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Using carboxyfluorescein diacetate succinimidyl ester to monitor intracellular protein glycation

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ABSTRACT

Protein glycation is a ubiquitous process involved in vascular complications observed in diabetes. Glyoxal (GO), an intracellular reactive oxoaldehyde that is one of the most potent glycation agents, readily reacts with amines present on proteins to produce the lysine-derived adduct carboxymethyllysine, which is a prevalent advanced glycation end-product (AGE). Our group previously showed that cell exposure to GO leads to an alteration in the cell contractile activity that could occur as a result of the glycation of various proteins regulating the cell contractile machinery. Here, we measured the extent of glycation on three functionally distinct proteins known to participate in cell contraction and cytoskeletal organization—Rho-kinase (ROCK), actin, and gelsolin (GSN)—using an assay based on the reaction of the cell membrane-permeable fluorescent probe carboxyfluorescein diacetate succinimidyl ester (CFDA–SE), which reacts with primary amine groups of proteins. By combining CFDA–SE fluorescence and Western blot detection, we observed (following GO incubation) increased glycation of actin and ROCK as well as an increased interaction between actin and GSN as observed by co-immunoprecipitation. Thus, we conclude that the use of the fluorescent probe CFDA–SE offers an interesting alternative to perform a comparative analysis of the extent of intracellular protein glycation in live cells.

An important aspect of uncontrolled glycemia occurring in type 1 and type 2 diabetes is the reaction of extracellular and intracellular proteins with glucose leading to the formation and accumulation of advanced glycation end-products (AGEs).[1] A large variety of spontaneous reactions occur between reducing sugars or glycating compounds derived from glucose (i.e., methylglyoxal, glyoxal [GO], and 3-deoxyglucosone) and primary amine groups of proteins [1–4], which lead to intermediate Amadori products and, over time, to heterogeneous AGEs. Glycation of intracellular proteins has an important impact on their related function, knowing that it affects their folding, solubility, or ability to bind to interacting partners [5–8].

We recently reported that, cell exposure to GO, an intracellular reactive oxoaldehyde responsible for the formation of the lysine-derived adduct carboxymethyllysine (CML) [7], was correlated with an increase in cell body stiffness and an alteration in the cell contractile activity. Here, we suggest that glycation of intracellular proteins involved in cell contraction could explain the observed alteration of the cell mechanical properties and function.

Cell contraction forces are driven by cyclic interaction between myosin and filamentous actin, which is regulated by the phosphorylation of the myosin light chain (MLC) subunit by both myosin light chain kinase (MLCK) and Rho-kinase (ROCK) [8,9]. ROCK is suspected to be affected by GO-mediated glycation given that its activity was reported to be enhanced in several diabetic models [10–12]. However, the presence of glycation on this important kinase remains to be demonstrated. Interestingly, several accessible lysine and arginine residues within the auto-inhibitory C-terminal moiety of ROCK or the kinase domain could be subjected to glycation [13], which could affect its folding or interaction with binding partners and potentially affect its enzymatic activity.

To support cell contraction, the organization and polymerization status of F-actin, acting as the main structural component for the cell contractile machinery, must be tightly regulated [14,15]. This regulation requires the interaction of actin with numerous proteins such as gelsolin (GSN), a potent actin filament capping and severing protein. It is known that the modulation of its interaction with actin has an impact on the regulation of the actin

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[1] *Abbreviations used:* AGE, advanced glycation end-product; GO, glyoxal; CML, carboxymethyllysine; ROCK, Rho-kinase; GSN, gelsolin; CFDA–SE, carboxyfluorescein diacetate linked to a succinimidyl ester moiety; CFSE, carboxyfluorescein succinimidyl ester; HEK293-AT_{1a}R, HEK-293A cells expressing the AT_{1a} receptor; FBS, fetal bovine serum; NG, normal glucose; AMG, aminoguanidine; DMSO, dimethyl sulfoxide; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; NIH, National Institutes of Health; EDTA, ethylenediaminetetraacetic acid; *p*-NPA, *p*-nitrophenylacetate; ANOVA, analysis of variance; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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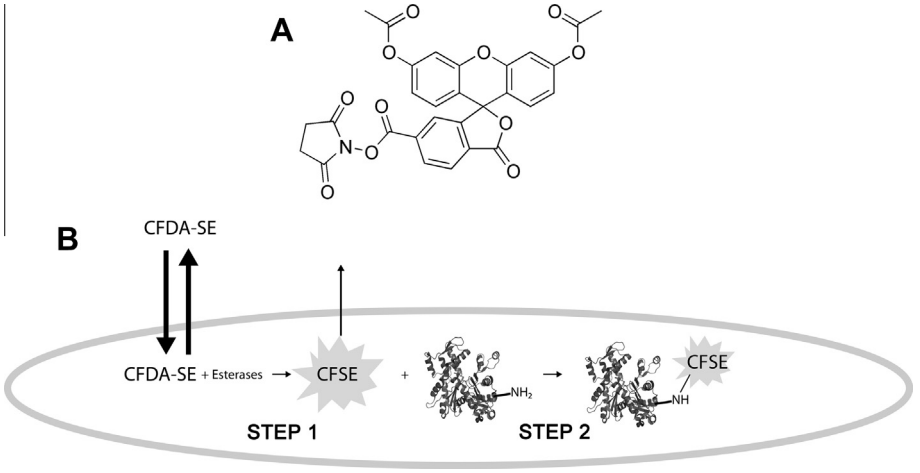

Fig.1. Mechanism involved in the fluorescent labeling of intracellular proteins using membrane-permeable CFDA–SE dye. (A) CFDA–SE structure (carboxyfluorescein diacetate succinimidyl ester). (B) CFDA–SE is first introduced into cell via diffusion across cell membrane in step 1. Enzyme reactions with cellular esterases cleave the acetate groups, resulting in highly fluorescent CFSE (carboxyfluorescein succinimidyl ester). In step 2, succinimidyl ester reacts covalently with intracellular proteins to anchor the carboxyfluorescein moiety, producing a stable fluorescent signal. Excess unconjugated reagent passively diffuses to the extracellular medium, where it can be washed away.

filament assembly/disassembly and compromises the forces and tension driving cell contractility [16–18]. It is worthy of notice that actin is particularly sensitive to glycation because it has a slow turnover and harbors several accessible lysine and arginine residues susceptible to being modified by glycating agents in vitro [19,20].

Intracellular glycation can be evaluated using high-performance liquid chromatography–mass spectrometry [21,22], using antibody against specific protein adducts such as CML [23], or by quantifying the autofluorescence properties of specific AGEs such as pentosidine [24]. Considering that most of these techniques are limited to the screening of a specific AGE or a group of AGEs sharing similar properties, there is a need to develop alternative approaches to evaluate global cellular protein glycation that is sensitive to all AGE types. Therefore, we have used the cell-permeant carboxyfluorescein diacetate linked to a succinimidyl ester moiety (CFDA–SE) to evaluate the extent of cell glycation. Briefly, this assay is based on the specific reactivity of the succinimidyl ester group of CFDA–SE with primary amine groups. CFDA–SE-modified proteins and unreacted CFDA–SE are nonfluorescent until the cleavage of their acetate groups by intracellular esterases, leading to the formation of the fluorescents carboxyfluorescein succinimidyl ester (CFSE)-modified proteins and unreacted CFSE that can diffuse freely back into the extracellular medium [25] (Fig. 1). CFDA–SE is used extensively in proliferation assays performed on live cells [26–29] by exploiting the fact that labeled proteins are inherited by daughter cells after either cell division or cell fusion. Here we posit that an increase in intracellular protein glycation should lead to decreased availability of primary amine groups and, thus, to a decrease in the fluorescence signal originating from CFSE-labeled proteins, allowing us to evaluate the global cell protein glycation. Moreover, by combining CFSE fluorescence and Western blot detection of specific proteins, we have focused on GO-induced glycation of three key intracellular proteins involved in the cell contractile functions: ROCK, actin, and GSN.

Materials and methods

Cell culture

A stably transfected human embryonic kidney cell line (QBI HEK-293A cells, Qbiogene, Carlsbad, CA, USA) expressing the AT_{1a} receptor (HEK293-AT_{1a}R), as described previously [30], was used. The clonal cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5 mM glucose, 2 mM L-glutamine, 2.5 μg/ml amphotericin B, 50 IU/ml penicillin, 50 μg/ml streptomycin (Wisent, St-Bruno, Québec, Canada), and 0.4 mg/ml G-418 (Gibco Life Technologies, Gaithersburg, MD, USA) at 37 °C in a 5% CO_2 incubator. Prior to experiments, cells were plated in 6-cm petri dishes in 5 mM glucose medium until confluence and were then exposed to specified conditions in FBS-free medium for 24 h. Normal glucose (NG) medium contained 5 mM glucose, whereas the GO (40% in H_2O, Sigma–Aldrich, Ontario, Canada)-enriched medium contained 1 mM GO and 5 mM glucose. Aminoguanidine (AMG; 296494, Sigma–Aldrich) was used at a concentration of 1 mM.

Fluorescence microscopy on CFSE-stained cells

A stock solution of CFDA–SE was prepared by dissolving CFDA–SE (Molecular Probes, Life Technologies, Thermo Fisher, USA) in dimethyl sulfoxide (DMSO; Sigma–Aldrich) at a concentration of 20 mmol/L. Living cells cultured in a petri dish were first washed twice with prewarmed Hank's balanced salt solution (HBSS; 20 mM Hepes, 120 mM NaCl, 5.3 mM KCl_2, 3 mM $CaCl_2$, 800 nM $MgSO_4$, and 5 mM glucose, 37 °C) and incubated at 37 °C in phosphate-buffered saline (PBS; Wisent) containing 0,3 μM CFSE for 15 min. Cells were then washed two times in HBSS and incubated for an additional 30 min at 37 °C in the dark to let CFDA–SE undergo acetate hydrolysis by endogenous cell esterases. CFSE staining was acquired using an inverted epifluorescence microscope (Axio Observer Z1, Carl Zeiss, Germany) equipped with an AxioCam MRm camera (Carl Zeiss). A light-emitting diode illumination system was used for detection of the fluorescein (excitation 470/40) in combination with a corresponding emission filter (530/40). The 10× and 100× objectives were used for fluorescence acquisition, and AxioVision LE software was used for image acquisition. All images were acquired under the same image attributes using the AxioVision software (Zeiss), and ImageJ software (National Institutes of Health, NIH) was used to quantify fluorescent signal intensities.

Esterase activities

Cells were washed two times with PBS, treated with extraction buffer (50 mM Hepes, 0,5% Nonidet-P40, 25 mM NaCl, 10% glycerol,