Localization of 2 11β-OH Steroid Dehydrogenase Isoforms in Aortic Endothelial Cells

Andrew S Brem, Robert B Bina, Thomas C. King, David J Morris

Abstract—11β-hydroxysteroid dehydrogenase (11β-HSD) is expressed in vascular smooth muscle cells (VSMC) but has not been reported to be present in vascular endothelial cells. This enzyme assists in regulating the cellular concentration of active endogenous glucocorticoids (GCs). We have observed that endothelium intact rat aortic rings express message for both Type 1 and Type 2 11β-HSD whereas primary cultures of VSMC express only mRNA for the Type I isoform. Since GCs diminish prostacyclin synthesis in endothelial cells, we hypothesized that 11β-HSD is present in vascular endothelial cells. In primary cultures of rat aortic endothelial (RAE) cells, mRNA from both isoforms of 11β-HSD could be detected by RT-PCR with higher levels of the Type 1 isoform. The oxo-reductase reaction “activating” 11-dehydro metabolites back to the parent steroid is the preferred enzyme direction (12 1 after a 120 minutes steroid incubation) in intact RAE cells. When RAE cells are grown in the presence of antisense oligonucleotides specific for Type 1 11β-HSD, oxo-reductase activity is decreased by approximately 50% but the dehydrogenase reaction, which activates endogenous GCs and is characteristic of the Type 2 isoform, is unaffected. Thus endothelial cells appear to express both isoforms of 11β-HSD, the Type 1 isoform dominates functioning in the oxo-reductase mode. Inhibition of the oxo-reductase reaction may lower the local concentrations of GC and indirectly allow for increased production of prostacyclin in endothelial cells. (Hypertension. 1998;31[part 2]:459-462.)

Key Words: endothelial cells • glucocorticoids • 11β-hydroxysteroid dehydrogenase • hypertension • corticosterone

GCs have long been implicated in various forms of human hypertension. While a high sodium intake clearly can worsen the hypertension seen in chronic GC exposure, the primary rise in blood pressure is not thought to be sodium or volume dependent. Rather, GC-induced hypertension appears to have its roots in altering the contractile responses within the vascular tree. VSMCs contain 11β-HSD, an enzyme responsible for the local metabolism of circulating GCs. This enzyme in vascular smooth muscle (VSM) has a lower apparent Km for corticosterone (200 nM) than the isoform found in liver (1.2 µM), but it uses NADP(H) as a cofactor and is bidirectional. Vascular 11β-HSD has the ability to deactivate (dehydrogenase, i.e., corticosterone → 11-dehydrocorticosterone) or reactivate (oxo-reductase, i.e., 11-dehydrocorticosterone → corticosterone) GCs. The physiological correlate of 11β-HSD direction has been shown in endothelium intact vascular rings, inhibiting the dehydrogenase reaction is associated with an enhanced contractile response to catecholamines and angiotensin II while blocking the oxo-reductase reaction is linked to an attenuated contractile response to these same agents.

Vascular endothelial cells contain a number of GC-sensitive mechanisms that directly affect the contractile response of adjacent smooth muscle cells. Specifically, the generation of prostacyclin and the activity of atrial natriuretic peptide-induced guanylate cyclase are both impaired in presence of excess GC. Thus vasorelaxing processes active in endothelial cells that normally would counterbalance circulatory vasconstrictors are blunted. Given the role GCs may play in altering endothelial function, the present studies have been designed to determine whether 11β-HSD exists in these cells and if so, what are its biological characteristics.

Methods

RAE Cell Cultures

RAE cells (passage 6) were originally purchased fromVEC TEC, Inc. which isolates these purified cells from rat aorta using a proprietary method. The RAE cells demonstrate the typical "cobblestone" morphology consistent with epithelial cells. In addition, these primary cells demonstrate the expected incorporation of acetylated low density lipoprotein (LDL) and expression of Factor VIII using polyclonal antibody (DAKO).

RAE cells were grown in DMEM (Gibco, BRL), containing 5% horse serum (Gibco, BRL) and 5% fetal bovine serum (Sigma Chemical) in an atmosphere of 5% CO₂ at 37°C.

Primary VSMC Cultures

The aorta from 8 previously sacrificed adult Sprague-Dawley rats were dissected under sterile conditions and associated fat and connective tissue removed. The aortae were next incubated at 37°C for 15 minutes with digestion mixture consisting of collagenase 2 (1 mg/ml), elastase 0.125 mg/ml, DMEM/F12 (Gibco BRL) with HEPES (pH 7.4), penicillin (100 µg/ml), and streptomycin (100 µg/ml) at the completion of the incubation, the adventitia was carefully stripped, the vessel longitudinally opened, and the luminal surface gently scraped with forceps to remove endothelial cells. The remaining tissue were then minced into 1 mm pieces and incubated with digestion mixture for an additional 90 minutes at 37°C using a shaking water bath. The
detection was stopped with 8 ml of DMEM/F12, 10% fetal calf serum, and 25 mmol/L HEPES. Following digestion, a suspension of smooth muscle cells was obtained. The cells were passed over a sieve and centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded, and the cells resuspended in DMEM/F12, 10% fetal calf serum, and 25 mmol/L HEPES. The cells were then plated at a concentration of 1x10^5 viable cells/cm^2 onto T-75 plates and placed in an incubator. The flasks were gassed with 5% CO_2, and were maintained at 37°C.

Cells studied up to 40 passages have shown to retain the expression of the factor, a feature unique to VSMCs.

**RNA Isolation**

Cells were lysed and RNA extracted by the addition of one ml RNAzol (CINNABOTECX) according to the manufacturer’s instructions. For isolation of RNA from endothelium intact rat aortas, associated fat and connective tissue were removed and the aortae were briefly rinsed. The adventitia was stripped and the aortae minced into one mm pieces and homogenized in RNAzol using a polytron.

**RT-PCR Methods**

Both 11β-HSD Type 1 and Type 2 primer sets were designed to span introns in the genomic sequence so that contaminating DNA would produce either a larger sized product (Type 2 11β-HSD, c.a. 900 bp) or no product (Type 1 11β-HSD) under standard PCR conditions. The primers for Type 1 amplify a 456 nucleotide product from cDNA beginning in the second coding exon. The primers for Type 2 amplify a 544 nucleotide product between nucleotides 648 and 1192 of the cDNA sequence. Total RNA (5 μg) from cultured rat VSMCs, RAE cells, rat kidney, or rat aorta was used for oligo dt primed cDNA synthesis with an Invitrogen kit using the manufacturer's instructions. The resulting cDNA was diluted to 50 μL and 0.25–5 μL was added to 50 μL PCR reactions containing primers specific for 11β-HSD Type 1 (+, GGC CGA TGT GGA RCT GTC 3.) and (i CTG GCA GGT CCA ACA GG 5.) and Type 2 (+, TGC AAG GTC GGA GGA 5.) and (1, GCC GAG GAC ACA GAG AGT GA 3.) and the intron 1 splice donor site (, ACA GTG CCA TGC 3.). A hot start technique was employed with Genetamp 1X PCR buffer II (Perkin-Elmer), 1.5 mmol/L MgCl2, and 200 μM Geneamp dNucleotide triphosphates (Perkin-Elmer), 5–10 μCi α-32P dCTP (Amersham) and 1.5 U of AmpliTaq DNA polymerase (Perkin-Elmer). Samples were overlaid with 50 μL mineral oil and subjected to 35 cycles of amplification (94°C for 1 minute, 59°C for 1 minute, 72°C for 2 minutes) with a final extension of 10 minutes at 72°C using a Perkin-Elmer 480 thermal cycler. PCR products were analyzed by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. Gels were then dried and autoradiographed. cDNA quality and quantity were monitored by amplifying rat GAPDH mRNA (data not shown).

**HPLC Identification of Steroids**

For experiments involving intact RAE cells, the cells were seeded uniformly and grown to confluence in 6-well plates, 36 mm in diameter per well (Corning-Costar), using DMEM/F12, 5% horse serum, and 5% fetal bovine serum. Before the experiment, the medium was removed and replaced with one ml of DMEM containing 1% serum. Tritiated corticosterone (DuPont NEN) or 11- dehydrocorticosterone at 10 nmol/1 was added to each well. The cells were then placed back in the incubator for the allotted time.

After incubation, the reaction was stopped with 4 mL of methanol and the sample analyzed using HPLC. The steroids present in the supernatant are separated by HPLC using a DuPont Zorbax C8 column eluted at 44°C at a flow rate of 1 mL/minute using 60% methanol for 10 minutes. The various steroid compounds were observed by monitoring radioactivity on-line with a Packard Radiomatic Flo-One/Beta Series A-500 counter (Meriden) connected to a Dell Optiplex 425 S/L computer running A505 Flo-One for Windows (version 2.0A) and identified by comparing the retention times to those of known standards.

**Antisense Oligonucleotide Inhibition of Gene Expression**

Antisense phosphorothioate oligonucleotides targeted to block 11β-HSD type 1 gene expression were obtained from Research Genetics. Two antisense oligomers complementary to 20 bp sequences spanning the ribosome binding/translation start site (+, CAT AAC TGC CGT CCA ACA CG 3.) and the intron 1 splice donor site (+, ACA GTG GTA CTC ACC TGG TC 3.) were used in separate experiments. Antisense oligomers (antisense 1: 1913 micrograms/ml [300 μmol/l] and antisense 2: 1923 micrograms/ml [300 μmol/l]) were directly added (10 μL/well) to preconfluent RAE cells cultured in standard culture media and remained in the medium for 72 hours. A phosphorothioate oligonucleotide of identical length but of arbitrary sequence was used as a negative control.9

**Statistics**

Where appropriate, data were analyzed by Student’s t test with P values of less than 0.05 being significant.

**Results**

**11β-HSD Expression in Vascular Tissue**

RNA was harvested from endothelium intact rat aortas, rat kidney, rat VSMCs, and rat endothelial cells. Using RT-PCR methodology with Type 1 primers, all the tissues examined strongly expressed message for Type 1 11β-HSD (Fig 1). In similar assays for Type 2 11β-HSD, rat kidney and endothelium intact aorta expressed the Type 2 isoform at approximately the same level. In contrast, rat VSMCs showed little or no detectable Type 2 message, while RAE cells had readily detectable Type 2 11β-HSD mRNA (Fig 2).

**11β-HSD Activity in Intact Endothelial Cells**

Endothelial cells were grown to confluence on 6 well plates, 36 mm in diameter per well. Confluent cells, when assayed, had an average protein concentration of 0.052±0.012 mg/ml (n=4;
11β-HSD type 2

Figure 2. Autoradiograph of 11β-HSD Type 2 Expression in Rat VSMCs, Rat Kidney, Rat Aorta, and Cultured Rat Endothelial Cells by RT-PCR. cDNA from each of the above was prepared as described in the methods. 0.25 to 5 µl of cDNA was amplified with 11β-HSD Type 2 specific primers and the products analyzed on a 2% agarose gel. cDNA quantity was monitored by amplifying rat GAPDH mRNA (data not shown). Molecular weight markers are provided for reference. Lane 1 rat VSM cells; lane 2 rat kidney (control); lane 3 rat aorta; lane 4 RAE cells. Note that 11β-HSD Type 2 mRNA is expressed in rat kidney and endothelium intact rat aorta at approximately the same level. Little or no expression is detected in rat VSM cells but lower levels of the Type 2 isoform expression are evident in RAE cells.

Effect of Type 1 11β-HSD Antisense
RAE cells expressed both Type 1 and Type 2 11β-HSD; Type 1 was the predominate isoform under these culture conditions. In order to determine whether the Type 2 isoform was functionally significant, endothelial cells were grown to confluence in the presence of 11β-HSD antisense oligomers. For these experiments, two different antisense oligomers were used and the results of each compared. As depicted in Fig 4, the o xo-reductase activity of RAE cells grown in the presence of the Type 1 antisense oligomers was decreased by nearly half but the dehydrogenase reaction, which inactivates endogenous GCs and is characteristic of the Type 2 isoform, was unaffected. Incubation of the RAE cells with an arbitrary phosphorothioate oligonucleotide as a control did not alter the o xo-reductase reaction (data not shown) and was not associated with any visible signs of toxicity.

Discussion
Previous studies of 11β-HSD conducted in vascular tissue have largely focused on the presence of this enzyme in the smooth muscle layer.5,6 Experiments performed in our laboratories and by others have clearly shown that the Type 1 isoform exists7 and is bidirectional, but has a significantly lower Km for corticosterone (approximately 200 nM)4 than that described for this isoform in liver (1-2 µM).5,6 A clear physiological role of 11β-HSD in vascular tissue in general is now emerging. Endothelium intact vascular rings incubated for 24 hours in the presence of physiological concentrations of corticosterone and an inhibitor of the dehydrogenase reaction demonstrate an enhanced contractile response to exogenously administered catecholamine and angiotensin II.5 Conversely, studies conducted in the same model but with 11-dehydrocorticosterone 200 nM and an inhibitor of the o xo-reductase reaction show an attenuated contractile response to these same agents.8 Thus the direction and vigor of GC metabolism in vascular tissue can influence the contractile response.

The role endothelial cells play in modulating vascular contractile responses is being increasingly appreciated. Both nitric oxide and prostacyclin are potent vasodilators and are produced by vascular endothelial cells. GCs have the potential to impair prostaglandin synthesis by inhibiting phospholipase A2 and thus blocking the generation of prostacyclin precursors.7,8 Perhaps as part of a potential feedback loop, phospholipase A2 itself rapidly inactivates the o xo-reductase reaction

Metabolism of Corticosterone [B] or 11-Dehydrocorticosterone [A] (10 nM) by Intact Endothelial Cells Cultured on 6 Well Plates

![Figure 3. 11β-HSD Activity in Intact RAE Cells. Cells were incubated with physiological concentrations of steroids for periods of up to 120 minutes. Cultured endothelial cells functioned largely in the o xo-reductase (11-dehydrocorticosterone → corticosterone) mode over the times tested.](http://hyper.ahajournals.org/Downloaded from hyper.ahajournals.org)
Limiting the formation of active GC from its corresponding 11-dehydro derivative. Implications drawn from this complex of biochemical reactions are clear. The presence and directional activity of 11β-HSD in endothelial cells could indirectly influence vascular contraction depending on whether or not GCs were allowed to be biologically operative.

We observed that endothelium intact rat aorta, but not primary cultures of rat VSMCs, expressed message for both isoforms of 11β-HSD. Given this finding, we hypothesized that Type 1 and Type 2 11β-HSD might be present in vascular endothelial cells. Our current studies are the first to establish the presence of both isoforms in unstimulated vascular endothelial cells. Type 1 predominates, functioning in the oxo-reductase mode. Experiments with Type 1 antisense oligomers support these findings, only the oxo-reductase reaction, a Type 1 function, appeared affected. The biological significance of both isoforms in endothelial cells remains to be established. The relative expression and activity of both endothelial isoforms, described in the present studies performed on unstimulated cultured cells, may likely change in vivo under different circumstances.

Krozowski and his associates have reported the presence of Type 2 isoform in human VSM by immunohistochemical techniques. However, this same group was unable to demonstrate the presence of Type 2 11β-HSD in rat aorta using Western blot analysis. In the present studies conducted in rat tissues, we have localized Type 2 in endothelial cells but not in VSMCs. Several factors could account for these observed differences. First, the relative sensitivities of RT-PCR and immunohistochemistry are different. Second, the vessel in which the Type 2 isoform was located was a human renal interlobular artery and not rat aorta. This raises the possibility that the appearance of the different isoforms may vary with species and/or the size of the vessel.

The function(s) of 11β-HSD in endothelial cells remain to be fully elucidated. By controlling the local concentration of GC, this enzymatic pathway could limit the generation of prostacyclin and the vasodilation associated with this agent. The potential roles these enzymes play in vascular endothelial and smooth muscle cells is a fertile area for future investigation.

**References**


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