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Hyperglycemia and redox status regulate RUNX2 DNA-binding and an angiogenic phenotype in endothelial cells

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ABSTRACT

Angiogenesis is regulated by hyperglycemic conditions, which can induce cellular stress responses, reactive oxygen species (ROS), and anti-oxidant defenses that modulate intracellular signaling to prevent oxidative damage. The RUNX2 DNA-binding transcription factor is activated by a glucose-mediated intracellular pathway, plays an important role in endothelial cell (EC) function and angiogenesis, and is a target of oxidative stress. RUNX2 DNA-binding and EC differentiation in response to glucose were conserved in ECs from different tissues and inhibited by hyperglycemia, which stimulated ROS production through the aldose reductase glucose-utilization pathway. Furthermore, the redox status of cysteine and methionine residues regulated RUNX2 DNA-binding and reversal of oxidative inhibition was consistent with an endogenous Methionine sulfoxide reductase-A (MsrA) activity. Low molecular weight MsrA substrates and sulfoxide scavengers were potent inhibitors of RUNX2 DNA binding in the absence of oxidative stress, but acted as antioxidants to increase DNA binding in the presence of oxidants. MsrA was associated with RUNX2:DNA complexes, as measured by a sensitive, quantitative DNA-binding ELISA. The related RUNX2 protein family member, RUNX1, which contains an identical DNA-binding domain, was a catalytic substrate of recombinant MsrA. These findings define novel redox pathways involving aldose reductase and MsrA that regulate RUNX2 transcription factor activity and biological function in ECs. Targeting of these pathways could result in more effective strategies to alleviate the vascular dysfunction associated with diabetes or cancer.

Introduction

Angiogenesis contributes nutrients and energy for tumor growth and is an essential component of wound healing (Potente et al., 2011). We, and others, have shown that rodents exhibit deficits in angiogenesis with age that account for the reduced growth of tumors and poor wound healing (Pili et al., 1994; Sato, 2003). One of the hypotheses proposed to explain these reduced angiogenic responses with aging is the elevation of ROS as a consequence of impaired antioxidant defenses (Lass et al., 1998). Angiogenesis is regulated by the coordinated expression of many cytokines, receptors, and transcription factors (DNA binding or associated factors) that control the expression of genes important in vascular endothelial cell (EC) migration, invasion, proliferation, and survival (Cao, 2014). One of these transcription factors, the Runt domain family member RUNX2, has been shown by our laboratory (Sun et al.,

Abbreviations: AR, aldose reductase; D-ELISA, DNA-based enzyme-linked immunosorbent assay; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; HAdEC, human microvascular adipose-derived ECs (HAdEC); HBME, human bone marrow microvascular ECs; HG, hyperglycemia; HMEC1, human microvascular dermal ECs (HMEC1); HRVT, Huvec retroviral telomerase; MetO, methionine sulfoxide; MsrA, Methionine sulfoxide reductase A; NAC, N-acetyl cysteine; PAO, phenyl arsine oxide; RIN-r, rat pancreatic ß-cells; ROS, reactive oxygen species; RUNX2, human Runt-related transcription factor; VAN, vanadate.

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2001) and others (Namba et al., 2000) to regulate EC migration, invasion, differentiation, proliferation, and survival. RUNX2 promotes angiogenesis through expression of angiogenic cytokines, matrix molecules, and metalloproteinase target genes (Bronckers et al., 2005; Qiao et al., 2006; Qiao et al., 2004; Sun et al., 2001; Sun et al., 2004). RUNX2 knockout mice exhibit defects in placental vessels (Komori et al., 1997), skin and hair follicle development (Glotzer et al., 2008), and fail to express VEGF in hypertrophic chondrocytes, resulting in lack of blood vessel infiltration and no bone formation (Zelzer et al., 2001). Adult RUNX2(+/−) heterozygote mice, but not young mice exhibit poor wound healing after bone marrow ablation (Juttner and Perry, 2007; Tsuji et al., 2004).

Oxidative stress in mammalian cells reflects a balance between the production of reactive oxygen species (ROS) and the activation of antioxidant pathways (Jones, 2008), which regulate angiogenesis (Brautigam et al., 2013; Sohal and Orr, 1998). ROS contribute to immune responses, act as essential physiological signaling molecules that regulate basic cell biology (Hamanaka and Chandel, 2010), and promote oxidative damage with age (Stadtman et al., 2005). Sources of ROS are numerous and include ligand/receptor activation (Sundaresan et al., 1996) and enzymatic generation (Leto and Geiszt, 2006) while ROS targets include all components of the cell including proteins, lipids, and nucleic acids (Kohen and Nyska, 2002; Wood et al., 2003). Cellular oxidant sensors and antioxidant defenses consist of DNA damage response enzymes (ribonucleotide reductases), superoxide dismutase and catalase, Keap1/Nrf1-induced activation of antioxidant genes (Kohen and Nyska, 2002), the thioredoxin/thioredoxin reductase complex, Ref1 response systems (Gius, 2004), and the protein repair enzymes Methionine sulfoxide reductases (MsrA; MsrB) (Oien and Moskovitz, 2008). The Msr enzymes not only act directly on oxidized methionine (MetO) residues to restore transcriptional activity of proteins such as p53 (Hanson et al., 2005; Nomura et al., 2009) but can also reduce overall cellular ROS by neutralizing H_2O_2 in the presence of sulfoxide antioxidant scavenger substrates (Weissbach et al., 2005). These enzymes play important roles in anti-oxidant defenses and oxidative stress-related neurological malfunction (Fomenko et al., 2009; Moskovitz et al., 2001; Moskovitz et al., 1995; Oien et al., 2008; Oien et al., 2010; Ortiz et al., 2011), controlling transcriptional activation (Agbas and Moskovitz, 2009), and targeting proteins for proteolytic degradation (Oien et al., 2009b; Stadtman et al., 2003). Therefore, identification of new target proteins that might be regulated by Msr antioxidant activity will be important in understanding how cellular ROS control cell signaling, transcriptional activation, enzymatic function, and aging.

RUNX2 expression is downregulated in hyperglycemic (HG) diabetic mice, which may account for the observed angiogenic dysfunction in these mice (Fowlkes et al., 2008; Lu et al., 2003). Recent findings from our laboratory revealed that RUNX2 is regulated by nutrients in the microenvironment and its DNA-binding activity is especially sensitive to glycemic status (D'Souza et al., 2009). HG inhibited, while antioxidants increased, RUNX2 activity and wound healing (D'Souza et al., 2009). Glucose activated RUNX2 DNA binding by increasing phosphorylation through the cdk pathway, which is less active in HG (Pierce et al., 2012). RUNX2 DNA binding may be sensitive to oxidation because of the presence of two conserved cysteine (Cys) residues in the Runt DNA binding domain that are necessary for correct protein folding and interaction with DNA (Akamatsu et al., 1997; Kurokawa et al., 1996). DNA binding is also controlled by the presence of a conserved Met residue in the Runt domain that mediates heterodimer formation with the RUNX2 binding partner, Cbfß, which enhances DNA binding ten-fold relative to the RUNX2 monomer (Tahirov et al., 2001). However, the possible role of HG stress in regulating the redox status of Met residues in RUNX2:Cbfß interactions has not been studied.

To test the hypothesis that HG activation of the AR pathway in ECs could lead to elevated oxidative stress and negative regulation of RUNX2, we examined EC redox status in HG and how this affects RUNX2 DNA binding. We found that HG regulated RUNX2 activation through the AR glucose utilization pathway by elevating redox stress. Maintenance of RUNX2 activity was mediated by the Met-specific MsrA system, which can restore global redox balance of Cys residues after oxidative stress and regulates the redox status of critical Met residue(s) in the DNA-binding Runt domain of RUNX2. The results from this study provide new evidence that RUNX2 activity is regulated by glycemic status and oxidative stress. This may encourage new approaches to modulate the redox status of ECs to inhibit tumor angiogenesis or alleviate the vascular dysfunction associated with diabetes (Cao et al., 2011).

Methods

Reagents

2,2-Bis(3-allyl-4-hydroxyphenyl) hexafluoropropane was prepared by dissolving hexafluorobisphenol A and K_2CO_3 in acetone (200 ml) and heating to reflux. After 30 min, allyl bromide was added drop wise and the resulting mixture was stirred at reflux for an additional 7 h and concentrated under reduced pressure. The remaining residue was taken up in ethyl acetate, washed with water and brine, dried over $MgSO_4$, and concentrated under reduced pressure. BCl_3 in dichloromethane was added drop wise to a solution of the crude 2,2-bis(4-allyloxyphenyl)hexafluoropropane in dry dichloromethane (100 ml) at −78 °C. The resulting mixture was allowed to reach room temperature and stirred for another 3 h. The reaction mixture was quenched with water (100 ml), extracted with chloroform, washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by flash chromatography to generate 2,2-bis(3-allyl-4-hydroxyphenyl) hexafluoropropane as a yellow oil. For DNA-binding assays, sulindac sulfoxide or sulfide was diluted from a 100 mM stock in 100% ethanol. Control treatment with ethanol alone (<0.1%) did not affect DNA binding.

Cell culture

Human bone marrow microvascular ECs (HBME) were obtained from Dr. Ken Pienta (University of Michigan); human microvascular adipose-derived ECs (HAdEC) were prepared by Dr. John McLenithan (NORC core facility at the University of Maryland); human microvascular dermal ECs (HMEC1) were obtained from the Centers for Disease Control and Prevention, Atlanta, GA; human retroviral telomerase (HRVT) ECs were prepared in our laboratory by infecting primary human umbilical vein endothelial cells (Huvec from Cell Systems, Kirkland, WA) with a retroviral vector expressing the human telomerase gene (hTert) from Dr. R. Weinberg (Counter et al., 1998); rat pancreatic ß-cells (RINr) were a generous gift from Dr. Jun Hayashi (A&G Pharmaceuticals, Columbia, MD). All cells, except HRVT ECs, were cultured in DMEM + 10%FBS (complete medium) (Qiao et al., 2006); HRVT ECs were cultured in endothelial basal media (EBM2) plus growth supplements and 2% FBS (Lonza, Gaithersburg, MD). In some experiments, cells were cultured in glucose and serum-free, phenol red-free, DMEM supplemented with pyruvate, $NaHCO_3$ (starvation medium). Cells were routinely tested for Ac-LDL uptake (fluorescence microscopy) and CD31 expression (FACS analysis) to confirm endothelial origin.

Measurement of redox status

For ROS measurements, cells were harvested, and replated for 24 h to 48 h in defined medium containing 5 mM or 25 mM glucose prior to preparation of nuclear extracts for use in Western blots. The aldose reductase (AR) inhibitors alrestatin (Tocris Cookson, Inc. Ellisville, MO) or ranirestat (AS-3201; Eisai Limited, United Kingdom) and the ROS quencher N-acetyl Cys (NAC; Sigma-Aldrich, St. Louis, MO), were used as antioxidants. Cellular ROS was measured using the CDCF-DA reagent (Enzo Life Sciences, New York, NY; #52103). Briefly, cells were