Liu Y, Mladinov D, Pietrusz JL, Usa K, and Liang M. Glucocorticoid Response Elements and 11β-Hydroxysteroid Dehydrogenases in the Regulation of Endothelial Nitric Oxide Synthase Expression

Supplement: Expanded Materials and Methods

Chronic monitoring of arterial blood pressure in rats and renal medullary interstitial infusion. Male Sprague-Dawley rats, 270 to 290g body weight, were used in these studies, which were approved by the Institutional Animal Care and Use Committee. Arterial blood pressure of uninephrectomized, conscious, freely moving rats was recorded from 9:00AM to noon each day using an arterial catheter chronically implanted in the femoral artery. Saline or drugs were continuously infused into the chronically implanted renal medullary catheter at a rate of 8.3 μl/min.

Cell culture. Human umbilical vein endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMEC-d) were obtained from Cambrex (East Rutherford, NJ) and cultured with the following modifications. To standardize the glucocorticoid content in the culture medium, FBS and cortisol supplementations provided by Cambrex were omitted (the basal medium did not contain any glucocorticoids according to Cambrex). Instead, a single stock of FBS containing less than 5 nmol/L cortisol/corticosterone according to our analysis was used throughout the study, which contributed less than 0.1 nmol/L of cortisol/corticosterone in the final medium. Cortisol (Sigma, St. Louis, MO) was dissolved in ethanol at 20 mmol/L, and added to EGM-2 or EGM-2MV to various concentrations as described in Results.

Aliquots of the media were analyzed before use to confirm the expected concentrations of cortisol. Cells between passages 3 and 6 were used for experiments at 80-90% confluency unless otherwise indicated.

Promoter and deletion segment constructs. Human genomic DNA was isolated from HUVEC cells with DNAzol (Invitrogen, Carlsbad, CA) and used as the template for PCR amplification of eNOS promoter segments. The eNOS promoter segments from -2091 bp, -848 bp, -430 bp, -168 bp, or -119 bp to +22 bp, relative to the transcription start site, were amplified using the pfu DNA polymerase (Stratagene, La Jolla, CA). The forward primers were 5'-CTCGAGAGTGGTGCAGGCAAGAAGACAAT-3' for the -2091 bp segment, 5'-TACATCTCGAGAACTGTAGTTTCCCTAGTCCCC-3' for -848 bp, 5'-TACATCTCGAGCCCTGCTACAGAAACGGTGCT-3' for -430 bp, 5'-TAACTCTCGAGGCGTGGAGCTGAGGCTTTAG-3' for -168 bp, and 5'-TTACTCTCGAGATTGTGTATGGGATAGGGGCG-3' for -119 bp. The reverse primer used for all five segments was 5'-ACTTGAAGCTTGTTACTGTGCGTCCACTCTGCTG-3'. The *Xho*I and *Hind*III linkers, together with its adjacent protection nucleotides, were included in the 5' end of the forward and reverse primers, respectively, for cloning purposes. The PCR products were recovered, digested with *Xho*I and *Hind*III, and cloned into the *Xho*I-*Hind*III site of pGL4.81[hRlucCP/Neo] (Promega, Madison, WI). This process generated constructs in which a promoter segment was linked to the renilla luciferase reporter gene. The insertions were verified by DNA sequencing. The reporter constructs, as well as the pGL2 control vector containing the firefly luciferease gene, were transformed into

competent E. Coli, propagated, and extracted and purified with the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA).

Site-directed mutagenesis. Site-directed mutagenesis was performed with the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene), following the protocol suggested by the company. The -2091 bp promoter construct was used as the template. The forward and reverse primers for mutagenesis at position -516 bp to -513 bp were 5'-CAAGGGTGGGGATCCAGGAacaaTTGTATGTATGGGGGGAGG-3' and 5'-CCTCCCCCCATACATACAAttgtTCCTGGATCCCCACCCTTG-3', respectively. The forward and reverse primers for mutagenesis at position -110 bp to -107 bp were 5'-CCTGTCCCATTGTGTATaaatTAGGGGCGGGGCGAGGGC-3' and 5'-GCCCTCGCCCCGCCCCTAatttATACACAATGGGACAGG-3', respectively. Another mutation construct was produced using the shortest, -119 bp promoter segment as the template. The forward and reverse primers for introducing mutations into the -110 bp to -107 bp positions in the short segment were 5'-GCCTCGAGATTGTGTATaaatTAGGGGCGGGGCGAGGGC-3' and 5'-GCCCTCGCCCCGCCCCTAatttATACACAATCTCGAGGC-3', respectively. Mutated bases were designated by lower case letters. Mutagenesis was verified by DNA sequencing. Reporter gene constructs containing mutated promoters were prepared as described above.

Transfection and reporter gene analysis. HUVEC at 60-70% confluency were transfected with reporter gene constructs using Lipofectin (Invitrogen), following the protocol suggested for HUVEC. Briefly, 2 μg of the pGL2 control firefly luciferase plasmid and 1 μg of the recombinant renilla luciferase plasmid were transfected into a 35

mm dish of HUVEC using 6 μl Lipofectin. After 3 hours, the transfected cells were treated with the ECM-2 medium without cortisol or with 100nM cortisol. After 45 to 48h, the cells were used for the measurement of renilla and firefly luciferase activities using the Dual-Luciferase Reporter Assay System (Promega) and Berthold LB953 AutoLumat Plus following the recommended protocols. The firefly luciferase activity was used to normalize the renilla luciferase activity to control for transfection efficiency.

RNA interference. SiRNAs were designed, synthesized, and transfected using Oligofectamine (Invitrogen) into HUVEC or HMEC-d (30-50% confluent). The sequences of siRNAs (5' to 3') used were as follows: siRNA targeting human 11β-HSD1, GCAGAGCAAUGGAAGCAUUdTdT and AAUGCUUCCAUUGCUCUGCdTdT; siRNA targeting human 11β-HSD2, ACGUGGGUCAGUGGGAAAAdTdT and UUUUCCCACUGACCCACGUdTdT; control siRNA targeting luciferase that had no sequence homology with any mammalian genes, CUUACGCUGAGUACUUCGAdTdT and UCGAAGUACUCAGCGUAAGdTdT.

Real-time PCR. Real-time PCR analysis was carried out using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) and the Taqman 5' nuclease method. The mRNA level was normalized to the internal control 18S rRNA. Sequences of the primers and probes used were: human eNOS forward, GCCAACGCCGTGAAGATC; reverse, CATACAGGATTGTCGCCTTCACT; probe, FAM-CGCCATCACCGTGCCCATGAG-TAMRA; rat eNOS forward, TGTTCCAGGCTACAATTCTTTCTGT; reverse, GTGTCCAGACGCACCAGGAT; probe, VIC-CAGAGCAGCAAATCCACCCGAGC-TAMRA; human guanylate cyclase 1B3 forward, CAACCTCCTCAACGACCTCTACA; reverse,

CAACAGTCTCCACCTTATAAACAAATG; human bradykinin receptor B1 forward, GCTCTTCCCTCAAAATGCTACG; reverse, GGAGGAAGACCAACAGGACAAA; human bradykinin receptor B2 forward, GAAGTTCAAGGAGATCCAGACAGAG; reverse, ATGAATAGCAGCAGCACAACCA.