Calreticulin Destabilizes Glucose Transporter-1 mRNA in Vascular Endothelial and Smooth Muscle Cells Under High-Glucose Conditions

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Abstract—Substrate autoregulation of glucose transporter-1 (GLUT-1) mRNA and protein expression provides vascular endothelial and smooth muscle cells a sensitive mechanism to adapt their rate of glucose transport in response to changing glycemic conditions. Hyperglycemia-induced downregulation of glucose transport is particularly important in protecting these cells against an excessive influx of glucose and consequently increased intracellular protein glycation and generation of free radicals; both are detrimental in the development of vascular disease in diabetes. We aimed to investigate the molecular mechanism of high-glucose–induced downregulation of GLUT-1 mRNA expression in primary bovine aortic vascular endothelial (VEC) and smooth muscle (VSMC) cell cultures. Using RNA mobility shift, UV cross-linking, and in vitro degradation assays, followed by mass-spectrometric analysis, we identified calreticulin as a specific destabilizing trans-acting factor that binds to a 10-nucleotide cis-acting element (CAE_{2181-2190}) in the 3'-untranslated region of GLUT-1 mRNA. Pure calreticulin accelerated the rate of GLUT-1 mRNA-probe degradation in vitro, whereas overexpression of calreticulin in vascular cells decreased significantly the total cell content of GLUT-1 mRNA and protein. The expression of calreticulin was augmented in vascular cells exposed to high glucose in comparison with low-glucose conditions. Similarly, increased expression of calreticulin was observed in aortae of diabetic Phascolomys obesus in comparison with normoglycemic controls. These data suggest that CAE_{2181-2190} calreticulin complex, which is formed in VSMC and VEC exposed to hyperglycemic conditions, renders GLUT-1 mRNA susceptible to degradation. This interaction underlies the process of downregulation of glucose transport in vascular cells under high-glucose conditions. (Circ Res. 2005;97:1001-1008.)

Key Words: calreticulin • glucose transporter-1 • hyperglycemia • mRNA turnover • vascular smooth muscle cells • vascular endothelial cells

Hyperglycemia is a major risk factor in the development of cardiovascular complications associated with the metabolic syndrome and diabetes.1,2 Chronic hyperglycemia alters the normal function of endothelial and smooth muscle cells in blood vessels, which undergo morphological and functional modifications, because of an excessive extra- and intracellular protein glycation and an uncontrolled production of free radicals. Collectively, these processes contribute to basement-membrane thickening, vascular occlusion, increased permeability, and initiation and progression of vascular disease and atherosclerosis.3–6

The transport of glucose across the plasma membrane of cells is the rate-limiting step for subsequent glucose metabolism. A family of glucose transporters (GLUTs) mediates the entry of glucose into cells by facilitated diffusion.7 Vascular endothelial (VEC) and vascular smooth muscle (VSMC) cells express predominantly the ubiquitous GLUT-1 and to a very small extent the insulin-sensitive GLUT-4.8 We showed that these cells autoregulate their rate of glucose transport in response to changes in ambient glucose levels; specifically, they reduce their rate of hexose transport by ≈50% on exposure to high-glucose levels. Concomitantly, total cell GLUT-1 (but not GLUT-4) protein and mRNA content, as well as its plasma-membrane abundance, are reduced to the same extent.8 This downregulatory mechanism enables the cells to sustain normal glucose metabolism and ATP production similar to cells exposed to normal glucose levels.9

The expression of GLUT-1 is regulated posttranscriptionally in various cell types under diverse physiological and pathophysiological conditions, such as glucose deprivation, inhibition of oxidative phosphorylation, cytokine-, hormone-, and metabolite-dependent stimuli, and neoplastic transformation.10–12 The 5' and 3' untranslated regions (UTR) of

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GLUT-1 mRNA are highly conserved among species. The 3′ UTR contains a unique and conserved 10-nucleotide (nt) cis-acting stabilizing element (5′-CCAACCACCTC; CAE2181 to 2190) in bovine GLUT-1.1,13,14 Recently, Boado et al showed that CAE2181-2190-dependent interactions stabilized GLUT-1 mRNA in rat glial C6 tumor cells deprived of glucose and exposed to hypoxic conditions.15 These studies point to CAE2181-2190 as a glucose-sensitive element in GLUT-1 mRNA. Yet, the 3′ UTR of GLUT-1 mRNA also contains AU-rich elements that may either stabilize or destabilize the entire molecule.16–18

Hitherto, the molecular mechanism regulating the turnover rate of GLUT-1 mRNA in vascular cells under hyperglycemic conditions has not been elucidated. This study shows that calreticulin, whose expression is augmented in vascular cells under high-glucose conditions, destabilizes GLUT-1 mRNA by its specific interaction with CAE2181-2190.

Materials and Methods

Cell Cultures

Primary cultures of bovine aortic endothelial and smooth muscle cells were prepared and characterized as described previously.8 GH354 cells (courtesy of Dr J. M. Wilson, University of Pennsylvania, Philadelphia) were grown as described.19

Methods

The preparation of RNA transcripts, expression vectors, and adenoviral constructs, along with detailed descriptions of in vitro degradation assays, RNA mobility shift assays, UV cross-linking assays, real-time PCR, cell extract preparation, Western blot analyses, and immunoprecipitation are given in the online data supplement available at http://circres.ahajournals.org.

Statistical Analysis

Statistical analyses were performed using the nonparametric Mann–Whitney test.

Results

High Glucose– and CAE2181-2190–Dependent Degradation of GLUT-1 mRNA Probes

The role of CAE2181-2190 in regulating bovine GLUT-1 mRNA stability in vascular cells was studied in an in vitro degradation assay. [32P]-Labeled 203-nt (5′ 2098 to 2300) and 203-ntCAE2181-2190 probes of the 3′ UTR of bovine GLUT-1 mRNA were incubated at 37°C with S100 cytosolic proteins of VSMC that had been maintained at 2 or 25 mmol/L for 14 hours. Samples were processed at the indicated times, as described in the online data supplement. B, VSMC were cotransfected with a GFP-expressing plasmid (pEGFP-N1), and the luciferase expression vector pGL2 without (V) or with insertions of the 203-nt or 203ΔCAE2181-2190 sequence of GLUT-1 mRNA in the 3′ UTR of the luciferase gene. The cells were then incubated at 25 mmol/L glucose for 48 hours. GFP-positive cells were collected by FACS, and the relative levels of endogenous GLUT-1 and luciferase mRNA were determined by real-time PCR, as described in the online data supplement. The expression of the each mRNA in control plasmid-transfected cells (V) was taken as 1 U. Means±SEM; n=3. *P<0.05 for difference from the respective control (V).

Figure 1. High-glucose VSMC cytosolic proteins destabilized GLUT-1 mRNA probes in a CAE2181-2190–dependent manner. A, [32P]-Labeled 203-nt and 203ΔCAE2181-2190-nt (203Δ10) probes were incubated with 0.1 μg of cytosolic proteins of VSMC that had been maintained at 2 or 25 mmol/L for 14 hours. Samples were processed at the indicated times, as described in the online data supplement. B, VSMC were cotransfected with a GFP-expressing plasmid (pEGFP-N1), and the luciferase expression vector pGL2 without (V) or with insertions of the 203-nt or 203ΔCAE2181-2190-nt sequence of GLUT-1 mRNA in the 3′ UTR of the luciferase gene. The cells were then incubated at 25 mmol/L glucose for 48 hours. GFP-positive cells were collected by FACS, and the relative levels of endogenous GLUT-1 and luciferase mRNA were determined by real-time PCR, as described in the online data supplement. The expression of the each mRNA in control plasmid-transfected cells (V) was taken as 1 U. Means±SEM; n=3. *P<0.05 for difference from the respective control (V).
protein concentrations (upper arrows), reaching maximum with 3 μg of extract, and a lower diffused band. The specificity of these binding interactions was investigated by adding 20- to 200-fold molar excess of the unlabeled 203-nt transcript or an unrelated transcript of 3′ UTR of parathyroid hormone mRNA to the reaction mixture (Figure 2B).20 As expected, only the former competed effectively with the labeled probe for binding of cytosolic proteins.

The capacity of low- and high-glucose cell extracts to interact with CAE2181-2190-containing probes are shown in Figure 3A. The extracts were prepared from VSMC and VEC that had been maintained at 2 or 25 mmol/L glucose for 14 or 20 hours, respectively. These were the minimal incubation periods at high glucose required to produce maximal binding interactions. The slower response of VEC agrees with our previous report on a delayed downregulatory response of these cells to high glucose.21 Figure 3A shows, in the REMSA of the [32P]-labeled 57-nt probe, 3 distinct complexes that were not apparent with the longer 203-nt probe. Similar REMSAs with CAE2181-2190-deleted probes were performed (Figure 3B): the intensities of the 2 upper complexes formed with the [32P]-labeled 57ΔCAE2181-2190 probe were significantly lower in comparison with those formed with the intact 57-nt probe. Yet, the binding interactions of the [32P]-labeled 203ΔCAE2181-2190 probe were similar to those detected with the intact probe, possibly attributable to additional binding sequences for other proteins in the longer 203-nt probe, which masked the effect of CAE2181-2190 deletion. Therefore, we used the 57-nt probe in subsequent experiments.

The molecular mass of the [32P]-labeled 57-nt probe VSMC protein complexes was determined by UV cross-linking analysis. Four complexes corresponding to 112-, 62-, 58-, and 43-kDa, the binding intensities of which were greater with high-glucose than low-glucose VSMC protein extracts, were depicted (Figure 4A, lanes 2 and 3). Unlabeled 57-nt transcript, added at a 2-fold molar excess before UV cross-linking, reduced significantly the formation of the 62- and the 58-kDa complexes and, to a lesser extent, the 112- and 43-kDa complexes (lane 4).

To elucidate which of these complexes required CAE2181-2190 for binding, 20- to 300-fold molar excesses of complementary oligonucleotides (ONDs) were hybridized to the [32P]-labeled 57-nt probe before UV cross-linking with high-glucose

Figure 2. VSMC cytosolic proteins bound the [32P]-labeled 203-nt probe in a protein concentration–dependent manner. A, Complexes formed between the [32P]-labeled 203-nt probe (lane 1, free probe) and increasing amounts of S100 protein extract prepared from VSMC, which had been maintained at 25 mmol/L glucose for 14 hours (lanes 2 through 8), were analyzed by REMSA, as described in the online data supplement. B, The same probe was incubated without (lane 1) or with 2 μg of protein extract, as in A, in the absence (lane 2) or presence of 20- to 200-fold molar excess of unlabeled 203-nt transcript (lanes 3 through 6) or unlabeled 3′ UTR of parathyroid hormone (PTH) mRNA (lanes 7 through 10).

Figure 3. High glucose increased the binding capacity of VSMC and VEC protein extracts with GLUT-1 mRNA probes in a CAE2181-2190–dependent manner. A, REMSA of [32P]-labeled 203- or 57-nt probes incubated with S100 cytosolic proteins (1 or 2 μg, as indicated) of VSMC or VEC, which had been maintained at 2 or 25 mmol/L glucose for 14 (VSMC) or 20 (VEC) hours. The left lane in each panel shows the free probe. B, REMSA of [32P]-labeled 57- and 203-nt probes (lanes 1 through 3 and 7 through 9, respectively) or probes lacking CAE2181-2190 (57Δ10 and 203Δ10; lanes 4 through 6 and 10 through 12, respectively) incubated without or with similar VSMC or VEC cytosolic proteins (1 or 2 μg). Arrows mark RNA–protein complexes.
VSMC extract (Figure 4B). OND2 was complementary to CAE2181-2190, whereas OND1 and -3 overlapped distal up- or downstream sequences within the probe (Figure 4B). The 58- and 43-kDa complexes were non–CAE2181-2190 dependent, because all ONDs weakly interfered with their formation. Because OND2 abolished effectively the formation of the 112- and 62-kDa complexes, both could be considered candidates for an interaction with CAE2181-2190. However, the 112-kDa complex was excluded, because its formation was also abolished by OND1. Hence, the 62-kDa complex was characterized as a CAE2181-2190–dependent complex.

Calreticulin Is a CAE2181-2190–Binding Protein That Destabilizes GLUT-1 mRNA

High-glucose VSMC protein extract was affinity chromatographed on a Heparin-Fractogel-EMD column with increasing concentrations of KCl. Figure 5A shows REMSA of each KCl-eluted fraction incubated with the [³²P]-labeled 57-nt probe. Of the various complexes formed with the original S100 extract (left arrows), 2 were significantly enriched in the 125 and 200 mmol/L KCl eluates (right arrows). Their respective molecular weights were resolved as 43 and 62 kDa in a UV cross-linking assay (Figure 5B).

The 125 and 200 mmol/L KCl eluates were concentrated 10-fold on an Amicon Ultra-4 centrifugal filter device (30-kDa cutoff) and run on SDS-PAGE. The 43- and 62-kDa bands were excised, processed, and analyzed by mass spectrometry. The results showed sequence homologies to rat aldolase A and bovine calreticulin, respectively (online Table I). The calculated molecular mass of bovine calreticulin is 46.6 kDa, but it resolves on gels as an ≈60-kDa protein, because of a large number of negatively charged carboxylic groups (pI=4.7). Because the data in Figure 4B showed that CAE2181-2190 was essential for the formation of the 62-kDa, but not the 43-kDa, complex, we investigated the role of calreticulin in the regulation of GLUT-1 mRNA stability.

The following experiments were conducted to ascertain whether calreticulin was the protein in the 62-kDa complex. Aliquots of high-glucose VSMC protein extract and its 200 mmol/L KCl eluate were incubated with the [³²P]-labeled 57-nt probe, UV-cross-linked, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was

![Figure 4](image1.png)

**Figure 4.** CAE2181-2190 formed a specific 62-kDa complex with VSMC cytosolic proteins. A, UV cross-linking assay of the [³²P]-labeled 57-nt probe without (lane 1) or with S100 cytosolic proteins (15 μg) prepared from VSMC that had been incubated at the indicated glucose concentrations for 14 hours (lanes 2 through 4). Lane 1, free probe; lane 4, competition with a 2-fold molar excess of unlabeled 57-nt transcript that was added to the reaction mixture before UV illumination. B, A similar UV cross-linking assay without (lane 2) or with hybridization of the probe with the indicated fold molar excess of complimentary oligonucleotide competitors OND1, -2, or -3, as described in the online data supplement. The sequences of the 57-nt probe and the ONDs are shown at the bottom; bold letters mark CAE2181-2190.

![Figure 5](image2.png)

**Figure 5.** Identification of calreticulin as CAE2181-2190–binding protein. A, S100 cytosolic extract (2 mg of protein) of VSMC, which had been maintained at 25 mmol/L glucose for 14 hours, was applied on a Heparin-Fractogel-EMD column and eluted with increasing concentrations of KCl, as described in the online data supplement. A, REMSA of KCl eluates incubated with [³²P]-labeled 57-nt probe. Lane 1, Free probe; lane 2, probe with the original S100 extract (1.5 μg of protein); lane 3, probe with 6 μL of the column flow-through; lanes 4 through 11, probe with 6 μL of KCl eluates. Complexes of the original S100 extract are marked by arrows on the left; right arrows mark complexes of the 125 and 200 mmol/L KCl eluates. B, UV cross-linking assay of the 125 and 200 mmol/L KCl eluates and the [³²P]-labeled 57-nt probe. Lane 1, Free probe; lane 2, probe with the original S100 protein extract (15 μg). Lanes 3 and 4, Probe with proteins eluted with 125 or 200 mmol/L KCl (10 μL), respectively.
first used for Western blotting of calreticulin and then taken for phosphorimaging to visualize $^{32}$P-labeled RNA–protein complexes. Figure 6A depicts 2 colocalized 62-kDa (calreticulin) bands, detected independently by each method of the same gel. In addition, Figure 6B depicts a specific binding interaction of the same probe with pure bovine calreticulin (lane 2), which disappeared in the presence of a 10-fold molar excess of unlabeled 57-nt sequence (lane 3) or following prehybridization of the probe with OND2 (lane 4).

Figure 6C compares UV cross-linking profiles of the labeled 57 nt with pure bovine calreticulin and with VSMC cytosolic proteins. Whereas the typical multicomplex profile was apparent with the latter (lane 2), calreticulin formed a single 62-kDa complex (lane 3), which was blocked by prehybridization of the probe with OND2 (lane 4). Because the anti-calreticulin antibody used interacted well with only denatured calreticulin, our attempts to supershift the 62-kDa complex in native REMSAs were unsuccessful. Instead, an aliquot of VSMC protein extract was UV cross-linked with the $^{32}$P-labeled 57-nt probe, immunoprecipitated with anti-calreticulin antibody under denaturing conditions, and electrophoresed. A single band corresponding to the same size as the complex with pure calreticulin was observed (compare lane 5 with 3). Prehybridization of the probe with OND2 before UV illumination completely eliminated the formation of this complex (not shown). No bands were detected following immunoprecipitation with rabbit nonimmune serum (not shown).

High-Glucose Augments Calreticulin Expression in Vascular Cells In Vitro and In Vivo

VSMC and VEC were incubated in high glucose for 24 or 48 hours, respectively, to induce maximal downregulation of glucose transport and GLUT-1 protein level in VSMC and VEC, respectively, as was shown recently.21 Figure 7A and 7B show a 1.8- to 2.0-fold increase in calreticulin expression and its plasma membrane abundance and reduced GLUT-1 levels in both types of cells exposed to high glucose in comparison with low glucose.

The physiological relevance of these findings in cell cultures was confirmed by determining calreticulin content in aortic segments isolated from normoglycemic and hyperglycemic Psammomys obesus. The respective blood glucose levels of the gerbils were $109 \pm 20$ (n=3) and $263 \pm 29$ (n=5).
mg/dL. Figure 7C shows a significant 1.99±0.28-fold increased calreticulin content in aortae of diabetic animals in comparison with their normoglycemic controls. However, because of the high level of GLUT-1 content in contaminating erythrocytes trapped within the aortic segments, the analysis of aortic GLUT-1 was unreliable. Yet, calreticulin expression in the erythrocytes was negligible (online Figure I).

Calreticulin Reduces GLUT-1 Expression in VEC and VSMC

Effects of overexpression of calreticulin on GLUT-1 protein level in VSMC and VEC were then determined. VSMC were transfected with GFP (pEGFP-N1) without or with calreticulin (pMCC-ZP-CTR)-expressing plasmids, as described in the online data supplement, and incubated at 2 or 25 mmol/L glucose for 48 hours. GFP-positive cells were collected by FACS, lysed, and taken for the indicated Western blot analyses, as described in the legend of Figure 7. Right, VEC were infected with adeno viral constructs expressing GFP or GFP and calreticulin (viral multiplicity of infection values, 85 and 240 pfu/cell, respectively). The cells were then exposed to 2 or 25 mmol/L glucose for 48 hours, lysed, and taken for the indicated Western blot analyses. B, Real-time PCR analysis of GLUT-1 mRNA in similarly infected VEC was performed as described in the online data supplement. Means±SEM (n=4 to 6). *P<0.05 for difference from control cells infected with the GFP-expressing virus and incubated at 2 mmol/L glucose. C, [35P]-Labeled 203-nt probes containing or deleted of CAE 2181-2190 (203Δ10) were incubated with 0.1 μg of protein of S100 protein extract of VSMC that had been maintained at 2 mmol/L glucose for 14 hours. Calreticulin (CTR) (2 ng) was added to the mixtures where indicated. The assay was performed as described in the legend to Figure 1. D, Summary of 3 similar experiments (○, 203-nt probe; ●, 203Δ10-nt probe and calreticulin; □, 203-nt probe and calreticulin). Means±SEM. *P<0.05 for difference from the respective control (at 0 hour).

VEC were infected with adenoviral vectors expressing GFP or both GFP and calreticulin and incubated at 2 or 25 mmol/L glucose for 48 hours. Figure 8A (right panel, lanes 1 and 2) shows an ~50% reduction in GLUT-1 content in control, GFP- expressing cells that were exposed to high glucose in comparison with the low-glucose incubation. Overexpression of calreticulin in cells exposed to 2 mmol/L glucose mimicked the effect of high glucose in control cells (compare lane 3 with 2). Overexpressed calreticulin reduced GLUT-1 levels in VEC exposed either to 2 or 25 mmol/L glucose (lanes 3 and 4).

GLUT-1 and calreticulin mRNA levels in similarly infected VEC were determined by real-time PCR. The level of calreticulin mRNA in cells infected with the calreticulin-expressing virus was 5.4±1.5- and 6.9±2.3-fold higher (n=6) under 2 and 25 mmol/L glucose incubation, respectively, in comparison with cells infected with control GFP-expressing virus. Figure 8B shows 40±17% reduction in GLUT-1 mRNA content in cells expressing GFP only and incubated at 25 mmol/L glucose in comparison with the low-glucose incubation. The level of GLUT-1 mRNA in cells infected with the calreticulin-expressing virus was reduced by 44±16% and 48±16% under 2 or 25 mmol/L glucose, respectively.

Finally, we repeated the in vitro degradation assay shown in Figure 1 and added pure calreticulin to low-glucose VSMC protein extract, which did not degrade the [32P]-labeled 203-nt probe (Figures 1 and 8C). Figure 8C and 8D shows that calreticulin promoted a rapid degradation of the intact probe but not of the [32P]-labeled 203ΔCAE2181-2190 probe.
**Discussion**

The mechanism of high glucose-induced downregulation of GLUT-1 mRNA expression in VEC and VSMC were investigated. As already reported, high-glucose incubation reduced both GLUT-1 mRNA and protein expression in VSMC and VEC. In vitro degradation assays and cell transfection with CAE-2181-2190-containing pGL2 plasmid indicate that CAE-2181-2190 in the 3' UTR of GLUT-1 mRNA acts as a destabilizing glucose-sensitive element. REMSeAs, UV cross-linking assays, and affinity chromatography purification of cell extracts, followed by mass-spectrometric analysis, identified calreticulin as a binding protein in the 62-kDa complex with this CAE. Overexpression of calreticulin in the cells significantly reduced GLUT-1 mRNA and protein content, whereas pure calreticulin promoted rapid degradation of CAE-2181-2190-containing transcripts. Furthermore, high-glucose incubations increased calreticulin expression in both types of cells. Similarly, increased calreticulin content was found in aortae of hyperglycemic *P. obesus* in comparison with their normoglycemic controls. These findings support a model in which calreticulin interacts with CAE-2181-2190 in the 3' UTR of GLUT-1 mRNA and destabilizes the entire transcript, leading to reduced GLUT-1 protein expression and plasma membrane localization and, subsequently, to downregulation of glucose transport. This adaptive mechanism protects vascular cells against damaging effects of an uncontrolled influx of glucose in face of hyperglycemia.

Early studies identified CAE-2181-2190 in GLUT-1 mRNA as a glucose-sensitive element, which formed an 80-kDa mRNA stabilizing complex in brain microcapillary endothelial cells and rat C6 glioma cells. However, no such VSMC- or VEC cytosolic extract–CAE-2181-2190 complex was detected in the present study. Because GLUT-1 mRNA expression was reported to be regulated in an organ-specific manner by diverse proteins, GLUT-1 mRNA–protein complexes in vascular cells may vary from those formed in other cell types and under various experimental or pathophysiological conditions. Moreover, other CAEs in the 3' UTR of GLUT-1 mRNA can also affect GLUT-1 mRNA stability in concert with CAE-2181-2190. Indeed, our findings on an increased expression of luciferase mRNA on transfection of VSMC with the 203CAE-2181-2190-inserted pGL2 vector (Figure 1B) indicate a role for additional stabilizing interactions within the deleted 203-nt sequence that became effective in the absence of CAE-2181-2190-mediated destabilizing effects.

Overexpression of calreticulin in vascular cells exposed to low glucose mimicked the effect of high-glucose incubation on GLUT-1 mRNA and protein levels (Figure 8A and 8B). Yet, the combined effect of high-glucose incubation and overexpression of calreticulin was not additive and was, in fact, comparable to that observed in control cells exposed to high glucose. Thus, it seems that the increased calreticulin content in cells exposed to high glucose was sufficient to induce maximal destabilization of GLUT-1 mRNA, whereas overexpressed calreticulin under these conditions was consequential. We showed that transfer of vascular cells from a high-glucose to lower glucose levels induced upregulation of glucose transport and GLUT-1 mRNA expression and that glucose-deprived cells increase the expression of GLUT-1 to ameliorate a metabolically challenging and stressful condition. We also showed that GLUT-1 was a stress-inducible protein belonging to the family of glucoregulated proteins. Thus, the combination of overexpression of calreticulin and high-glucose incubation of the vascular cells could markedly reduce GLUT-1 content and cause glucose deprivation–like effects, followed by a stress reaction aimed to increase GLUT-1 gene transcription and/or expression of GLUT-1 mRNA–stabilizing factors. Other mechanisms could also explain the lack of additive effects of calreticulin overexpression and high-glucose incubations on GLUT-1 mRNA and protein in the cells.

Other studies also found specific RNA–calreticulin interactions. Destabilization of angiotensin-1 receptor mRNA in VSMC induced calreticulin binding to an AU-rich element in the 3' UTR. Calreticulin inhibited the translation of C/EBP transcription factors by its interaction with GCU repeats of C/EBPα/β mRNAs. In addition, rubella virus replication and/or localization in infected cells was controlled by calreticulin interactions with the 3' terminal of the viral RNA. These studies and the present results indicate the lack of a consensus RNA sequence for calreticulin binding. Indeed, computer-based predictions (http://www.bioinfo.rpi.edu/~zuker/mrna) of the 2D structure of the CAE-2181-2190 region in the 3'-UTR GLUT-1 mRNA and of the abovementioned RNA regions point to a stem-loop structure, rather than a consensus sequence, as a potential target for calreticulin binding.

The mechanism of high glucose–dependent augmented expression of calreticulin in vascular cells has not yet been investigated. We have found that high-glucose levels increase the expression of the enzyme 12-lipoxygenase and the production of its metabolite 12-hydroxyeicosatetraenoic acid (12-HETE) in vascular cells. This eicosanoid mediates high-glucose–induced reduction in GLUT-1 mRNA and protein levels and downregulation of hexose transport. Others have shown that peroxisome proliferator–activated receptors interact with various HETEs to promote gene transcription. These studies and present results allude to a peroxisome proliferator–activated receptor interaction with 12-HETE in the mechanism mediating augmented calreticulin expression in vascular cells.

Since the discovery of calreticulin more than 30 years ago as a calcium-binding protein in the sarcoplasmic reticulum, it has become clear that this is a multifunctional protein, whose subcellular localization is not restricted to the sarcoplasmic reticulum. Among other functions, calreticulin chaperones proteins in the endoplasmic reticulum, activates integrins, regulates cell adhesion, induces NO synthesis, and regulates steroid-sensitive gene expression. The present results on high glucose–dependent increased expression of calreticulin in VEC and VSMC and aortae of diabetic *P. obesus* (Figure 7A through 7C) suggest a role for calreticulin in modifying cell-matrix interactions, chaperoning and targeting of over-glycosylated, and regulating NO production in diabetic blood vessels.

Our results also indicate that the gluconeogenic enzyme aldolase A might interact with the 57-nt probe of the 3'-UTR region of GLUT-1 to form a non-CAE-2181-2190-dependent 43-kDa complex (Figure 4 and 5). This enzyme was found to
bind specifically to the 3′ UTR of myosin heavy chain mRNA and possibly target or anchor it to subcellular compartments of myosin synthesis by association with the cytoskeletal framework.\textsuperscript{35} It remains to be investigated whether aldolase A shares a similar function in subcellular targeting of GLUT-1 mRNA in vascular cells.

In summary, this study shows a central role for calreticulin in the mechanism of downregulation of GLUT-1 mRNA in vascular cells under high-glucose conditions, which ultimately protect the cells against excessive glucose influx and its deteriorating effects.

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Materials and methods

Materials

Glucose-free Dulbecco’s modified Eagle’s medium (DMEM), newborn calf serum (NBCS), fetal calf serum (FCS) and bovine fibronectin were from Beth-Haemek Biological Industries. PerkinElmer supplied \(\alpha-^{32}\text{P}\)rUTP (222 MBq/mmol). Oligonucleotide primers were synthesized by Sigma-Aldrich Israel. Heparin-Fractogel\textsuperscript{®}-EMD was purchased from Merck. Protein A Sepharose\textsuperscript{™}-CL-4B beads were from Amersham Pharmacia Biothech. ZipTip-C18 column were purchased from Millipore. T7 RNA polymerase, random primers, \(Pfu\) DNA polymerase, restriction enzymes, ligases, pGEM-T Easy vector, pGL2 luciferase expression vector, RNsae A, RNase T1 and RNasin were purchased from Promega. Real-time PCR reagents were purchased from Applied Biosystems. AdEasy\textsuperscript{™} Kit was from Q-Biogene. Roche Applied Science supplied baker’s yeast tRNA. The pEGFP-N1 vector was from Clonthech Laboratories. Bovine calreticulin, protease inhibitor cocktail, Sephadex\textsuperscript{®}-G-50 and monoclonal mouse anti-\(\alpha\)-smooth muscle actin- and mouse anti-tubulin (clone 1A4) antibodies were from Sigma-Aldrich. Polyclonal rabbit anti-calreticulin antibody was from Stressgen Biotechnologies. Rabbit anti-GLUT-1 polyclonal antibody was donated by Dr. H.-G. Joost (German Institute of Human Nutrition, Bergholz-Rehbrücke, Germany).

Animals

Male diabetes-prone *Psammomys obesus* (150-200 g, 14-16 week old) were fed a low energy diet (digestible energy 2.4 kcal/g, Kafolk, Petach-Tikva, Israel) to maintain normoglycemia. Diabetes was induced by feeding animals a regular rodent chow,
considered high-energy diet for *P. obesus* (3.3 kcal/g, Harlan-Teklad, Wilmington), for 3-4 weeks. The latter group developed a Type 2 diabetes-like syndrome and hyperglycemia due to β-cell dysfunction, as recently described.\(^1\)\(^2\) Segments of descending aortae were dissected from anesthetized animals (ketamine hydrochloride, Parke-Davis), cleaned of non-vascular tissue and frozen in liquid N\(_2\). Blood glucose levels were determined in blood samples with a glucometer (Accutrend Sensor; Roche Diagnostics, Mannheim, Germany). The animal studies were approved by the animal care and use committee of the Hebrew University and Hadassah Medical Organization.

**Preparation of vascular cell-protein fractions**

S100 cytosolic protein extracts of VEC and VSMC were prepared as described.\(^3\) Lysates of VEC and VSMC for Western blot analyses were prepared as described.\(^4\) Aortic protein extracts from *Psammomys obesus* were prepared as described by Langdown *et al.*\(^5\)

**Western blot analyses**

Western blot analysis of GLUT-1 was performed as previously described.\(^6\) All other antibodies were used for Western blot analyses according to suppliers’ protocols. Surface biotinylation of vascular cells and isolation of biotinylated proteins were performed as described previously,\(^4\) followed by Western blot analysis of cell-surface localized calreticulin in purified biotinylated proteins.

**Probes and RNA transcripts**

RNA isolation from cells, cDNA synthesis, using 0.15 µg random primers, and PCR, using *Pfu* DNA polymerase, were performed as described.\(^6\) The sense oligonucleotide primer for the nt-203 sequence (5’-2098-2300) was designed to contain 26 bases at the
5’-end, which encoded the T7 RNA polymerase start site, and 18 nucleotides (bold letters) corresponding to nt 5’-2099-2116 of bovine GLUT-1 mRNA (Genebank accession number: M60448; 5’-GCATGCTAATACGACTCACTATAGGG-GGCCACCCCTTCTATGGG). The antisense primer was an 18-mer oligonucleotides (5’-TGGCCCTCAGTACAAAGG), corresponding to bases 5’-2283-2300. The 57-nt sequence (5’-2160-2216) was synthesized similarly by PCR, using a sense oligonucleotide primer that contained 26 bases that encode to the T7 RNA polymerase start site and 18 nucleotides (bold letters) corresponding to 5’-2160-2177 of GLUT-1 mRNA (5’-GCATGCTAATACGACTCACTATAGGGCCTATTCCCGTCTC-TTCC). The antisense primer (5’-GGTCTCAGGCAAGGAAAG) corresponded to nt 5’-2199-2216 of the mRNA.

The nt-203 probe deleted of CAE_{2181-2190}, (nt-203ΔCAE_{2181-2190}) was prepared following a stepwise PCR of two fragments of nt-203: the first was synthesized with the sense primer described above and an antisense primer containing an EcoR I restriction site replacing the CAE_{2181-2190} sequence (5’-AGGAATTCATAGGAAGAGCGGAA-TAG). The second fragment was synthesized with the sense primer 5’-AGGAATTCACCTTTCTTGCCGTAGAC, which contained the EcoR I restriction site, and the antisense primer described above. The two PCR products were digested with EcoR I and ligated with T4 ligase into a pGEM-T-Easy vector. The nt-57 deleted of the CAE_{2181-2190} (nt-57ΔCAE_{2181-2190}) was prepared by PCR of nt-203Δ CAE_{2181-2190}, using the same sense and antisense primers described above for preparation of the full 57-nt sequence. In vitro transcription of probes was performed according to the T7 RNA polymerase supplier’s instructions. Sephadex G-50 spin columns were used to purify the
probes. $[^{32}\text{P}]$-Labeled probes were prepared by adding 2.15 TBq of $[^{\alpha-32}\text{P}]$rUTP to the reaction mixture. Sequence analyses were performed at the DNA analysis Unit of the Hebrew University, Jerusalem, Israel.

**Construction of pGL2 plasmids and cell transfection**

Various sequences of the GLUT-1 mRNA 3’UTR were inserted into the luciferase expression vector pGL2 at a unique pfM1 site located at nt-2,235 in the 3’-UTR of luciferase gene. The 203- and 203ΔCAE2181-2190-nt of bovine GLUT-1 3’-UTR were generated by PCR using specific primers (*Sense*: 5’-ATGCCAACCTATGGGCCACCCCTTCTATGGGC; *Antisense*: 5’-GTACCATAGG-TTGGTGCCCTCAGTACAAAGG) containing the pfM1 restriction site (bold letters). The primers contained three unrelated nucleotides at the 5-end to facilitate restriction endonuclease digestion. The PCR product was digested with pfM1 and ligated to pfM1-linearized pGL2 plasmid with T4 ligase. Inserts were confirmed by DNA sequencing using a 23-mer primer corresponding to nucleotide 5’-2,142-2,164 of the pGL2 vector (5’-GAGATTTAAAGCTCTAAGGTAAA). Cells were co-transfected with 4 μg of GFP-expressing plasmid (pEGFP-N1) and 10 μg pGL2 control or modified plasmid by calcium phosphate precipitation followed by a hyperosmotic shock (10% glycerol in PBS, 2 min at room temperature). Following a 48-h incubation period at 25 mmol/L glucose, the cells were detached with trypsin and GFP-positive cells were collected by FACS.
In-vitro RNA degradation assay

Labeled RNA probes (10^5 cpm) were incubated with 0.1 µg protein of S100 extracts in a final volume of 20 µl of a buffer containing (in mmol/l) 10 Hepes, pH 7.9, 3 MgCl₂, 40 KCl, and 1 DTT, and supplemented with 5% (v/v) glycerol. The samples were incubated at 37ºC up to 12 min. The incubation was terminated at various time intervals by the addition of phenol/chloroform. Aliquots were enriched with yeast tRNA, as described by Tsukamoto et al., and electrophoresed on native 4% PAGE, followed by film autoradiography and densitometric analysis.

RNA Electrophoretic Mobility Shift Assay (REMSA)

The [³²P]-labeled probes of (4×10⁴ cpm) were incubated with aliquots of S100 protein extracts of VEC and VSMC for 10 min at 4ºC in a final volume of 20 µl of REMSA buffer containing (in mmol/l) 10 Hepes, pH 7.9, 3 MgCl₂, 40 KCl and 1 DTT, supplemented with 5% (v/v) glycerol, 10 units of RNasin and 0.25 µg of tRNA. Samples were run on a native PAGE (4% polyacrylamide:bisacrylamide, 40:1) at 4ºC. RNA-protein complexes were visualized by film autoradiography and/or phosphorimaging (Bio-Imaging Analyzer, Bas 1000, Fujix, Kanagawa, Japan) of the dried gel.

UV cross-linking assay

Aliquots of S100 protein extracts (15 µg) of VEC or VSMC were mixed in REMSA buffer with [³²P]-labeled probes (10⁶ cpm), in the absence or presence of unlabeled RNA competitors, and incubated for 10 min at 4ºC. When complementary ONDs were used, the order of addition was as follows: the [³²P]-labeled probe was preincubated with an ODN at 65ºC for 3 min, followed by a 10 min incubation at 22ºC. Aliquots of S100
extracts were then added to the mixture and incubated further for 10 min at 4°C. All mixtures were irradiated with a UV lamp (254 nm) for 5 min, digested by RNsae A and RNase T1, mixed with SDS sample buffer and boiled for 5 min. Samples were separated by 12% SDS-PAGE. The gels were dried and RNA-protein complexes were visualized by film autoradiography.

**Heparin column chromatography**

Swelled Heparin-Fractogel® EMD beads were mixed (1:10) in a buffer containing 20 mmol/l KH₂PO₄, pH 6.85, and 0.02% (w/v) sodium azide, and used for columns. The columns were equilibrated with 10 volumes of a buffer containing (in mmol/l) 5 Tris-HCl, pH 7.5, 10 KCl, 1.5 MgCl₂, 0.01 EDTA, 0.05 DTT and 0.5 PMSF, and supplemented with 2.5% (v/v) glycerol. Aliquots of S100 protein extract (2 mg) were applied on the column and let to equilibrate for 10 min at 4°C, followed by a 10-volume pre-elution wash with the same buffer. Stepwise elution was then carried in 3 volumes of the same buffer supplemented with increasing concentrations of KCl (10-225 mmol/L).

**Mass spectrometry**

Protein bands were excised from gels following an SDS-PAGE, reduced with DTT and acetylated by iodoacetamide, followed by trypsin grade digestion, as described. The peptide mixture was extracted from gel slices with 60% (v/v) CH₃CN and 1% (v/v) CHOOH mixture and evaporated to dryness. The peptide mixture was solid phase extracted on ZipTip-C18 column and nanosprayed directly into QTOF-II MS system (MicroMass). MS spectra of each peak were collected and analyzed using the MicroMass Biolynx package. Database searches were performed with the Mascot package (Matrix Science). Searches of sequences were also performed manually with the Wisconsin

**Immunoprecipitation of calreticulin**

The anti-calreticulin antibody used recognizes the protein in its denatured form. An aliquot of S100 protein extract (50 µg) of VEC was mixed with the $[^{32}\text{P}]$-labeled 57-nt probe (2.5 x $10^6$ cpm) and UV cross-linked as described above. SDS was added to the mixture to a final concentration of 2% (w/v) followed by denaturation at 99°C for 5 min. The mixture was diluted 1:25 in 100 mmol/l Tris-HCL, pH 8.0, 100 mmol/l NaCl, 1 mmol/l EDTA and 1% (v/v) of Triton X-100. Anti-calreticulin antibody or rabbit non-immune serum were added (1:500 dilution) with swelled Protein A Sepharose™ beads, and the mixture was incubated overnight at 4°C. The beads were then washed 3 times with the same buffer and the bead pellet was resuspended in SDS-loading buffer, boiled for 5 min and the supernatant was taken for SDS-PAGE.

**Plasmid-driven overexpression of calreticulin**

The calreticulin expression plasmid (pMCC-ZP-CTR) in the sense orientation was prepared by subcloning the *XhoI-Smal* fragment of a pSVL-CRT plasmid containing calreticulin cDNA (courtesy of Dr. M. Michalak, University of Alberta, Edmonton, Canada) into a pMCC-ZP plasmid containing a CMV promoter. Cells were transfected by calcium phosphate precipitation with 10 µg of pMCC-ZP control plasmid or the calreticulin expression plasmid together with 4 µg of the CMV-GFP expression vector pEGFP-N1, followed by a hyperosmotic shock (10% glycerol in PBS, 2 min at room temperature). After a 48 h incubation period in complete DMEM supplemented with 2 or
25 mmol/l glucose the cells were detached, collected and GFP-positive cells were sorted and collected by FACStar-Plus cell sorter (Becton Dickinson). Protein lysate of the GFP-positive cells were prepared as described.\textsuperscript{6}

**Preparation of recombinant adenovirus**

Adenovirus construct expressing calreticulin and GFP was prepared by homologous recombination in bacteria, as described.\textsuperscript{10} Briefly, the cDNA encoding calreticulin was inserted into pAdTrack-CMV into the \textit{Xho I}/\textit{Sal I} sites. The resultant plasmid, pAdTrack-CMV-calreticulin, was then recombined with pAdEasy-1 (carrying the Adeno-5 genome) in BJ5183 bacteria. Small colonies were picked and positive clones were selected by size analysis of plasmid DNA on agarose gel. The recombinant plasmid was linearized with \textit{Pac I} and transfected with lipofectamine into GH354 cells for a high-yield production of the virus.\textsuperscript{11} The control virus expressing only GFP was kindly provided by Dr. H. Giladi (Goldyne Savad Institute of Gene Therapy, Hadassah University Hospital, Jerusalem, Israel). Titers of the viral stocks were determined by plaque assay on GH354 cells and were $2.5 \times 10^9$ pfu/ml for both. Over 90\% of infected cells expressed GFP, as was determined by fluorescent microscopy (data not shown).

**Real-time PCR**

RNA isolation from VEC and cDNA and synthesis of bovine GLUT-1 cDNA were described above. Real-time PCR was performed in ABI PRISM\textsuperscript{®} 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer’s guidelines. Oligonucleotide primers were designed using Primer Express program (Applied Biosystems) and were synthesized by Sigma-Aldrich Israel. Primer sequences are given in Online Table 1. PCR was performed in 20 µl volumes containing 1xSyBr Green
Master Mix and 500 nmol/l sense and antisense primers and GLUT-1 cDNA (25 ng) or 18S rRNA cDNA (10 pg). The thermal cycling program consisted of 2 min at 95°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. A cycle threshold (Ct) was calculated for each sample using GeneAmp5700 software. Dissociation curves, to detect non-specific amplification, were performed. Results were normalized against those obtained for 18S rRNA.
Supplemental Results

GLUT-1 and calreticulin expression in *Psammomys obesus* erythrocytes

We estimated the volume of blood trapped in the dissected segments of *Psammomys obesus* aortae after extensive washings be less than 5 µl. Since erythrocytes express extremely high level of GLUT-1 in their plasma membrane, even a small number of contaminating erythrocytes could greatly distort the results of aortic GLUT-1 determination. Therefore, we performed Western blot analyses of GLUT-1 and calreticulin in a lysate prepared from washed erythrocytes of *P. obesus* (Online Fig. 1). While the expression of calreticulin in erythrocytes was negligible, the content of GLUT-1 was found exceptionally high. These results clarify the reason for the unreliable determination of GLUT-1 Western blotting of aortic lysates. Moreover, since erythrocytes express negligible amount of calreticulin, these results validate the accuracy of the determination of aortic calreticulin (Fig. 7C in the article).

Mass spectrometric analysis

The following are the peptide sequences obtained from the mass-spectrometric analyses of the 62- and 43-kDa bands excised from SDS-PAGE.

**The 62-kDa complex**

IDNSQVESGSLEDWDFLPK; SGTIFDNFLITNDEAYAAEFGNETWGVTK; LFPAGLDQTDHMDEYNYIMFGPDICGPGTK;

Bovine calreticulin; NCBI accession S43376.

**The 43-kDa complex:**

VDKVVGPLLNTQGETTTQGLDGLSER; IGEHTPSLAMENANVLR;
GILAADESTGSIAK; LQSIGTENTEENRR; ADDGRPFQVIK;
ALANSLACQGK; QLLLTAADDR.

Rat aldolase A; NCBI accession P05065
References


8. Tsukamoto H, Boado RJ, Pardridge WM. Differential expression in glioblastoma multiforme and cerebral hemangioblastoma of cytoplasmic proteins that bind two
different domains within the 3'-untranslated region of the human glucose transporter 1 (GLUT1) messenger RNA. *J Clin Invest.* 1996;97:2823-2832.


Online Figure legend

**Online Figure 1** Western blot analyses of GLUT-1 and calreticulin in *Psammomys obesus* erythrocytes. Blood was collected into a test tube containing sodium citrate buffer. Erythrocytes were washed 3 times in PBS and resuspended in the original volume with PBS. A 50-µl sample of washed erythrocytes was then centrifuged and the pellet was suspended in 300 µl of lysis buffer. Following incubation at 4°C for 30 min and centrifugation, samples of soluble proteins corresponding to the indicated volume of whole blood were taken for Western blot analyses of GLUT-1 and calreticulin.

**Online Figure**

![Western blot analyses of GLUT-1 and calreticulin in Psammomys obesus erythrocytes](image)

Online Figure 1
### Online Table 1  Primer sequences for Real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genebank Accession Number</th>
<th>Primer Sequences</th>
</tr>
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| Bovine GLUT-1       | M60448                    | Sense: 5'-TTCATTGTGGGCGATGTGCTT  
                         |              | Antisense: 5'-AACCAGGAGCCACGGTGAAGA                   |
| Luciferase          | X65326                    | Sense: 5'-CCGCTGAATTGGAATCGATATT                     |
|                     |                           | Antisense: 5'-GGAAGTTCACCGGCGTCAT                     |
| Bovine calreticulin | S43376                    | Sense: 5'-CTACGCTCTGTCGGCCAGAT                       |
|                     |                           | Antisense: 5'-AGCGTCTGACCCCTGTTGCT                    |
| Bovine 18S rRNA     | AF176811                  | Sense: 5'-CGGCTACCACATCCAAAGGAA                      |
|                     |                           | Antisense: 5'-GGCCTCGAAAGAGTCTGAT                     |