Adenoviral Overexpression of the Glutamylcysteine Ligase Catalytic Subunit Protects Pancreatic Islets against Oxidative Stress

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Phuong Oanh T. Tran‡, Sarah M. Parker‡, Eric LeRoy‡, Christopher C. Franklin§‡‡, Terrance J. Kavanagh‡, Hao Zhang‡, Huorang Zhou‡, Portia Vliet‡, Elizabeth Oseid‡, Jamie S. Harmon‡, and R. Paul Robertson‡***

From the ‡Pacific Northwest Research Institute, Seattle, Washington 98122 and ‡Department of Pathology, NEIHS Center of Ecogenetics and Environmental Health, National Institutes of Health, Department of Environmental Health, Departments of Medicine and Pharmacology, University of Washington, Seattle, Washington 98195, and ‡‡‡Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Denver, Co 80262

The catalytic subunit of glutamylcysteine ligase (GCLC) primarily regulates de novo synthesis of glutathione (GSH) in mammalian cells and is central to the antioxidant capacity of the cell. However, GCLC expression in pancreatic islets has not been previously examined. We designed experiments to ascertain whether GCLC is normally expressed in islets and whether it is up-regulated by interleukin-1β (IL-1β). GCLC expression levels were intermediate compared with other metabolic tissues (kidney, liver, muscle, fat, and lung). IL-1β up-regulated GCLC expression (10 ng/ml IL-1β, 3.76 ± 0.86; 100 ng/ml IL-1β, 4.22 ± 0.88-fold control) via the p38 form of mitogen-activated protein kinase and NFκB and also increased reactive oxygen species levels (10 ng/ml IL-1β, 5.41 ± 1.8-fold control). This was accompanied by an increase in intraislet GSH/GSSG ratio (control, 7.1 ± 0.1; 10 ng/ml IL-1β, 8.0 ± 0.5; 100 ng/ml IL-1β, 8.2 ± 0.5-fold control; p < 0.05). To determine whether overexpression of GCLC increases the antioxidant capacity of the islet and prevents the adverse effects of IL-1β on glucose-induced insulin secretion, islets were infected with an adenovirus encoding GCLC. IL-1β significantly decreased glucose-stimulated insulin secretion (control, 123.8 ± 17.7; IL-1β, 40.2 ± 3.9 microunits/ml insulin/islet). GCLC overexpression increased intraislet GSH levels and partially prevented the decrease in glucose-stimulated insulin secretion caused by IL-1β. These data provide the first report of GCLC expression in the islet and demonstrate that adenoviral overexpression of GCLC increases intracellular GSH levels and protects the beta cell from the adverse effects of IL-1β.

Glutamylcysteine ligase (GCL)1 is the primary and rate-limiting enzyme responsible for de novo synthesis of intracellular glutathione (GSH). This enzyme catalyzes ATP-dependent ligation of L-glutamate and L-cysteine to form γ-glutamyl-L-cysteine, which undergoes another ATP-dependent ligation with glycine catalyzed by glutathione synthetase to form the final GSH product (1, 2). GCL is a heterodimer, composed of a light regulatory subunit as well as a heavy catalytic subunit. Although both are required for optimal enzyme activity, overexpression of the catalytic subunit GCLC alone is sufficient to increase enzyme activity significantly over control levels (1). The regulation of GCL expression and activity has been studied in many other cell types, including mesangial and endothelial cells, but not the pancreatic islet. Long term exposure to high glucose levels decreases GCLC expression in mesangial, retinal Muller, and endothelial cells, which results in a decrease in GSH levels (3–5). Long term exposure to high glucose conditions inhibits endothelial cells from responding to cytokine exposure with an increase in GCL expression and activity (3). That the pathogenesis of diabetes mellitus involves interactions with IL-1β and oxidative stress secondary to chronic hyperglycemia (6, 7) raises the questions of whether GCL is normally expressed in the islet and whether overexpression of GCL protects the beta cell from oxidative stress.

We have reported that prolonged hyperglycemia or shorter exposure to ribose, a stronger reducing sugar, stimulates the formation of reactive oxygen species (ROS) in pancreatic islets (6) and causes beta cell dysfunction. These excessive increases in intraislet ROS levels cause decreases in insulin mRNA levels, insulin content, and glucose-stimulated insulin secretion. GSH plays a central role in the endogenous cellular response against oxidative stress by acting directly as an antioxidant to reduce reactive oxygen species and as a substrate for the antioxidant enzyme, glutathione peroxidase (1, 8–10). We have also reported that reducing intraislet GSH levels by the GCL inhibitor buthionine sulfoximine augments the adverse effects of ROS on beta cell function (6). These observations are clinically relevant because decreases in GSH levels or the ratio of reduced to oxidized glutathione (GSH/GSSG) measured in the red blood cells of type 2 diabetic patients correlate with worsening of the diabetic state (11–13).

The studies described here were designed to establish whether GCLC is normally expressed in the islet and to determine whether adenoviral overexpression of GCLC in pancreatic islets prevents beta cell dysfunction caused by oxidative stress. Using interleukin-1β as an inducer of oxidative stress, we examined 1) the level of GCLC expression in control islets and islets exposed to IL-1β, as well as the consequences of

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1 The abbreviations used are: GCL, glutamylcysteine ligase; GCLC, glutamylcysteine ligase catalytic subunit; ROS, reactive oxygen species; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; RT, reverse transcription; PGE2, prostaglandin E2; AdV, adenovirus.

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** To whom correspondence should be addressed: Pacific Northwest Research Institute, 720 Broadway, Seattle, WA 98122. Tel.: 206-726-1210; Fax: 206-726-1217; E-mail: rpr@pnri.org.

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IL-1β-dependent changes in GCLC on ROS production, intraislet GSH levels, and insulin secretion; 2) whether IL-1β effects on GCLC gene expression depend on p38 MAPK and NFκB activation; and 3) whether adenoviral overexpression of GCLC increases intraislet levels of GSH and mitigates the adverse effects of IL-1β-induced oxidative stress on glucose-induced insulin secretion.

**EXPERIMENTAL PROCEDURES**

**Materials**—Adenovirus and antibodies for GCLC were obtained from the Department of Pathology and the NIEHS (National Institutes of Health) Center of Ecogenetics and Environmental Health, Department of Environmental Health at the University of Washington, Seattle. RT-PCR probes, primers, and reagents were purchased from Applied Biosystems (Foster City, CA). IL-1β was obtained from R&D Systems (Minneapolis, MN). All other reagents were obtained from Sigma.

**Isolation and Culture of Wistar Rat Islets**—Islets from the pancreata of male Wistar rats were isolated and cultured as described previously (6, 14).

**Measurements of Intracellular ROS Levels**—Intracellular levels of ROS were detected by flow cytometric analysis using a fluorescein-labeled dye, dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, OR), as described previously by our laboratory (6). The acetoxymethyl ester derivative readily permeates cell membranes and is trapped within the cell upon cleavage by esterases. Oxidation by reactive oxygen species converts the dye from its nonfluorescent to its fluorescent form. Briefly, islets were cultured with 4 μM dichlorodihydrofluorescein diacetate in phosphate-buffered saline for 1 h at 37 °C. After incubation with the dye, cells and islets were washed with phosphate-buffered saline to prevent measurement of any extracellular reaction between the dye and H2O2 released by the cells into the medium. Islets were then dispersed using trypsin, and intracellular peroxide levels were measured with the EPICS® XL-MCL flow cytometer controlled by the System II software (Beckman-Coulter). Results were calculated as fold difference from control untreated islets.

**GCLC Adenovirus Infection**—Adenovirus encoding human GCLC was obtained from the Department of Pathology at the University of Washington. Propagation of the adenovirus was performed as described by Franklin et al. (15) and He et al. (16) by the Pacific Northwest Research Institute Adenoviral Core. Isolated rat islets were cultured in the presence of adenovirus (at a final concentration of 10⁷ plaque-forming units/islet) in RPMI media supplemented with 10% fetal bovine serum for 24 h at 37 °C. Ascertainment of adenoviral penetration into beta cells within the core of the islet was performed by infection with virus encoding green fluorescent protein, insulin immunostaining, and detection via confocal microscopy (6). Beta cell toxicity of the adenovirus encoding GCLC was assessed by determining glucose-induced insulin secretion after infection with various concentrations of virus.

**Real Time Fluorescence-based RT-PCR Analysis of GCLC Expression Levels**—Total RNA was extracted from rat islets, and one-step RT-PCR was carried out using the Gold RT-PCR kit from PerkinElmer Life
NF-κB analysis was used to measure cytosolic levels of the p50 subunit of GSSG)/GSSG. Treatment of islets with 10 ng/ml IL-1β for 24 h caused a decrease in the p50 subunit, indicating NF-κB dissociation from IκB and translocation into the nucleus. *, p < 0.01, n = 3. Cytosolic levels of the p85 subunit of phosphoinositide-3 kinase did not change significantly from control (C).

Fig. 2. IL-1β up-regulation of GCLC expression involves activation of p38 MAPK and NFκB. A, exposure to IL-1β (10 ng/ml) for 24 h significantly increased GCLC expression levels over control. Inhibition of p38 MAPK activation by 1 μM SB203580 (SB) prevented IL-1β from increasing GCLC expression. *, p < 0.01, n = 3. B, Western blot analysis was used to measure cytosolic levels of the p50 subunit of NFκB. Treatment of islets with 10 ng/ml IL-1β for 24 h significantly increased GCLC expression levels over control. Inhibition of p38 MAPK activation by SB203580 ( SB) prevented IL-1β from increasing GCLC expression. *, p < 0.01, n = 3. Cytosolic levels of the p85 subunit of phosphoinositide-3 kinase did not change significantly from control (C).

Fig. 3. Effect of IL-1β on intracellular ROS levels. Islets were cultured overnight in the presence of either 10 ng/ml IL-1β or 10 μM PGE2. IL-1β increased ROS levels significantly over those measured in untreated cells, but PGE2 did not. As a positive control, islets were also treated for 2 h with 100 μM H2O2. *, p < 0.05 compared with untreated control, n = 3.

Fig. 4. Adenoviral overexpression of GCLC in islets. Islets were infected with adenovirus encoding for GCLC for 16 h prior to experimentation. Glucose-induced insulin secretion was evaluated to determine optimal viral concentrations that would not interfere with normal islet function. After the 16-h infection period, islets were resuspended in fresh medium, and a 1-h static incubation (Cont) was begun. Western blot analysis of protein collected at the same time showed a substantial increase in GCLC protein levels in islets infected with the GCLC adenovirus but not the control (C) luciferase virus at a concentration 10^7 plaque-forming units (pfu)/islet.

**Table I**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>IL-1β</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 ng/ml</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>GSH</td>
<td>19.29 ± 0.9</td>
<td>21.99 ± 1.8a</td>
<td>26.27 ± 1.8a</td>
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<tr>
<td>GSSG</td>
<td>21.0 ± 0.3</td>
<td>2.21 ± 0.3</td>
<td>2.58 ± 0.3</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>7.1 ± 0.1</td>
<td>8.0 ± 0.5a</td>
<td>8.2 ± 0.5a</td>
</tr>
</tbody>
</table>

*a p < 0.05 compared with control n = 5. GSH/GSSG = (GSH − 2GSSG)/GSSG.

Sciences and an ABI Prism 7700 Sequence detector equipped with a thermocycler (Taqman™ technology) and a cooled charge-coupled device camera to detect fluorescence emission over a range of wavelengths (500–650 nm) as described previously by our laboratory (14). Probe and primers designed to detect the control gene β-actin as described previously (6).

**Western Analysis of GCLC Protein Levels**—Total protein was collected from islets following incubation under different experimental conditions using cell lysis buffer containing 1% Nonidet P-40, 140 mM NaCl, 10 mM Tris, 1 mM CaCl2, 1 mM MgCl2, and 10% glycerol along with protease inhibitors. A total of 30 μg of protein was run on SDS-PAGE, and immunoblotting was performed according to the standard protocol. In addition to measuring the protein levels of GCLC, Western analysis was also performed to detect the protein levels of the p85 subunit of phosphoinositide-3 kinase or α-tubulin to correct for protein loading. Protein levels were analyzed using the Optiquant analysis program (Packard Instrument Co.).

**GSH/GSSG Measurements in Rat Islets**—The levels of GSH and GSSG were measured in isolated rat islets using the GSH/GSSG 412 kit (Oxis Research, Portland, OR). After incubation under experimental conditions, islets were collected and washed once with potassium phosphate buffer. Islets were divided equally into samples for GSH and GSSG measurements in an assay kit buffer and analyzed per the manufacturer’s instructions.

**Insulin Secretion Studies**—Following incubation under experimental conditions, islets were placed into static incubation wells containing reverse primer, TGCTCTGCGATGTGAATCC. As a control for RNA quantity between different samples, RT-PCR was also carried out using probes and primers designed to detect the control gene β-actin as described previously (6).
**TABLE II**

Effect of GCLC overexpression on GSH/GSSG levels

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>Lac</th>
<th>Lac + IL</th>
<th>GCLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>13.46 ± 2.0</td>
<td>13.75 ± 2.7</td>
<td>16.53 ± 3.1*</td>
<td>14.46 ± 2.4*</td>
</tr>
<tr>
<td>GSSG</td>
<td>3.61 ± 0.7</td>
<td>3.64 ± 0.8</td>
<td>4.02 ± 0.8</td>
<td>3.7 ± 0.9</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>1.7 ± 0.1</td>
<td>1.8 ± 0.03</td>
<td>2.1 ± 0.1*</td>
<td>1.9 ± 0.05*</td>
</tr>
</tbody>
</table>

\* *p < 0.05 compared with control. GSH/GSSG = (GSH - 2GSSG)/GSSG.

**RESULTS**

**GCLC Expression Level and IL-β Regulation of GCLC Expression, GSH/GSSG Ratios, and Intraislet ROS Levels**—The expression level of GCLC mRNA in pancreatic islets was less than that found in kidney and liver, and greater than that observed in lung, subcutaneous fat, and skeletal muscle (Fig. 1A). Western analysis for GCLC protein was generally in agreement except that lung content was greater than islet content (Fig. 1B).

**Effect of IL-1β on GCLC Gene Expression and Its Dependence on p38 MAPK and NFκB Interaction**—IL-1β (10 ng/ml) increased GCLC expression (Fig. 2A) as well as GSH levels (Table I) and ROS levels (Fig. 3). Co-incubation with 1 μM SB203580, which prevents activation of the p38 form of MAPK, inhibited IL-1β-induced increases in GCLC expression (Fig. 2A). IL-1β (10 ng/ml) decreased cytosolic levels of the p50 form of NFκB, indicating dissociation of the NFκB-IκB complex and translocation of active NFκB into the nucleus (Fig. 2B). Cytosolic levels of the p85 subunit of phosphoinositide-3 kinase used as a control did not change significantly from control following treatment with IL-1β. Western analysis for GCLC protein/α-tubulin in three experiments revealed the following levels: control, 0.79 ± 0.11; IL-1β, 0.87 ± 0.04; SB203580, 1.29 ± 0.01; and IL-1β + SB203580, 0.95 ± 0.16; mean ± S.E., density light units. None of these was significantly different.

**Effects of Adenoviral Overexpression of GCLC on IL-1β Decreases in Insulin Secretion**—To determine whether increased GCLC expression causes an increase in GSH levels and prevents the inhibitory effects of oxidative stress on beta cell function, islets were infected with adenovirus encoding the GCLC catalytic subunit. Islets were infected overnight with adenovirus at various concentrations to determine the optimal viral concentration to use. Glucose-induced insulin secretion was used to evaluate potential toxicity of the infection. Infection with adenovirus up to a concentration of 10^7 plaque-forming units/islet did not adversely affect glucose-stimulated insulin secretion. Infection with adenovirus encoding GCLC partially prevented the inhibitory effects of IL-1β. Western analysis for GCLC protein/α-tubulin in three experiments revealed the following levels: control, 0.79 ± 0.11; IL-1β, 0.87 ± 0.04; SB203580, 1.29 ± 0.01; and IL-1β + SB203580, 0.95 ± 0.16; mean ± S.E., density light units. None of these was significantly different.

**Decreased Insulin Secretion**—How does increased GCLC expression prevent the inhibitory effects of IL-1β on glucose-stimulated insulin secretion? The effect of IL-1β (10 ng/ml) on glucose-stimulated insulin secretion (Fig. 4) or the morphology of the islet as determined by light microscopy. Protein expression levels of GCLC by Western blot analysis were significantly increased in islets infected with the adenovirus encoding for GCLC (Fig. 4). Overexpression of GCLC increased overall activity of GCLC as evidenced by an increase in the ratio of...
GCLC Protects Pancreatic Islets against Oxidative Stress

Exposure of endothelial cells to high glucose conditions decreases GCL activity significantly from that found in cells cultured under low glucose conditions (3). Cytokines increase GCLC expression and activity in endothelial cells, and this response is inhibited by prior long term exposure to high glucose conditions (3). The latter finding is similar to the effect of IL-1β up-regulation of GCLC expression that we observed. Long term exposure to high glucose conditions decreases GCLC expression in mesangial as well as retinal Muller cells resulting in a decrease in GSH levels (4, 5). Decreased GSH levels in erythrocytes from hyperglycemic type 2 diabetics are also accompanied by a decrease in GCLC activity (13).

IL-1β has a well established role as an inducer of chronic inflammation. Increases in islet IL-1β during the development of diabetes in an animal model have been reported (7). We reported that specific inhibitors of cyclooxygenase-2 and PGE2 production prevent IL-1β from inhibiting glucose-induced insulin secretion in isolated islets (14). However, the effect of IL-1β that inhibits insulin secretion via ROS formation we identified in the current study appears to be independent from PGE2 production because PGE2 did not increase ROS levels. The effect of IL-1β that is mediated by PGE2 to inhibit glucose-induced insulin secretion may be more potent than the corresponding ROS-mediated effect of IL-1β because GCLC overexpression only partially prevented the IL-1β inhibitory effect on insulin secretion, whereas treatment with specific cyclooxygenase-2 inhibitors completely prevented IL-1β-induced inhibition of glucose-induced insulin secretion (14).

Our results suggest that ROS production caused by exposing islets to IL-1β modulates glucose-induced insulin secretion downward and that the level of endogenous GCLC serves to act as a brake on this modulation by virtue of its ability to increase GSH and decrease intraislet ROS levels (Fig. 6). The clinical implications of our findings relate to the potential participation of oxidants in the pathogenesis of diabetes mellitus. In type 1 diabetes IL-1β is generally regarded as playing an important role in beta cell destruction. In type 2 diabetes chronic exposure to high glucose levels is accompanied by oxidative stress and appears to contribute to the ongoing decline in beta cell function (21). That high glucose levels favor production of IL-1β within pancreatic islets (7) suggests the hypothesis that the cumulative effects of chronic hyperglycemia and local cytokine production may contribute to ongoing destruction of beta cells in both forms of diabetes. Our previous report of the protective effects of glutathione peroxidase overexpression (6) and the beneficial effects of GCLC overexpression that we now report suggest that enhancing endogenous islet antioxidant enzyme activity may offer a therapeutic strategy for protection against ongoing beta cell damage secondary to oxidative stress in diabetes mellitus.

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