Differential Regulation of Early Growth Response Gene-1 Expression by Insulin and Glucose in Vascular Endothelial Cells

Rukhsana N. Hasan, Sharbani Phukan, Shuko Harada

Objective—Early growth response gene (Egr)-1 is a key transcription factor involved in vascular pathophysiology. Its role in diabetic vascular complications, however, remains unclear. Because hyperinsulinemia and hyperglycemia are major risk factors leading to diabetic vascular complications, we examined the effect of insulin and glucose on Egr-1 expression in murine glomerular vascular endothelial cells.

Methods and Results—Insulin or glucose, when added separately, increased egr-1 mRNA levels and promoter activity, as well as Egr-1 protein levels in nuclear extracts. When insulin was added to cells preincubated with glucose, the two had an additive effect on Egr-1 expression. Furthermore, vascular endothelial growth factor receptor-1 (flt-1) and plasminogen activator inhibitor-1, two known Egr-1-responsive genes, were also upregulated in the presence of insulin or glucose. An investigation into the underlying molecular mechanisms demonstrated that insulin, but not glucose, increased Egr-1 expression through extracellular signal-regulated kinase 1/2 activation, which is consistent with our previous reports. In contrast, inhibition of protein kinase C by phorbol ester or by the specific protein kinase C inhibitor chelerythrine chloride downregulated glucose-induced, but not insulin-induced, Egr-1 expression.

Conclusions—Differential regulation of Egr-1 expression by insulin and glucose in vascular cells may be one of the initial key events that plays a crucial role in the development of diabetic vascular complications. (Arterioscler Thromb Vasc Biol. 2003;23:988-993.)

Key Words: Egr-1 ■ vascular endothelial cells ■ insulin ■ mitogen-activated protein kinase ■ protein kinase C

Elevation of plasma glucose concentrations is a characteristic phenomenon of diabetes. Plasma insulin concentrations are also generally elevated to compensate for elevated glucose concentrations, either endogenously from the β-cells or exogenously by injection of insulin, even though the increase in plasma insulin levels varies depending on the relative severity of insulin deficiency and insulin resistance. Hyperglycemia and hyperinsulinemia are important factors leading to vascular complications such as atherosclerosis or diabetic nephropathy in obese and diabetic patients.

The migration and proliferation of vascular smooth muscle cells within the intimal layer of arterial wall is a common feature of vascular pathogenesis. Various growth factors and cytokines, produced locally by vascular endothelial cells and macrophages, and extracellular matrix proteins play an important role in inducing cell migration and proliferation in these cells. Vascular endothelial cells, on the other hand, are located at the interface between circulating blood and peripheral tissues and are exposed to high levels of insulin and glucose in diabetic patients. Vascular endothelial cells, although classically considered non–insulin responsive, maintain a balance in the vasculature between growth promotion and inhibition, vasoconstriction and vasodilatation, and anticoagulation and procoagulation. Hyperinsulinemia associated with insulin resistance correlates with endothelial dysfunction, which could contribute to atherosclerosis in obese or diabetic subjects. However, the direct role of hyperglycemia and hyperinsulinemia in the pathogenesis of diabetic vascular complications remains elusive.

Early growth response gene-1 (egr-1), also known as Krox-24, zif/268, NGFI-A, and TIS8, was originally identified as one of the immediate early genes associated with cell proliferation and differentiation. Egr-1 is an 80- to 82-kDa zinc finger transcription factor, which activates the transcription of many genes, including platelet-derived growth factor (PDGF) A and B, tissue factor (TF), plasminogen activator inhibitor (PAI)-1, vascular endothelial growth factor (VEGF) receptor-1 (flt-1), insulin-like growth factor (IGF)-2, tumor necrosis factor (TNF)-α, interleukin (IL)-2, intracellular cell adhesion molecule (ICAM)-1, and egr-1 itself, often via binding to the GC-rich consensus 5’ GGG(T/G)GGGCG. Egr-1 expression is upregulated after vascular injury or fibroblast growth factor stimulation, and Egr-1 was found to be involved in profound chemotactic and mitogenic effects.
which may contribute to the structural remodeling that typically occurs in the pathogenesis of vascular disease.7 Furthermore, McCaffrey et al8 demonstrated sustained elevated Egr-1 expression levels, along with an increase in the expression of several Egr-1–regulated proteins in atherosclerotic lesions, which suggests that Egr-1 is involved not only in acute conditions such as vascular injury but also in chronic conditions such as atherosclerosis. Elevated Egr-1 expression and transcriptional activation mediated by Egr-1 resulting in altered gene expression patterns seem to be one of the initial key steps in coordinating the cellular events that result in vascular lesions. Therefore, we examined the effect of glucose and insulin, two major factors leading to diabetic vascular complications, on Egr-1 expression in vascular endothelial cells derived from mouse glomeruli (glomerular endothelial cells [GEtCs]).

Methods

Cell Culture and Glucose/Insulin Treatment

Murine GEnCs, provided by Dr F.N. Ziyadeh,9 were cultured in DMEM containing 25 mM D-glucose (glucose) and 10% FBS, but without insulin or any insulin-like molecules. Cells were ~70% to 80% confluent were serum-deprived in DMEM containing 2 to 5.5 mM glucose and 0.1% BSA, but without FBS, for 18 to 24 hours. When cells reached confluence, they were treated with glucose and insulin. Cells were incubated with 25 mM glucose or 17 to 100 mM insulin or were preincubated with 25 mM glucose/17 mM insulin followed by the addition of insulin and additionally incubated (see Results and figure legends for incubation times).

Northern Blot Analysis

Total RNA (15 µg) was analyzed by Northern blot as described.10 Radiolabeled signal was measured by PhosphorImager and quantified with ImageQuant 5.2 software (both from Molecular Dynamics).

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA (1.5 µg) was reverse transcribed with SuperScript II reverse transcriptase (RT). RT products (1 µL) were amplified by polymerase chain reaction (PCR) with 2 U Platinum Taq DNA polymerase and the specific forward and reverse primers for egr-1 (forward, 5'- CTCCCGGCTGCTTCTACTCTC; reverse, 5'-TCCGGGAT- CATCTTCTCCCTTGTG); flt-1 (forward, 5'-CCCCCGGGATGTCGA- CACCTGA; reverse, 5'-GTCGCCCTCCTTGTTTACTG); PAI-1 (forward, 5'-AGCGCGCACAGGCTGCTTC; reverse, 5'- CCGAGGTCTGGAGTCGTTTG), and α-tubulin (forward, 5'- AGTCCAGACAAACTCGT; reverse, 5'-CCTGAGAACACT- CCCCCTCT). Primers (Integrated DNA Technologies) were based on sequences obtained from GenBank. Number of PCR cycles for each gene was chosen so that it was within the linear range of amplification (egr-1, 25 cycles; flt-1, 37 cycles; PAI-1, 30 cycles; α-tubulin, 23 cycles). PCR products were analyzed by agarose gel electrophoresis and photographed under UV with a Kodak EDAS-290 digital camera system. Images were quantified with ImageQuant 5.2 software. All reagents were from Life Technologies.

Plasmids and Luciferase Assay

Mouse egr-1 sequences −903 to +65 bp and −395 to +65 bp from egr-1 CAT constructs, kindly provided by Dr J.G. Monroe,12 were subcloned in pGL3 basic vector (Promega) to generate p903-luc and p395-luc, respectively. Sequence and insert orientation were verified by sequencing analysis. Using FuGene-6 transfection reagent (Roche), 60% to 70% confluent cells in a 24-well plate were cotransfected with the respective egr-1-luc plasmid and control vector pHRL-TK (Promega). At 18 hours after transfection, cells were serum-deprived for 24 hours and then treated with glucose and insulin. Luciferase activity in cell lysate was measured using Dual-Luciferase Assay Kit (Promega) and Lumat LB9501 luminometer. Transfection efficiency was determined by an independent transient transfection using pEGFP-C3 (Clonetech) and was ~25%.

Western Blot Analysis

Nuclear extracts were prepared as described.12 For all other analyses, equal amounts of lysates were subjected to SDS-PAGE and Western blot analysis either directly or after immunoprecipitation with specific antibodies and Trisacryl (Pierce).10

Insulin Binding Assay

GEnCs (1×10⁵ cells/mL) or H35 cells in Krebs Ringer Mops buffer (containing 2 mM glucose and 1% BSA) were incubated with 3.6 nmol/L ¹²⁵I-insulin in the presence or absence of 4 µmol/L unlabeled insulin or IGF-1 for 30 minutes at 37°C. The amount of ¹²⁵I-labeled insulin associated with cells was determined as described.13

Results

Insulin and Glucose Additively Increase egr-1 mRNA Levels

We first examined the effect of glucose and insulin on variations in egr-1 mRNA levels by Northern blot analysis. Serum-deprived cells were incubated with 25 mM/L glucose, 17 mM/L insulin, or 17 mM/L insulin in the presence of 25 mM/L glucose. Figure 1A shows time course and Figure 1B summarizes fold induction of egr-1 mRNA levels (normalized by α-tubulin, internal control) from 7 independent experiments. Both insulin and glucose increased egr-1 mRNA levels by 3.07±0.60-fold and 2.84±0.21-fold at 30 minutes, respectively but with different time courses. Insulin-induced egr-1 expression was transient, peaking at 30 minutes, whereas egr-1 induced by glucose, remained elevated until 120 minutes. Interestingly, when insulin was added to cells that were preincubated with 25 mM/L glucose for 60 minutes, egr-1 expression levels were higher (7.90±1.40-fold) than those in cells incubated with either insulin or glucose alone.

The dose-response curve for insulin demonstrated that even 0.1 mM/L insulin increased egr-1 expression, suggesting that cells were very sensitive to insulin (see http://atvb.ahajournals.org). Moreover, 25 mM/L glucose enhanced insulin-induced egr-1 expression at all concentrations of insulin. However, the magnitude of insulin-induced increase in egr-1 expression was similar in the presence of 5.5 or 25 mM/L glucose, suggesting that glucose and insulin had an additive effect on egr-1 expression. In addition, we observed similar levels of insulin-induced egr-1 expression in the presence of 15 and 25 mM/L glucose, suggesting that even a moderate elevation in glucose concentration was enough to enhance insulin-induced egr-1 expression (not shown).

We then investigated if the enhancement of insulin-induced egr-1 expression in presence of glucose was specific to glucose. Cells grown in 5.5 mM/L glucose were incubated with different metabolizable and nonmetabolizable monosaccharides. When added separately, L-glucose, D-sorbitol, or 2-deoxyglucose neither increased egr-1 expression nor enhanced insulin-induced egr-1 expression (Figure 1C), suggesting that the effect seen with glucose was specific to glucose.

Insulin and Glucose Increase egr-1 Promoter Activity and Its Transcriptional Activity

To determine if egr-1 was upregulated at the promoter level, we performed reporter gene assay using egr-1 promoter-
two Egr-1-responsive genes, flt-1 and PAI-1,14–16 by RT-PCR. Both flt-1 and PAI-1 were upregulated in the presence of glucose or insulin (Figure 3B). Insulin-induced egr-1 and flt-1 expression peaked at 30 minutes, whereas the expression of PAI-1 was maximal at 60 minutes. Glucose by itself, or along with insulin, increased both flt-1 and PAI-1 expression modestly yet consistently. These results suggest that the Egr-1 protein in the nucleus was transcriptionally active.

Mechanisms Involved in Egr-1 Upregulation by Insulin and Glucose

The next question addressed was how glucose enhances insulin-induced egr-1 expression. We first sought to confirm that the effect of insulin on egr-1 expression is initiated through the insulin receptor (IR), not through the IGF-1 receptor (IGF-1R). The amount of insulin associated with GEnCs, as determined by insulin binding assay, was 6.04 fmol/10^6 cells, which was lower than that observed in H35 rat hepatoma cells (13.6 fmol/10^6 cells). However, the binding was displaced by excess unlabeled insulin (the binding decreased to 33.6±1.84% of that without excess insulin) but not by excess IGF-1 (100.8±23.1% of that without excess IGF-1), suggesting that in GEnCs, insulin bound specifically to IR, not to IGF-1R (see http://atvb.ahajournals.org).

Subsequently, we examined if glucose modified insulin-induced protein tyrosine phosphorylation of various signaling proteins (see http://atvb.ahajournals.org). Insulin increased tyrosine phosphorylation of a 180-kDa protein with maximum phosphorylation at 10 minutes independent of glucose concentrations. IR β subunit was also phosphorylated maximally, but weakly, at 1 minute because of relatively low expression levels of IR. Increase in tyrosine phosphorylation of Shc in response to insulin or glucose treatment was not observed as determined by immunoprecipitation with anti-Shc antibodies followed by Western blotting with antiphosphotyrosine antibodies (data not shown). To identify the 180-kDa tyrosine-phosphorylated protein as IRS-1 or IRS-2, lysate from insulin-treated cells was immunoprecipitated with anti-IRS-1 or anti-IRS-2 antibody (see http://atvb.ahajournals.org).
reporter gene assays with egr-1 results suggest that insulin, but not glucose, induces ERK1/2 activation after insulin treatment (Figure 4B). These itself actually decreased ERK1/2 activity and did not enhance 5.5 mmol/L and 25 mmol/L glucose (Figure 4B). Glucose by insulin increased phosphorylated ERK1/2 levels in both glucose-induced

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Figure 4A) downregulated basal as well as glucose/insulin-induced egr-1 expression. However, when MAP kinase activity was determined in terms of phosphorylation of ERK1/2, insulin increased phosphorylated ERK1/2 levels in both 5.5 mmol/L and 25 mmol/L glucose (Figure 4B). Glycine by itself actually decreased ERK1/2 activity and did not enhance ERK1/2 activation after insulin treatment (Figure 4B). These results suggest that insulin, but not glucose, induces egr-1 expression through ERK1/2 activation.

Lastly, we examined if protein kinase C (PKC) is involved in insulin- or glucose-induced egr-1 expression. Downregulation of PKC by pretreatment with phorbol 12-myristate 13-acetate (PMA) for 24 hours significantly downregulated glucose-induced egr-1 expression but had no effect on insulin-induced egr-1 expression (Figure 5A). Likewise, in reporter gene assays with egr-1 promoter luciferase construct, preincubation with 0.5 μmol/L chelerythrine chloride, a specific inhibitor of the classical PKC isoforms, inhibited glucose-induced egr-1 promoter activity but had no significant effect on insulin-induced promoter activity (Figure 5B). Western blot analysis with phosphospecific PKC (pan) antibodies revealed increased phosphorylation levels of PKC in the presence of 25 mmol/L glucose (Figure 5C), suggesting that PKC activated by glucose is involved in upregulation of egr-1 expression. Although insulin also increased phospho-PKC levels, this increase was not involved in insulin-induced egr-1 expression, because PKC inhibitors did not inhibit it. Taken together, these results indicate that insulin and glucose differentially regulate egr-1 expression, with glucose mediating its effects through one of the classical PKC isoforms and insulin acting through the ERK1/2 pathway.

Discussion

Hyperinsulinemia and hyperglycemia are two major risk factors for vascular complications in obese and diabetic patients. In this study, we demonstrated that insulin or high concentrations of D-glucose increased egr-1 expression at three distinct levels, ie, mRNA levels (Figure 1), Egr-1 protein levels in nuclear extracts (Figure 3A), and egr-1 promoter activity (Figure 2). Interestingly, when insulin was added to cells preexposed to glucose, the two had an additive effect on egr-1 expression. Recently, Gousseva et al17 demonstrated that insulin increased egr-1 promoter activity and cell proliferation in bovine aortic smooth muscle cells. However, unlike our study, they did not observe the effect of glucose on egr-1 promoter activity. This could be a cell line–specific effect (smooth muscle versus endothelial cells, bovine versus murine). Different experimental parameters used in the two studies could also explain the discrepancy in results; Gousseva et al incubated cells with glucose for 24 hours, which could have led to a loss of the glucose effect. In our study,
glucose incubation was much shorter (1 to 7 hours). Furthermore, we analyzed egr-1 promoter activity using luciferase reporter gene-based assay, which is more sensitive than the chloramphenicol acetyl transferase–based assay used by Gousseva et al.

The molecular mechanisms leading to insulin-induced Egr-1 expression have not been completely resolved yet. Based on insulin binding study and dose-response curve, we confirmed that insulin effect on Egr-1 expression was mediated through IR and not IGF-1R. We previously reported that in 32D cells, tyrosine phosphorylation of IR and Shc followed by MAP kinase activation, but not IRS-1 phosphorylation and P13'-kinase activation, led to Egr-1 expression.10 However, studies with embryonic fibroblasts from IRS-1–deficient mice indicated that an IRS-1 mediated pathway was necessary for Egr-1 expression.18 The apparent discrepancy between these studies may be attributable to a difference in the amount and distribution of signaling molecules in different cell types. In GEnCs, IRS-1 seemed to be the primary signaling protein tyrosine-phosphorylated in response to insulin. Even though IRS-2 and Shc were expressed in these cells, insulin did not increase tyrosine phosphorylation of these proteins. This could be attributable to intracellular localization of these molecules; IRS-2 and Shc are mainly cytosolic, whereas IRS-1 is membrane associated and accessible to IR.19 Another possibility is that IRS-1 binds to IR with a higher affinity compared with IRS-2 or Shc20,21 and therefore is a preferential substrate for IR. IRS-2 or Shc may become important only in the absence of IRS-1. In all cases, MAP kinase activation was one of the common pathways, if not the only one, leading to Egr-1 expression.

We observed an increase in egr-1 expression at glucose concentrations as low as 15 mmol/L (not shown), and L-glucose, D-sorbitol, or 2-deoxy-glucose did not increase basal or insulin-induced egr-1 expression (Figure 1C). Therefore, the effect was glucose-specific and not induced by osmotic change. This pathway leading to egr-1 expression by glucose has not been identified. Although MEK inhibitors inhibited glucose-induced egr-1 expression, we ruled out the involvement of ERK1/2, because glucose did not increase ERK1/2 activity. Possible explanations for this inhibition could be that a kinase downstream of MEK other than ERK1/2 is involved, MEK inhibitors are inhibiting other signaling pathways, or low levels of MAPK activation are required for basal egr-1 expression and another signaling pathway mediates glucose-induced increase in egr-1 expression. We therefore examined PKC and p38 activation by glucose. Rolli et al.22 reported that stress-induced Egr-1 stimulation was mediated by CRE through p38 MAP kinase activation, whereas PKCα and PKCβ were reported to be involved in hypoxia-induced Egr-1 expression.23,24 In GEnCs, p38 MAP kinase was not activated by glucose (not shown). Because downregulation of PKC with PMA or chelerythrine chloride significantly decreased glucose-induced egr-1 expression but had no effect on insulin-induced egr-1 expression, we concluded that glucose regulated egr-1 expression through PKC-dependent pathway (Figures 5A and 5B). These results suggest that insulin and glucose differentially regulate Egr-1 expression, with glucose mediating its effects through one of the classical PKC isoforms and insulin acting through the ERK1/2 pathway and possibly through distinct promoter regions.

Our studies were conducted in cultured GEnCs, the endothelial cells from capillary vessels in glomeruli. These cells were established as a cell line without SV40 transformation and show characteristic features of vascular endothelial cells such as cobblestone appearance and factor VIII expression.9 Pinkney et al.25 suggested that peripheral capillary endothelium was the primary origin of circulating markers that were produced by endothelial dysfunction. They pointed out that microalbuminemia was a powerful risk factor for cardiovascular disease and that endothelial dysfunction in capillary beds may be important for the development of atherosclerosis in large arteries. Therefore, the change that occurs in GEnCs may be one of the major and the earliest markers that indicate vascular complications.

Finally, we have demonstrated that glucose and insulin increased the expression of two Egr-1–responsive genes, flt-1 and PAI-1 (Figure 3B). We also observed a modest but consistent increase in the expression levels of PDGF-A and TF, two other Egr-1 target genes (not shown). These, along with several other genes, have been implicated in pathogenesis of vascular disease. Flt-1 binds not only to VEGF but also to its homologs placental growth factor and VEGF-B. A
recent study on the mouse model of ischemic heart and limb demonstrated that treatment with placental growth factor enhanced revascularization in ischemic tissues and blocking FGF-1 signaling with anti-FGF-1 antibodies strongly inhibited atherosclerotic plaque formation, suggesting an important role of FGF-1 in angiogenesis and arteriogenesis.26 Growing evidence indicates that PAI-1, as part of the fibrinolytic system, plays an important role in the vascular remodeling process underlying atherosclerosis and restenosis.27 TF, the primary cellular initiator of blood coagulation cascade, is expressed in human atherosclerotic plaques and may play an important role in thrombotic complications associated with plaque rupture.28 Likewise, PDGF-A upregulation in vascular tissue has been implicated in the initiation and progression of vascular occlusive lesions.4,29 Finally, sustained elevated levels of Egr-1 and many of the Egr-1–responsive genes have been reported in human atherosclerotic tissue.6 Collectively, our findings, along with evidence published by other groups that demonstrate a coordinated and increased expression of Egr-1–responsive genes relevant to atherogenesis, underscore the significance of Egr-1 in the pathology of the disease and strongly support the hypothesis that upregulation of Egr-1 by insulin and glucose in vascular endothelial cells may be one of the initial events that play a crucial role in the development of vascular complications in diabetes. However, although cultured cells are particularly helpful in dissecting the mechanisms and signaling pathways that regulate gene expression, we need to be cautious with the interpretation of the results thus obtained. As is the case with most studies based on cultured cells, the results reported here may need to be further evaluated in the endothelium derived from primary cultures as well as in animal models.

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Figure I.

Dose response curve for the insulin effect on egr-1 expression in GEnCs. Cells were incubated with 0.1 – 100 nM insulin in presence of 5.5 or 25 mM glucose. egr-1 mRNA levels were determined by Northern blot analysis and expressed as fold induction over control, untreated cells (no insulin, 5.5 mM glucose). α-tubulin was used as internal control. Data shown are representative of three independent experiments.
The amount of $^{125}$I-insulin associated with GEnCs. Cells were incubated with 3.6 nM $^{125}$I-insulin in the absence (No addition) or presence (+36 nM insulin) of 36 nM unlabeled insulin, 4 µM unlabeled insulin (+4 µM insulin) or 4 µM unlabeled IGF-1 (+4 µM IGF-1) for 30 min at 37°C. Bars represent the amount of $^{125}$I-insulin associated with GEnCs (mean ±SEM of triplicate samples), which was determined as described in the Methods section. Data shown are representative of three independent experiments.
**Figure III.**

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**Effect of insulin and glucose on protein tyrosine phosphorylation in cell lysates and on tyrosine phosphorylation of IRS-1 and IRS-2 in immunoprecipitates.** **A**, Cells were preincubated with 5.5 mM or 25 mM glucose for 60 min followed by incubation with 100 nM insulin for 0-20 min. Cell lysates were subjected to Western blot analysis with anti-phosphotyrosine (αPY; Transduction Laboratories). Molecular weights are indicated on the left. IR: insulin receptor, pp120: proteins at around 120 kDa that were constitutively tyrosine phosphorylated, NS: non-specific band. **B**, Cell lysates from the same incubation were immunoprecipitated with IRS-1 or IRS-2 antibodies and subjected to Western blot analysis with anti-phosphotyrosine (αPY), anti-IRS-1 or anti-IRS-2 antibodies (Upstate Biotechnology Inc).