Cyanate attenuates insulin secretion in cultured pancreatic β cells

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Abstract. The vast majority of long-term complications in transplanted patients are associated with cardiovascular disease. Previously, an alternative and dominant mechanism for cyanate formation in atherosclerotic lesions has been discovered. This study was designed to determine the effect of cyanate on insulin secretion in cultured pancreatic β cells (INS-1 cells). The cytotoxicity of cyanate was determined by 3-(4,5-Desethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Insulin secretion was measured by ELISA in cyanate-treated INS-1 cells. Reactive oxygen species (ROS) generation was also determined by measuring the fluorescent oxidized product of 2,7-dichlorefluorescein in cyanate-treated INS-1 cells. FACS analysis was carried out to determine the effect of cyanate on the apoptosis of INS-1 cells. Firstly, we found that cyanate, within concentration ranges in which no cytotoxic effect was observed (0.01, 0.1 and 1.0 mM), decreased insulin secretion dose-dependently in both non-glucose-stimulated and glucose-stimulated INS-1 cells. Cyanate at a 1.0 mM concentration inhibited insulin secretion by more than 50% in non-glucose-stimulated cells and glucose (5 and 10 mM)-stimulated cells. Cyanate, however, did not affect ROS generation. Furthermore, no pro- or anti-apoptotic effect was observed in cyanate-treated INS-1 cells. The results in this study suggest the possible inhibitory effect of cyanate on insulin secretion in INS-1 pancreatic β cells. The inhibitory effect was not mediated either by ROS generation or by apoptosis. Further studies to determine the underlying mechanisms will be of benefit.

Introduction

At physiological pH and body temperature, 0.8% of the molar concentration of urea is spontaneously converted to cyanate (1). Cyanate subsequently reacts irreversibly with the N-terminal groups of amino acids, peptides and many proteins by a process known as carbamylation (2-4). Accordingly, the potential role of cyanate and carbamylation has to date been investigated only in the context of uremia (2,5-7). Proteins isolated from patients with chronic renal failure and end-stage renal disease have been shown to be easily carbamylated due to the urea-derived high concentrations of cyanate.

In a previous study, the discovery of an alternative and quantitatively dominant mechanism for cyanate formation at the sites of inflammation was reported. The study clearly proves that cyanate (OCN^-) is produced by myeloperoxidase (MPO), a heme protein derived from leukocytes, and that cyanate-mediated protein carbamylation occurs under physiologically relevant conditions (8).

It is well known that MPO is catalytically active in chronic inflammatory diseases, such as atherosclerosis, in an H_2O_2-dependent manner (9,10). Elevated levels of MPO have been observed in both type 1 and 2 diabetes mellitus (11). The role of MPO-generated cyanate, however, is largely unknown. Therefore, the present study was designed to determine the effect of cyanate on pancreatic β cells.

Materials and methods

Cell culture. The INS-1 glucose-sensitive pancreatic cell line was maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 in RPMI (WelGENE, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum (WelGENE), 1% sodium pyruvate and 0.35% mercaptoethanol.

Cell viability. Cell viability of INS-1 cells was determined with 3-(4,5-Desethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA) assay to the colored formazan product by mitochondrial lactate dehydrogenase activity in viable cells. Briefly, after incubating INS-1 cells with cyanate at different concentrations, MTT solution was added to a final concentrion of 0.5 mg/ml, and the cells were further incubated for 4 h. The dark blue formazan crystals formed in intact cells were solubilized with DMSO and absorbance at 492 nm was measured with a microplate reader.

Flow cytometry analysis. INS-1 cells were suspended in 100 µl of PBS, and 100 µl of 95% ethanol were added while vortexing. The cells were incubated at 4˚C for 1 h, washed with PBS and resuspended in 250 ml of propidium iodide (PI) solution (0.1% NP40, 0.1% Trisodium citrate, 50X PI) together with 50 µg/ml of RNase. Incubation was continued at 4˚C for 20 min. The stained cells were analyzed by FACScan (Becton-Dickinson, Franklin Lakes, NJ, USA).

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Measurement of reactive oxygen species (ROS). INS-1 cells (10,000 cells/well) seeded in 96-well plates were grown overnight. The cells were loaded with 30 mol/l 2,7-dichlorefluorescein diacetate (Sigma; St. Louis, MO, USA) for 45 min at 37˚C in culture medium. The cells were washed with phosphate-buffered saline solution containing 1% fetal bovine serum. Fluorescence was measured at 485-nm excitation and 535-nm emission every 5 min for 60 min at 37˚C using a VICTOR3 multilabel counter (PerkinElmer, Boston, MA, USA). The changes in relative fluorescence unit (RFU) from zero time were expressed as δ RFU.

Enzyme-linked immunosorbent assay. Secreted insulin was measured using 25 µl of diluted (x10) supernatant from each well (100,000 cells/ml; 200 µl) of 96-well plates using the Insulin (Rat) Ultra Sensitive ELISA kit from ALPCO Diagnostics (Windham, NM, USA). In brief, 25 µl standard, control and unknown were incubated separately with 75 µl conjugate solution, containing insulin antibody, for 120 min at room temperature. Plates were washed 6 times manually and allowed to react with 3,3',5,5'-tetramethylbenzidine peroxidase substrate for at least 15 min at room temperature. Reactions were stopped by the addition of 50 µl stop solution. Finally, bi-chromatic absorbance measurement with reference at 650 and 450 nm was performed using a Benchmark Biorad Microplate spectrophotometric reader (BioRad, Sunnyville, CA, USA).

Statistical analysis. The results were expressed as the means ± SD. Statistical evaluation of a significant difference between means was performed with Student's t-test. P-values of <0.05 were considered to indicate statistically significant differences.

Results

Effect of cyanate on cytotoxicity. First, we determined whether cyanate affects cell viability by MTT assay and found that cyanate at concentrations up to 1 µM did not affect cell viability significantly (Fig. 1).

Effect of cyanate on insulin secretion. The effect of cyanate on insulin secretion was assessed by ELISA assay. As shown in Fig. 2, cyanate decreased insulin secretion significantly in a dose-dependent manner. Cyanate at 0.01 µM attenuated insulin secretion by 6±11% and at 1 µM by 59±14% (P<0.01) compared to the control. When INS-1 cells were stimulated with glucose (5 and 10 G), insulin secretion in INS-1 cells was also significantly reduced dose-dependently by cyanate treatment (5 G, P<0.05; 10 G, P<0.005).

Effect of cyanate on ROS generation. To determine the underlying mechanism of the inhibitory effect of cyanate on insulin secretion, ROS generations by cyanate in INS-1 cells were determined. As shown in Fig. 3, no difference in ROS generation in cyanate-treated cultured pancreatic β cells was observed compared to the contol.

Effect of cyanate on apoptosis. To determine whether the inhibitory effect of cyanate on insulin secretion was due to apoptosis, we executed FACS analysis and observed that cyanate did not induce apoptosis in INS-1 cells (Fig. 4).

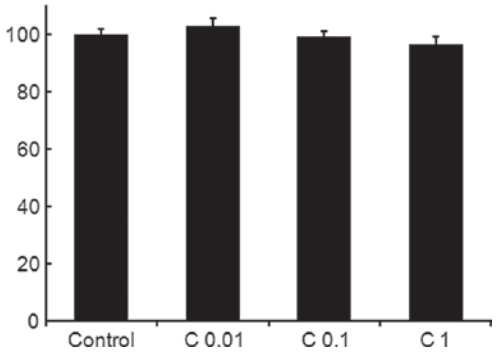

Figure 1. Effect of cyanate on the viability of INS-1 cells. The cells were incubated for 4, 12, 24 and 48 h in 96-well microplates with various concentrations of cyanate (0.01, 0.1 and 1 µM). The cytotoxity of cyanate on the INS-1 cells was evaluated using the MTT cell proliferation assay kit and expressed as a percentage of viable cells compared to the control. Data are presented as the means ± SD of 3 different experiments performed in triplicate.

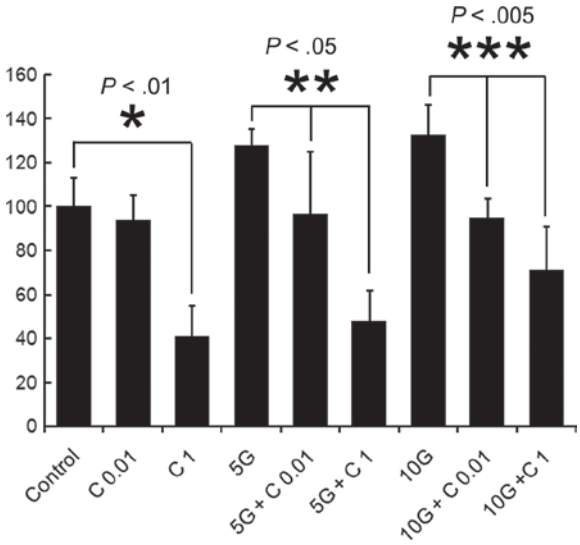

Figure 2. Effect of cyanate on insulin secretion. Secreted insulin was measured using the Insulin ELISA kit. Cells were stimulated with glucose (5 and 10 mM) and then treated with cyanate (0.01 and 1 µM). After 24 h, the culture media were collected and analyzed for insulin. The samples were processed according to the manufacturer's instructions. Each column represents the means ± SD of 3 separate experiments performed in duplicate.

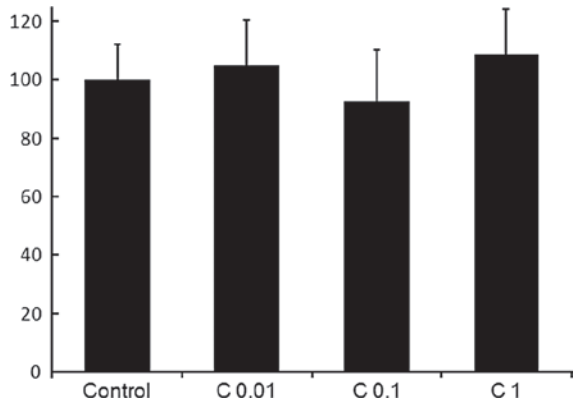

Figure 3. Effect of cyanate on reactive oxygen species (ROS) generation. INS-1 cells treated with 0.01 µM (C 0.01), 0.1 µM (C 0.1) and 1 µM cyanate (C 1) were incubated for 24 h and then the amount of generated ROS was measured. Each column represents the means ± SD of 4 separate experiments performed in duplicate.

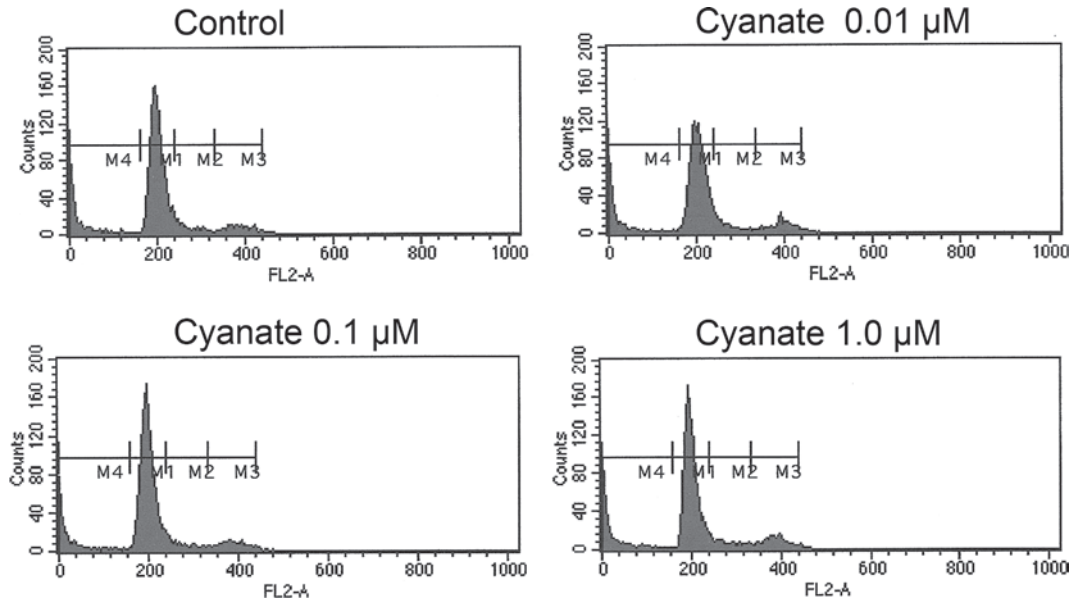

Figure 4. Effect of cyanate on apoptosis. INS-1 cells were treated with 0.01 μM (C 0.01), 0.1 μM (C 0.1) and 1.0 μM cyanate (C 1) and then incubated for 24 h. Apoptosis was measured.

Discussion

Although evidence has undoubtedly proven that cyanate is produced by the leukocyte heme peroxidase MPO via the $MPO/H_2O_2/SCN^-$ system and MPO-generated cyanate catalyzes protein carbamylation (8), the potential biological role for cyanate in human disease has to date been investigated only in the context of uremia (2,3).

Very few studies concerning the biological function of cyanate have been reported. Cyanate reacts with proteins, including hemoglobin, low density lipoprotein (LDL) and ceruloplasmin (4,13,14). Cyanate-catalyzed carbamylated LDL has been reported to induce proliferation and increase adhesion molecule expression in human coronary artery smooth muscle cells (15). The post-translational binding of cyanate to lysine ε-NH_2 groups in collagen has been demonstrated to impair the oxidative functions of polymorphonuclear neutrophils (16). We recently reported that carbamylated LDL increases ROS and apoptosis via the lectin-like oxidized LDL receptor-mediated pathway in human umbilical vein endothelial cells (17), and carbamylated albumin stimulates microRNA-146 in human renal cell carcinoma (18).

MPO is a heme protein abundantly present in neutrophils, monocytes and certain tissue macrophages, and has emerged as a potential participant in the development of atherosclerosis (19). Increasing evidence supports the involvement of MPO in the pathogenesis of coronary artery disease (20-23). Evidence also suggests that type 2 diabetes mellitus is associated with increased levels of MPO (24).

In the present study, we show that cyanate attenuates insulin secretion in INS-1 pancreatic β cells and that this inhibitory effect of cyanate on insulin secretion is not mediated via apoptosis or ROS generation. Since elevated levels of MPO have been observed in both type 1 and type 2 diabetes mellitus (11,24), and cyanate is produced under physiological conditions from MPO, the findings in the present study suggest the possible involvement of cyanate or a cyanate-mediated as yet unidentified carbamylated protein in the development or progression of diabetic complications in cardiovascular disease. Further studies would elucidate the underlying inhibitory mechanisms of cyanate on insulin secretion and identify the involved proteins.

Acknowledgements

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