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Original Contribution

Activation of promoter activity of the catalytic subunit of γ-glutamylcysteine ligase (GCL) in brain endothelial cells by insulin requires antioxidant response element 4 and altered glycemic status: Implication for GCL expression and GSH synthesis

J. William Langston [1], Wei Li [1], Lynn Harrison, Tak Yee Aw *

Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center at Shreveport, Shreveport, LA 71130, USA

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ABSTRACT

Our recent finding that insulin increased the expression of the glutamate–cysteine ligase catalytic subunit (GCLc) with coincident increases in GCL activity and cellular glutathione (GSH) in human brain microvascular endothelial cells (IHECs) suggests a role for insulin in vascular GSH maintenance. Here, using IHECs stably transfected with promoter–luciferase reporter vectors, we found that insulin increased GCLc promoter activity, which required a prerequisite increase or decrease in medium glucose. An intact antioxidant response element-4 was essential for promoter activation, which was attenuated by inhibitors of PI3-kinase/Akt/mTOR signaling. Interestingly, only under low-glucose conditions did promoter activation correlate with increased GCLc expression and GSH synthesis. Low *tert*-butylhydroperoxide (*t*BH) concentrations similarly mediated promoter activation, but the maximal activation dose was decreased 10-fold by insulin. Insulin–*t*BH coadministration abrogated the low or high glucose requirement for promoter activation, suggesting possible ROS involvement. ROS production was elevated at low glucose without or with insulin; however, GSH increases were not inhibited by tempol, suggesting that ROS did not achieve the threshold for driving GCLc promoter activation and de novo GSH synthesis. The minor effect of pyruvate also ruled out a major role for hypoglycemia (±insulin)-induced metabolic stress on GSH induction under these conditions.

Regulation of cellular glutathione (GSH) levels can occur through redox cycling between glutathione disulfide (GSSG) and reduced GSH, enzymatic and nonenzymatic conjugation to various electrophilic and xenobiotic compounds, protein thiolation, and transmembrane flux [1]. However, de novo synthesis is by far, quantitatively, the most important pathway of controlling cellular GSH concentration. GSH biosynthesis is carried out by two ATP-dependent enzymatic processes beginning with the formation of γ-glutamylcysteine; this rate limiting step is catalyzed by γ-glutamylcysteine ligase (GCL) with subsequent addition of glycine catalyzed by glutathione synthetase [2].

GCL is a heterodimeric holoenzyme composed of a catalytic (GCLc) and a modulatory (GCLm) subunit. Although GCLc alone possesses all of the catalytic activity of the enzyme and can function monomerically in vivo, dimerization with GCLm lowers the K_m for glutamate and ATP and increases the K_i for GSH feedback inhibition [3]. Thus holoenzyme formation is one way of increasing GCL activity and GSH synthesis, whereas posttranslational phosphorylation [4] and feedback inhibition by GSH [5] serve to decrease enzyme activity. The primary means by which GCL activity and GSH synthesis are increased is through transcriptional upregulation of one or both subunits. In 1997, Mulcahy et al. cloned and characterized roughly 4.2 kb of the human GCLc promoter and found that both constitutive and electrophile-induced promoter activity was dependent on an intact antioxidant response element 4 (ARE4) [6].

A number of cardiovascular diseases are characterized by decreased endothelial-dependent vascular reactivity associated with endothelial oxidative stress stemming from increased enzymatic generation of reactive oxygen species (ROS) [7]. Coincident with this increase in ROS production is a decrease in endothelial antioxidant capacity reflected in decreased aortic GSH content in animal models of hypertension [8], atherosclerosis [9], and diabetes [10]. An associated attenuation in vascular insulin signaling [11] suggests that insulin could play an important role in maintaining normal endothelial GSH redox homeostasis. Previous work from our laboratory has shown that insulin mediated a transcriptional upregulation of GCLc and increased GSH synthetic capacity in a brain microvascular endothelial cell line (IHEC) cultured under conditions of chronic high glucose (7 days) [12] or acute low glucose (8 h) [13]. We therefore hypothesized that insulin signaling increases GCLc promoter activity in IHECs under conditions of altered glycemic status. Using IHECs stably transfected with promoter–luciferase reporter vectors, we show here the requirement of ARE4 within the GCLc promoter for its induction by insulin as well as by *tert*-butylhydroperoxide (*t*BH). Furthermore, our results are consistent with

* Corresponding author. Fax: +1 318 675 6005.
E-mail address: taw@lsuhsc.edu (T.Y. Aw).
[1] These authors contributed equally to this work.

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a role for the PI3-kinase/Akt/mTOR pathway in the insulin effect under hypo- and hyperglycemic status. Interestingly, despite insulin-induced GCLc promoter activation at low and high glucose, corresponding increases in endothelial GCLc expression and de novo GSH synthesis occurred only under low-glucose conditions. Moreover, although ROS production was elevated by hypoglycemia, the requirement of low glucose for the insulin-induced promoter activation and cellular GSH synthesis appeared to be ROS-independent.

Methods

Reagents

Medium 199, hygromycin B, *t*BH, porcine insulin, insulin–transferrin–sodium selenite solution, rapamycin B, leupeptin, aprotonin, phenylmethylsulfonyl fluoride (PMSF), L-buthionine-(*S,R*)-sulfoximine (BSO), pyruvate, and tempol were purchased from Sigma–Aldrich (St. Louis, MO, USA). LY294002, okadaic acid, and Akt inhibitor (SH-5) were purchased from Calbiochem (San Diego, CA, USA). Dihydrorhodamine 123 (DHR) was from Molecular Probes (Eugene, OR, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Atlanta, GA, USA). Glucose-free M199 powdered medium was from US Biologicals (Swampscott, MA, USA). Anti-GCLc rabbit polyclonal antibody was obtained from Neo-Markers (Fremont, CA, USA) and anti-actin mouse monoclonal antibody from BD Biosciences (San Jose, CA, USA). The pGL3Basic vector (pGL3B) and luciferase assay kit were purchased from Promega (Madison, WI, USA). pGL3B vectors containing the human GCLc promoter and various truncations of this promoter were kindly provided by Dr. Jeffrey Johnson, University of Wisconsin (Madison, WI, USA).

Creation and culture of stable cell lines

An immortalized human brain endothelial cell line (IHEC) was kindly provided by Dr. Danica Stanimirovic (Cellular Neurobiology Group, Institute for Biological Sciences, NRC, Canada) and propagated by Dr. J. Steven Alexander at LSUHSC (Shreveport, LA, USA). IHECs were cultured in M199 medium with 10% FBS, 1% insulin–transferrin–sodium selenite solution, and 1× antibiotic/antimycotic (complete M199 medium) at 37 °C in 5% CO_2. IHECs were used for the generation of stable cell lines expressing various luciferase vectors containing different portions of the human GCLc promoter. The vectors were constructed by Mulcahy et al. [6]. In brief, 4.2 kb of the human GCLc promoter (from −3802 to +465, where +1 is the transcription start site) was cloned into the pGL3B luciferase reporter construct. A series of vectors containing truncations of this promoter sequence was generated via progressive deletion from its 5′ end [6]. Additionally, a vector was created that contained the full-length, 4.2-kb GCLc promoter with a single T→G mutation in the ARE4 located approximately 3.1 kb upstream of the transcription start site [6] (Fig. 1). In this study, the following vectors were used in the generation of stable IHEC lines: the fully functional GCLc promoter; the truncated promoter sequences −2752 to +465, −814 to +465, and −511 to +465; and the ARE4 point mutant. The resultant IHEC clones expressing these vectors are herein referred to as WT GCLc cells, −2752 GCLc cells, −814 GCLc cells, −511 GCLc cells, and ARE4 mut GCLc cells, respectively. Generation of the lines was as follows: 1.0×10^6 IHECs were resuspended in 100 μl Amaxa Nucleofector solution with 2.0 μg of luciferase vector and 0.2 μg of a hygromycin resistance vector (pTK-Hyg). After nucleofection, the cells were seeded into three 100 mm dishes and cultured in complete M199 medium containing 100 μg/ml hygromycin B and cultured at 37 °C and 5% CO_2. After 4–6 weeks, isolated colonies were removed from the dishes and expanded under identical conditions with the exception that hygromycin B was decreased to 50 μg/ml. Clones were verified by measuring luciferase activity on a Promega GloMax 20/20 luminometer. Experiments were performed in M199 base medium supplemented with 1× antibiotic–antimycotic and 50 μg/ml hygromycin B only. M199 base medium was used in all experiments for serum starvation and incubations unless otherwise stated.

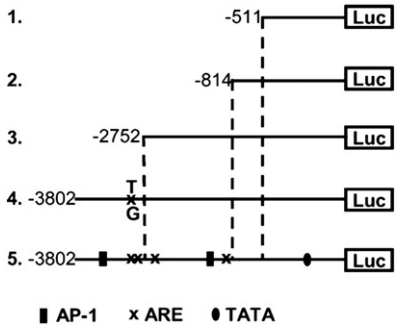

Fig. 1. Luciferase reporter constructs used to generate stable cell lines. A 4.2-kb fragment of the human GCLc promoter was used to generate a series of luciferase reporter constructs. The diagram of the vector containing the full-length, fully functional promoter (No. 5) shows the locations of several AP-1 sites and AREs 1–4 (numbered in descending order from 5′ to 3′). Vector 4 represents the full-length promoter with a single nucleotide mutation in ARE4, and vectors 1–3 represent three truncated versions of the full-length promoter.

Assessment of luciferase activity

For all experiments, IHEC lines were grown to confluency in either six-well or 35-mm tissue culture plates. At the indicated times posttreatment, cells were washed three times with PBS and lysed in 1× lysis buffer. Cell lysates were centrifuged at 12,000 rpm for 2 min to remove particulate matter, and a 20-μl aliquot of each sample was taken for protein quantitation (Bio-Rad RcDc protein assay). Luciferase activity was measured using 20 μl of each sample. All samples were measured in duplicate and luciferase activity was normalized to protein content (relative light units/mg protein). Luciferase activity at a given time point was expressed as a ratio of the activity in serum-starved cells before treatment (time 0). The various treatment conditions and cell incubations were as follows.

tBH treatment

Confluent IHEC monolayers were serum starved for 12 h in M199 base medium and then treated with 0.5–50 μM *t*BH in fresh base medium.

Insulin treatment under various glucose conditions

For experiments at normal glucose, confluent IHECs were serum starved overnight and then treated with or without 100 nM insulin in fresh M199 base medium containing 5.5 mM glucose for 8 h. For experiments in elevated glucose, cells were serum starved overnight in M199 medium supplemented with 25 mM glucose and thereafter incubated for 8 h with or without 100 nM insulin in fresh M199 medium with 25 mM glucose. Low-glucose conditions were achieved as follows. Confluent IHECs were serum starved for 8 h, at which time fresh M199 base medium was added, and cells were incubated for an additional 12 h to allow for glucose depletion. At 12 h, medium glucose was approximately 3.5 mM (63 mg/dl) [13]. Cells in reduced glucose medium were then treated with or without 100 nM insulin for 8 h. For inhibitor studies, IHECs were exposed to 10 μM LY294002, 10 μM SH-5, or 10 nM rapamycin for 30 min before and over the duration of insulin treatment.

Insulin and tBH cotreatment

Confluent monolayers were serum starved overnight and then treated with *t*BH (0.5–50 μM) with or without 100 nM insulin in fresh M199 base medium for 8 h.