An aliquot of 20 µM oxidant-sensitive probe dicholorodihydrofluorescein diacetate (H_2DCFDA, Sigma Chemical Co., St. Louis, MO, USA) was added to the cells and incubated at 37 °C for 30 min. As H_2DCFDA enters cells, intracellular esterase activity removes the diacetate group and the resulting H_2DCF reacts with any ROS in the cell and fluoresces. After incubation with the dye the cells were washed once with PBS and fluorescence was measured using the plate reader at filter settings of 485 nm excitation and 528 nm emission. Results are expressed as % of control.

Cell viability assay

Cells plated in 96 well plates after reaching confluence were treated with ketones for 24 h. After treatment, Alamar Blue (Invitrogen, Carlsbad, CA) reagent was added and incubated at 37 °C in the dark for 3.5 h. Absorbance was read at 590 nm using a plate reader.

NADPH oxidase activity

NADPH oxidase activity of the cell lysates was determined following the method of Abid et al. [31]. HUVEC were grown to subconfluence and treated with ketones for 24 h. After treatment the cell lysates were quantified using the BCA method. Protein samples (50 µg) diluted in a reaction mixture containing 250 mM HEPES (pH 7.4), 120 mM NaCl, 5.9 mM KCl, 1.2 mM $MgSO_4(7H_2O)$, 1.75 mM $CaCl_2(2H_2O)$, 11 mM glucose, 0.5mM EDTA, and 5 µM lucigenin were loaded onto a white 96 well plate. NADPH (100 µmol/L) was added to cell lysates and luminescence was measured every min for 15 min using a Synergy HT microplate reader. Results are expressed as % of control.

Monocyte-HUVEC adhesion assay

HUVEC were plated and allowed to grow to confluent monolayers. They were treated accordingly with AA (4 mM), BHB (4 mM or 12 mM), or HG (25 mM) for 24 h. Monocytes were labeled with 8 µM CellTracker

Green (CMFDA; Invitrogen, Eugene, OR) and then treated similarly along with HUVEC. After 24 h, $1X10_6$ monocytes were added to the endothelial monolayers and incubated at 37 °C for 45 min. The nonadherent cells were washed away with EC media and collected. Both adherent cells and nonadherent cells were lysed in 0.2% Triton X for quantification. The fluorescent intensity of the monocytes added to the monolayer (input) as well as that of the nonadherent cells was measured at excitation 485 nm and emission 528 nm. Results are expressed as % of control.

Western blotting

After treatment the cells were lysed in RIPA buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS) supplemented with protease and phosphatase inhibitors (1 mM PMSF, 5 µg/mL leupeptin, 2 µg/mL aprotinin, 1 mM EDTA, 10 mM NaF, and 1 mM $NaVO_4$). Lysates were then centrifuged for 15 min at 13,000 rpm at 4 °C. Supernatants were collected and the protein concentrations were determined using a BCA assay kit (Pierce/Thermo Scientific, Rockford, IL). An aliquot of ~20 µg protein from each sample was prepared in SDS buffer with or without BME, loaded onto an 8% Tris-SDS acrylamide gel, run, and transferred to a nitrocellulose membrane followed by blocking with 1% BSA prepared in TBS-T for 1 h. The blot was incubated with an appropriate primary antibody overnight followed by washing and 1 h incubation in HRP-conjugated secondary antibody. Protein bands were detected using ECL detection reagents (PerkinElmer, Boston, MA) and exposed on blue X-ray film (Phenix Research Products, Candler, NC).

Quantitative PCR of NOX4 mRNA Expression

RNA extraction from HUVEC was performed using TRIzol reagent (Invitrogen). The concentration and quality of the extracted RNA were determined on a NanoDrop spectrophotometer (Thermo Scientific). High Capacity RNA-To-cDNA kit (Invitrogen) was used to synthesize cDNA. QPCR was performed using a 7900HT Real Time PCR system and software (Applied Biosystems) using the primer/probe set Hs00418356_m1 for NOX4 and Hs02758991_g1 for GAPDH (Invitrogen) respectively. The relative amount of mRNA was calculated using the relative quantification (ΔΔCT) method.

Data were analyzed with Sigma Plot statistical software using one way analysis of variance (ANOVA, SPSS, Chicago, IL, USA). A p value of 0.05 or less was considered significant.

Results

Effect of ketones and high glucose in inducing oxidative stress in HUVEC

Figures 1A and 1B illustrate the effect of ketones and high glucose (HG) on ROS levels and NOX4 expression in HUVEC respectively. Physiologically, individual ketone body concentration under diabetic conditions varies. BHB is found at concentrations 2 to 3 times greater than that of AA [3]. Depending on the severity of insulin deficiency, the ketone levels, especially the AA-to- BHB ratio, can vary in T1D patients anywhere from 1:1 to 1:4 due to the impaired utilization of BHB as well as the inability of the extrahepatic peripheral tissues to interconvert BHB to AA [2, 3, 32]. Endothelial cell treatment with ketones was carried out in the following manner: AA was administered at 4 mM and BHB at 12 mM, while the concentration of HG used was 25 mM. The effect of the combination of AA and BHB (in 1:3 ratio) in inducing ROS levels was comparable or higher to that seen with HG, but the presence of HG along with ketones enhanced the effect of ketones even further. Increases in ROS, as seen in Figure 1A, and NOX4 (Fig. 1B) were more pronounced in the presence of ketones and HG compared to those of either ketones or HG alone.

Effect of ketones on ICAM-1 upregulation and monocyte adhesion in the presence or absence of high glucose

Ketone treatment increases ICAM-1 expression in HUVEC after 24 h. HG and ketones both increase ICAM-1, but in combination drive the expression even higher (Fig. 2A). To evaluate the role of ketone and HG induced ICAM-1 in monocyte adhesion we performed a monocyte-endothelial adhesion assay. Monocytes (THP-1) and HUVEC were treated similarly but separately and then incubated together. As expected, we saw an enhanced adherence of

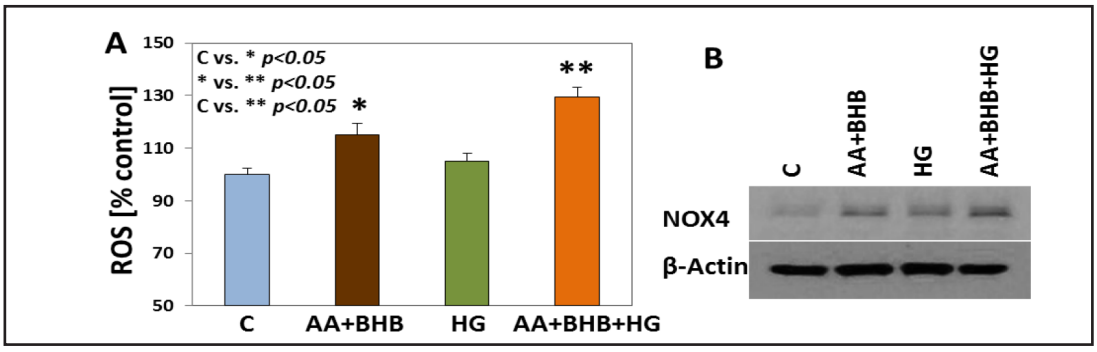

Fig. 1. HUVEC were treated with ketones (AA-4 mM and BHB-12 mM), HG (25 mM), or ketones+HG. The production of ROS and the expression levels of NOX4 were determined using DCFDA and Western blotting respectively as shown in *A* and *B.* Values are mean ±SE (n=3).

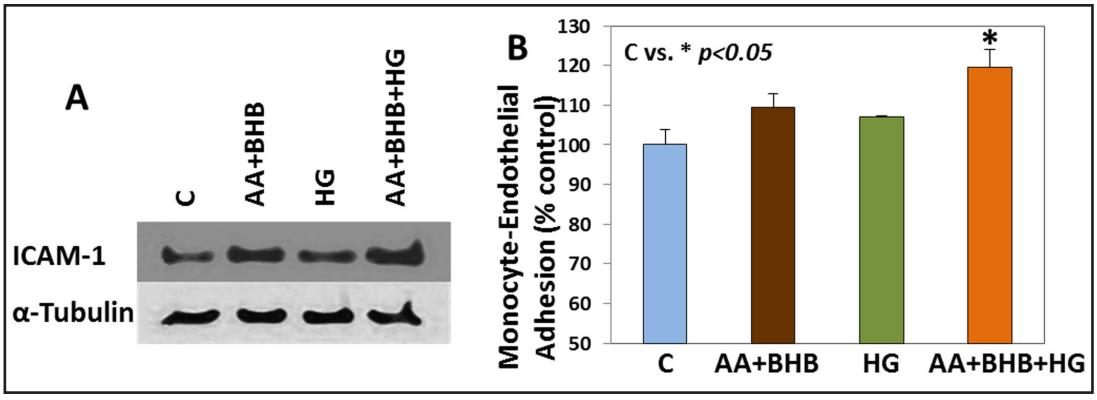

Fig. 2. HUVEC were treated with ketones, HG, or ketones+HG; the expression levels of ICAM-1 were determined using Western blotting (*A).* Adherence of THP-1 monocytes to HUVEC is shown in panel *B.* Values are mean ±SE (n=3).

monocytes to HUVEC in the presence of both ketones and HG. The percentage of adherence was comparatively lower in either HG treated or ketone treated cells (Fig. 2B).

Effect of NOX4 knockdown in ketone and high glucose treated HUVEC

To investigate the role of NOX4 in bringing about ketone and HG induced oxidative stress, we used NOX4 specific siRNA to knockdown the enzyme in HUVEC. The knockdown efficiency of NOX4 siRNA is shown in Figure 3A. Complexes of NOX4 siRNA and lipofectamine were allowed to form in culture dishes and then cells suspended in serum free media were added to this mixture. Once we had confluent monolayers we used them to perform ROS assays. Similarly treated cells were used to extract protein for Western blotting to look at ICAM-1 expression and to perform ROS and adhesion assays. Results demonstrate a decrease in ROS levels (Fig. 3C) in NOX4 knockdown cells that were treated with ketones, HG, or ketones + HG. This inhibition in ROS production also prevented increases in ICAM-1 expression (Fig. 3B) as well as monocyte-endothelial adhesion with NOX4 knockdown (Fig. 3D), suggesting that NOX4 is involved in bringing about ketone induced oxidative stress, which is activating downstream signaling pathways that potentiate the adherence of the monocytes to the endothelial cells.

Effect of AA VS BHB on HUVEC

To distinguish and determine the effects of AA and BHB separately we treated HUVEC with 4 mM of either AA or BHB. We observed that the effect of AA was much greater than that

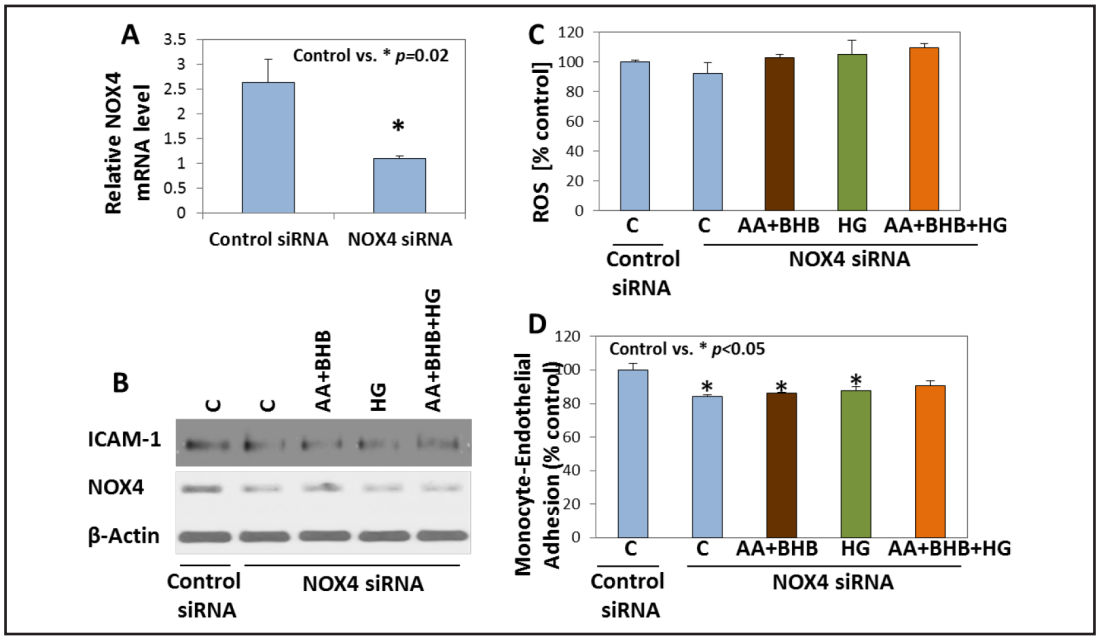

Fig. 3. NOX4 knocked down in HUVEC is shown in *A,* which represents the expression level of NOX4 mRNA. *B* shows the expression of NOX4 and ICAM-1 in NOX4 knockdown HUVEC that were treated with ketones, HG, or ketones+HG. ROS levels and the adhesion of monocytes to HUVEC in NOX4 knockdown cells are shown in panel *C* and *D* respectively. Values are mean ±SE (n=3).

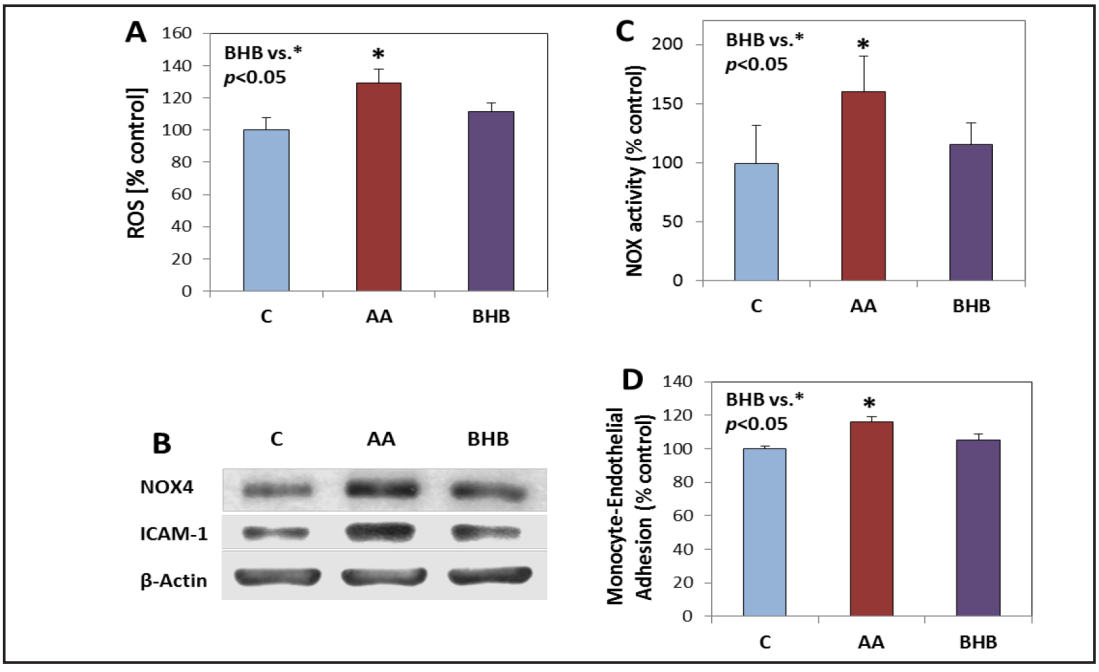

Fig. 4. ROS levels in HUVEC treated with either 4 mM acetoacetate (AA) or β-hydroxybutyrate (BHB), shown in panel *A*. The expression levels of ICAM-1 and NOX4 are shown in panel *B*. The activity of NADPH oxidases measured with lucigenin is shown in panel *C.* The adhesion of monocytes to HUVEC is shown in panel *D*. Values are mean ±SE (n=3).

of BHB in the upregulation of ROS (Fig. 4A). Similarly, NOX4 and ICAM-1 upregulation (Fig. 4B) and an increase in monocyte-endothelial adhesion (Fig. 4D) were seen in the presence of AA but not that of BHB. It appears that AA can activate the NADPH oxidases (Fig. 4C). Their

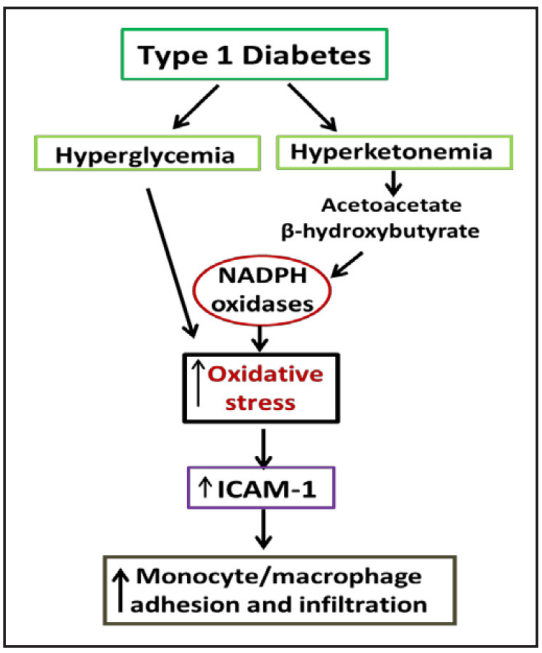

Fig. 5. Proposed mechanism by which T1D increases the risk of endothelial cell injury. This schematic diagram represents the events that follow diabetic hyperketonemia and hyperglycemia. An increase in ROS production leads to the upregulation of the downstream signaling pathways and an increase in the expression of adhesion molecules. This eventually causes the adhesion of circulating blood cells to the endothelium, followed by infiltration, and thereby increasing the risk of plaque formation and development of atherosclerosis. This study proposes that ketones induce ROS production by upregulating NOX.

activity was measured in HUVEC after 24 h treatment with either AA or BHB and there was a significant increase in the NADPH oxidase activity in the AA treated cells, while BHB failed to activate them. Overall, the changes observed with AA were significant ($p<0.05$) compared to those of control or BHB treated cells, suggesting that AA is the key player in mediating ketone induced oxidative stress.

Discussion

Several factors, including hyperglycemia, oxidative stress, and vascular inflammation contribute to the development of endothelial dysfunction in diabetes. Elevation in ICAM-1 levels is a known biomarker that can contribute to the monocyte adhesion to the endothelium and the progression of endothelial dysfunction and microvascular disease [33, 34]. Type 1 diabetes is associated with higher incidence of microvascular disease. NADPH oxidases (NOX) are the major source of oxidative stress in the arterial wall. Several studies have proposed that the upregulation of NOX contribute to the pathology of the vascular disease [35]. This study reports for the first time that elevated levels of ketones can upregulate NOX4 leading to increased ROS levels in HUVEC, and the effect was far greater when ketones were present along with HG. Knocking down NOX4 reduces the ketone and HG induced increases in ROS and ICAM-1 and suggests that NOX4 plays an important role in mediating the oxidative stress caused by ketones. Heightened oxidative stress found in diabetic patients can induce pro-inflammatory cytokines, increase adhesion molecule expression, and activate transcription factors, which in turn can cause cellular injury and contribute to the onset of complications associated with diabetes [25, 26]. We believe that ketones, by upregulating NOX4, are causing increases in ROS, which can activate various signaling pathways leading to an increase in the expression of ICAM-1 and thereby facilitating in adhesivity of monocytes, a surrogate biomarker of endothelial dysfunction. The expression of ICAM-1 on endothelial surface plays a critical role as it facilitates in the recruitment, attachment, and intravasation of monocytes that can lead to the initiation and progression of plaque formation in the vessel walls contributing to development of atherosclerosis.

Previous studies have demonstrated that the ketones, especially AA, can generate superoxide radicals and induce cytokine and adhesion molecule expression, and that ketonemic diabetics have high levels of oxidative stress compared to those of normoketonemic

diabetic patients [13, 14, 16, 20, 24, 36-54]. We observed that AA was significantly able to upregulate NOX4 expression and NADPH oxidase activity, while BHB failed to produce any adverse effects in HUVEC. This suggests that AA is the key contributor to the induction of oxidative stress. Even though we know that AA is not the ketone body that is primarily elevated in hyperketonemia (the concentration of BHB is at least 2 to 4 times higher than AA), it is still the one reported to remain in circulation for a prolonged period of time (causing considerable oxidative damage), as AA is not cleared out as rapidly as BHB [3]. Thus, ketone body acetoacetate is the key player that can independently induce changes in oxidative stress and adherence of monocytes.

This study demonstrates for the first time that ketones induce NOX4 upregulation and cause increased oxidative stress using HUVEC cell culture model. Results of this study suggest that high ketone levels in addition to high glucose levels can greatly increase the risk of developing cellular and organ damage in T1D. We provide a novel biochemical mechanism by which frequent episodes of hyperketonemia can act as a pro-oxidant and contribute to the excess vascular disease present in T1D. This finding will stimulate the study of new drugs targeting inhibition of NOX in preventing higher risk of complications associated with T1D.

Abbreviations

AA (acetoacetate); BHB (β-hydroxybutyrate); HG (high glucose); HUVEC (human umbilical vein endothelial cells); ICAM-1 (intercellular molecule-1); NOX (NADPH oxidases); NOX4 (NADPH oxidase 4); ROS (reactive oxygen species); T1D (type 1 diabetes); T2D (type 2 diabetes).

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Disclosure statement

The authors have declared that no conflict of interest exists.

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