Mammalian Bufadienolide Is Synthesized From Cholesterol in the Adrenal Cortex by a Pathway That Is Independent of Cholesterol Side-Chain Cleavage

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Abstract—An increasing body of evidence suggests that an endogenous mammalian bufadienolide (BD) may be involved in the regulation of Na\(^+\),K\(^+\)-ATPase activity and the pathogenesis of arterial hypertension. We developed a purification scheme for marinobufagenin (MBG), an amphibian cardiotonic BD, and applied it to purify and characterize material in human plasma, culture medium conditioned by Y-1 adrenocortical cells, and rat adrenal tissue. MBG immunoreactivity purified from plasma and measured by ELISA showed important similarities (chromatography and antibody cross-reactivity) to material secreted into cell culture medium by Y-1 cells. This observation indicates that circulating mammalian BD may have an adrenocortical origin. Release of mammalian BD from adrenocortical cells grown in the absence of exogenous cholesterol was reduced by treatment of cultures with mevastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. Supplementation of the serum and cholesterol-free cell culture medium with the LDL fraction of human plasma increased the production of MBG material in the presence of mevastatin, supporting its origin from cholesterol. We used Y-1 cell lines transfected with genes shown to inhibit steroidogenesis through cholesterol side-chain cleavage (Y-1/DAX and Y-1/RIAB) to investigate the dependence of MBG biosynthesis on side-chain cleavage. Our results indicate that the mammalian BD is synthesized in the adrenal cortex from cholesterol and shares important similarities with the amphibian BD MBG, that its biosynthesis is independent of transfer of cholesterol to the side-chain cleavage enzyme complex mediated by steroidalogenic acute regulatory protein, and that neither cAMP nor protein kinase A appears to be a critical component of the pathway controlling its biosynthesis. (Hypertension. 2000;36:442-448.)

Key Words: preeclampsia • adrenal gland • chromatography • steroids • bufadienolides • cholesterol

The membrane-bound Na\(^+\) pump, Na\(^+\),K\(^+\)-ATPase, conserves a binding site for cardiotonic steroids that inhibit the enzyme. This binding site is exploited therapeutically by the use of drugs such as the plant-derived digitalis glycosides. Structurally related cardiotonic steroid bufadienolides (BDs) extracted from amphibian venom are used in traditional Asian medicine.1 BDs contain a 6-membered lactone ring in the C17 composition of the steroid nucleus, replacing the 5-membered lactone characteristic of cardenolides, such as digitalis compounds and ouabain.2 Growing evidence supports the presence of BD compounds in mammals: Lichtstein and colleagues3,4 have isolated bufalin from human cataractous lens tissue3 and have found bufalin-like immunoreactive material in rat adrenal glands.4 A steroid containing a 6-membered lactone, incompletely identified as 3\(\beta\),14\(\alpha\),20:21-bufadienolide, was identified in extracts of human placenta.3

We have reported that cultured adrenocortical (AC) cells secrete a compound recognized by antibodies to the bufadienolide proscllaridarin A.5 Sich et al6 have demonstrated a proscllaridarin A immunoreactive substance in human plasma. A similar substance has been reported to be present in bovine adrenal extracts.8 Bagrov et al9 have reported the isolation and purification of material identical in a number of critical properties, including mass spectrum, with marinobufagenin (MBG), a major cardiotonic steroid component in the venom of *Bufo marinus*.

Endogenous mammalian inhibitors of Na\(^+\),K\(^+\)-ATPase have been proposed to participate in mammalian Na\(^+\) homeostasis and may play an important role in the pathogenesis of many forms of human and experimental arterial hypertension.10,11 However, the identity, origin, and biosynthetic pathways of these compounds in mammals remains to be
fully understood. Analogous to phytoestrogens, some studies suggest that the diet may be a source of such material in plasma and urine,\textsuperscript{12–18} whereas others have proposed that the hypothalamus,\textsuperscript{19–21} placenta,\textsuperscript{2,22} or adrenal cortex\textsuperscript{23–25} may be the source of the endogenous circulating Na\textsuperscript{+} pump inhibitor. We have demonstrated that Y-1 murine tumor AC cells grown in defined serum-free conditions produce a material that shows important similarities to MBG.\textsuperscript{26} Compared with plant-derived cardenolides, which have been proposed as endogenous mammalian inhibitors of Na\textsuperscript{+},K\textsuperscript{+}-ATPase (e.g., ouabain\textsuperscript{27} and digoxin\textsuperscript{28}), BDs have some attractive properties as endogenous inhibitors of Na\textsuperscript{+},K\textsuperscript{+}-ATPase. For example, bufalin induced positive inotropic and pressor responses, associated with a prominent natriuretic effect that was much stronger than the effect of equimolar concentrations of ouabain.\textsuperscript{29} Another BD, MBG, exhibits a greater affinity for the α\textsubscript{1} subunit of Na\textsuperscript{+},K\textsuperscript{+}-ATPase.\textsuperscript{30,31} which is the main Na\textsuperscript{+} pump isoform in renal tubules and vascular sarcolemma. Plasma MBG immunoreactivity (MBG-IR) is increased in several volume-expanded hypertensive states in humans and in animal models of hypertension.\textsuperscript{32–35} In our recent work,\textsuperscript{32} we showed that MBG-IR is elevated in plasma in women with normotensive pregnancy and in those with preeclampsia (2-fold and 10-fold, respectively, versus age-matched healthy women).

In the present study, we have further characterized and compared the chemical properties of MBG-like material in human preeclamptic plasma and in culture medium conditioned by Y-1 AC cells. Furthermore, we have used the cell culture system to demonstrate and investigate the biosynthetic pathway by which this material is synthesized in defined cell culture conditions.

Methods

Cell Culture

Mouse AC tumor cells (Y-1) were purchased from American Type Culture Collection. They were grown in T-75 flasks in defined serum-free medium (HyQ-CCM5, HyClone). Cells were grown to confluence, divided, and replated at 50% confluent density. The medium was replaced every 24 hours. All medium was collected and stored frozen at −20°C before analysis. Some studies used Y-1 cell lines stably transfected to express the human transcription factor DAX-1 (Y-1/DAX cell line) or the dominant-negative regulatory subunit of protein kinase A (PKA) (Y-1/R1AB cell line). The steroidogenic phenotypes of these cell lines have been fully characterized elsewhere.\textsuperscript{36}

Human Subjects

Plasma was obtained by plasmapheresis from young pregnant female subjects (n = 6) with pregnancy-induced hypertension (preeclampsia) who were admitted to Snegirev Obstetric Hospital (St. Petersburg, Russia). Subjects were included in the study after providing informed consent. The study protocol was approved by the Research Council of Ott Institute of Obstetrics and Gynecology. Preeclampsia was diagnosed according to the criteria of the American College of Obstetrics and Gynecology. This definition includes a diastolic blood pressure of at least 90 mm Hg or a systolic blood pressure of at least 140 mm Hg; a rise in the former of at least 15 mm Hg or in the latter of 30 mm Hg on at least 2 occasions ≥6 hours apart; or proteinuria, edema, or both, induced by pregnancy after the 20th week of gestation and sometimes earlier.\textsuperscript{37} None of the subjects had ever taken digitalis drugs. Venous plasmapheresis samples were collected during the first of 4 sessions of plasmapheresis and stored frozen at −20°C before extraction and analysis.

Purification of BD From Human Plasma and Y-1–Conditioned Medium

A purification scheme for MBG-IR material was developed, and MBG-IR material in Y-1–conditioned medium and human plasma was partially purified by using this scheme with some modifications for each of these sources. Techniques used include the following: (1) deproteinization by addition of ethanol and boiling, followed by centrifugation; (2) back-extraction of ethanol into chloroform; (3) normal phase (silica gel) chromatography; (4) analytical thin-layer chromatography to examine mobility properties of purified material; and (5) reversed-phase high-performance liquid chromatography (HPLC) on semipreparative and analytical columns. The purpose was to determine whether the same material (with the same chromatographic properties) that was present in conditioned Y-1 AC cell culture medium was also present in the tissue of origin in intact animals (adrenal gland) and whether it enters the circulation (human plasma). We have used the MBG immunoassay to monitor the purification of these samples.

Steroid Extraction

Plasma or Y-1–conditioned culture medium (4 L each) was deproteinized by boiling with 2 L of ethanol. The supernatant was collected after the cooling and centrifugation of the mixture. The protein residue was then washed with ~3 vol of 80% ethanol. The washed protein residue was centrifuged, and the supernatants were combined. The steroid fraction of this supernatant pool was extracted into 2.5 L of chloroform, and the extract was dried under vacuum.

Silica Column Chromatography

The resulting material was dissolved in a minimal volume of ethyl acetate (insoluble material was removed by centrifugation) and applied onto a glass column (25 mm×28 cm) packed with silica gel (Daviesil, 150 Å). The column had been prewetted and rinsed with ethyl acetate before the sample was applied. Eight 15-minute fractions (flow rate 2 mL/min) were eluted with ethyl acetate. Then a further 30-minute fraction was eluted with ethyl acetate/ethanol (1:1). Finally, a 30-minute fraction was eluted with ethanol. MBG-IR was measured by ELISA. Fractions containing MBG-IR were combined and dried under vacuum.

Thin-Layer Chromatography

Glass sheets precoated with silica gel (Whatman No. 60 F254, thickness 0.25 mm, Fisher Scientific) were used for thin-layer chromatography. The plates were developed at room temperature with ethyl acetate. MBG was detected by fluorescence during exposure to UV light.

High-Performance Liquid Chromatography

HPLC purification of MBG-IR from plasma and Y-1–conditioned medium included 2 chromatographic steps: (1) MBG-IR containing fractions from silica column were combined, dried, dissolved in 1 mL 40% acetonitrile (insoluble material was removed by centrifugation), and injected into a Rainin Dynamax reversed-phase C18 semipreparative HPLC column (22 mm ID×30 cm long). The column was eluted with 45% acetonitrile (flow rate 10.8 mL/min) over 80 minutes, at which time this solvent was progressively replaced (over 20 minutes) with a solvent containing 100% acetonitrile, and the column was eluted with 100% acetonitrile for 20 minutes. The UV absorbance of column effluent was monitored at 300 nm. Forty fractions (3 minutes each) were collected and dried under vacuum, and aliquots of each fraction were examined for MBG by ELISA. (2) Samples containing MBG-IR were combined, dried, dissolved in 25% acetonitrile, and injected into an analytical HPLC column (C18, 4.6 mm ID×30 cm long, Rainin Dynamax). A gradient was developed in which the starting solvent (25% acetonitrile) was progressively replaced with a solvent containing 40% acetonitrile over 40 minutes and increased to 100% acetonitrile in the
following 10 minutes. Then the column was eluted with 100% acetonitrile for 30 minutes. The flow rate was 1 mL/min. The resulting fractions were collected, and an aliquot of each fraction was examined by MBG ELISA.

**Immunoaassays**

Progesterone was measured by radioimmunoassay as previously described.\(^3\) MBG cross-reactive material in HPLC and in silica column fractions was measured by ELISA. Aliquots of each HPLC fraction were dried under vacuum and reconstituted in an assay buffer (0.1 mol/L PBS buffer, pH 7.4) before assay. Samples were tested during ELISA for their ability to inhibit the binding of rabbit antibody to solid-phase bound MBG (immobilized conjugate of MBG-3-glycoside to RNase, 0.2 µg of conjugate in 0.1 mL of bicarbonate-buffered saline per well). We added 20 µL of MBG standards and unknown samples to the coated wells, followed by 80 µL of MBG antibody. After 1 hour of incubation, the wells were washed 3 times with 0.9% NaCl containing 0.05% Tween 20, after which 100 µL of secondary antibody was added (goat anti-rabbit IgG peroxidase, Boehringer-Mannheim). After 1 hour of incubation, the wells were washed 3 times with the wash solution, and peroxidase substrate was added (TMB Microwell Peroxidase Substrate System, Kirkegaard and Perry Laboratories). Optical density was read at 450 nm. MBG was purified from the venom of *Bufo marinus* as described.\(^3\) The structure of this steroid is shown in Figure 1. The sensitivity of the immunoassay was 0.01 nmol/L. The cross-immunoreactivity of MBG antibody was as follows: MBG, 100%; progesterone, 30%; and pregnenolone, 0.1%. We did not analyze this material in the present study. Three adjacent fractions (fractions 5 to 7) from the plasma extract (B) contained MBG-IR.

**Statistical Analysis**

The results are expressed as mean±SEM. Statistical significance was determined by \(t\) test when simple comparisons across 2 groups were made. When multiple comparisons were required, ANOVA was performed. With the Fisher least significant difference test was performed. A value of \(P<0.05\) was considered to be significant.

**Results**

**Purification and Characterization of MBG-IR Material**

In the present study, we show that MBG-IR materials partly purified from human plasma, Y-1 murine AC cell culture medium, and normal rat adrenal glands all share important similarities with each other and with MBG purified from toad venom. These include elution in normal phase silica chromatography (Figure 2), extraction into organic solvents, migration in thin-layer chromatography (Figure 3), and elution in reversed-phase HPLC (Figures 4 and 5). Furthermore, purified material interacted with MBG antibodies (Figure 6), and analysis of this interaction by serial dilution suggested close homology between interaction of MBG standard with the antibody and partially purified material. It is possible that the

\[\text{MBG (3β,5β-dihydroxy-14-15-epoxy bufadienolide).}\]

![Figure 1](image1.png)

**Figure 1.** Structure of MBG (3β,5β-dihydroxy-14-15-epoxy bufadienolide).

initial preparation of MBG standard from toad venom was chemically modified during purification, which included an analytical HPLC step performed in the presence of trifluoroacetic acid. It is evident in mass spectroscopy that the MBG purified in this way exists, at least in part, as an MBG-trifluoroacetic acid adduct (data not shown). No trifluoroacetic acid was used in the purification of material from plasma and Y-1 medium, so perhaps the presence of such adducts is responsible for the difference in cross-reactivity observed between the standard and these samples (Figure 6). A subsequent preparation of MBG was used in the comparison of cross-reactivity of partially purified material from Y1/neo (control cell line, transfected with neomycin resistance selection vector only), Y1/DAX, and Y1/RIAB, and close parallelism was observed under these conditions (see below).

**Biosynthesis Pathway of MBG in the Adrenal Gland**

Y-1 mouse AC tumor cells constitute a model for the study of the biochemical mechanisms of steroid hormone production in the adrenal gland.\(^4\) To probe the dependence of MBG biosynthesis on cholesterol side-chain cleavage, we used 3 genetically modified Y-1 clones. The first (a control clone), Y-1/neo, is transfected with the selection vector (neomycin

![Figure 2](image2.png)

**Figure 2.** Distribution of MBG-IR in silica column chromatography. MBG-IR was measured by ELISA. In Y-1 medium extract, MBG-IR was observed in fractions 5 to 7 and in fraction 9 (A). Material in fraction 9 did not coelute with authentic MBG, and this peak of MBG-IR could be due to the ability of MBG precursors to interact with MBG antibody. We did not analyze this material in the present study. Three adjacent fractions (fractions 5 to 7) from the plasma extract (B) contained MBG-IR.

![Figure 3](image3.png)

**Figure 3.** Migration of authentic MBG and material in human plasma extract after fractionation on silica gel column in thin-layer chromatography system. Fractions 5 to 7 contain bands with radiofrequency identical to that of authentic MBG. (Data for Y-1 medium are not present because the amount of material present was smaller than the limit of detection by ambient UV light exposure).
resistance) only. The second, Y-1/DAX, contains the same vector but is also transfected to express the human DAX-1 gene. DAX-1 is a transcription factor that downregulates the expression of a number of proteins involved in adrenal steroidogenesis.\(^{36,41}\) Another Y-1 transformant line, Y1/RIAB, is transfected with a neomycin selection vector and a vector expressing the dominant-negative form of the PKA regulatory subunit.

In both Y-1/DAX and Y-1/RIAB, the cholesterol transfer protein, steroidogenic acute regulatory protein (StAR), which is responsible for the regulated flow of cholesterol to the inner mitochondrial site of side-chain cleavage,\(^{42}\) is undetectable at the level of both RNA and protein.\(^{36,41}\) Furthermore, expression of the cytochrome P-450 complex responsible for side-chain cleavage (P450scc) is reduced severalfold.\(^{36}\) The cAMP-dependent signaling pathway, which plays a major role in initiating steroidogenesis by activating side-chain cleavage, remains functional in Y-1/DAX cells but not in Y-1/RIAB.\(^{36}\) Both Y-1/DAX-1 and Y-1/RIAB cell lines show markedly reduced production of progesterone compared with Y-1/neo, but the production of MBG by these transformed lines was at the same levels as in Y-1/neo, the control line (Figure 7). In Y-1/neo, a 10-fold increase of progesterone production was detected after 2 hours of stimulation of the cAMP pathway by forskolin. At the same time, the level of progesterone production after 2 hours of forskolin stimulation in Y-1/DAX and Y-1/RIAB cells was not changed. However, the level of MBG production in all Y-1–derived cells did not significantly change after 2 hours of stimulation by forskolin (Figure 8). To determine whether MBG-IR material produced by these cells lines shares properties in common with authentic MBG, we partly purified this material from conditioned medium and fractionated it by HPLC. MBG-IR was found to elute in the same fractions as authentic MBG. Serial dilutions of HPLC-purified MBG-IR were compared with authentic MBG for their ability to compete for binding to the MBG antibody, and very similar dilution curves were observed (Figure 9).

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**Figure 4.** Fractionation of MBG-IR on a semipreparative reversed-phase C18 HPLC column (isocratic elution 45% acetonitrile, flow rate 10.8 mL/min). A, Y-1 extract: MBG eluted with retention time 12 to 17 minutes. B, Plasma extract: MBG eluted with retention time 9 to 15 minutes. The broader peak in the plasma sample corresponds with a higher amount of MBG-IR.

**Figure 5.** Fractionation of MBG-IR on analytical reversed-phase C18 HPLC column in linear gradient of acetonitrile (flow rate 1 mL/min). A, Y-1 extract: MBG eluted in fractions 8 and 9. This corresponds to 36% to 38% acetonitrile. MBG std indicates MBG standard. The majority of MBG-IR from plasma (B) eluted in fraction 10. This corresponds to 38% to 40% acetonitrile. The difference in retention time between these 2 samples could be due to normal small variations in the HPLC system (back pressure, batches of solvent, or preconditioning time after column storage), which may result in slightly different distribution of MBG-IR in HPLC fractions between runs separated by significant periods of time (days or weeks).

**Figure 6.** Interaction of purified MBG from plasma (A), Y-1–conditioned medium (●), and authentic MBG (□) with MBG antibody in ELISA. Serial dilutions of purified MBG-IR from Y-1–conditioned medium and plasma were compared with authentic MBG for their ability to compete for binding to MBG antibody. There was an equivalent of 0.05 mL of plasma (*) and 1 mL of Y-1 medium (**) in serial 1:2 dilutions of samples.

**Figure 7.** Progesterone (A) and MBG (B) production in Y-1–derived clones. Cells were plated in a 12-well culture plates and were grown to confluence in regular medium. Then they were washed and incubated for 2 hours in serum-free medium. The histograms show the mean of 6 duplicated experiments. **P=NS vs control (Y-1/neo).
These experiments indicate that MBG production is independent of STAR-mediated transfer of cholesterol to the inner mitochondrial membrane. Therefore, it is unlikely that P450scs is involved in the MBG biosynthesis pathway. These findings raise the question of whether cholesterol is a precursor in the biosynthesis pathway of MBG. Treatment of cultures growing in the absence of exogenous cholesterol with mevastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the major regulatory enzyme in cholesterol neogenesis, results in significant reduction of production of both progesterone and MBG (Figure 10). Addition of LDL cholesterol to the cholesterol-free growth medium of Y-1/neo reduced the inhibition of MBG release occurring in the presence of mevastatin.

Discussion

Recently, several pieces of evidence have suggested that mammals produce an endogenous cardiotonic steroid that is more similar in its properties to BDs (cardiotonic steroids best known through their presence in venoms of amphibia from the family Bufonidae) than to the plant-derived cardiotonlides typified by digoxin and ouabain. An important issue in this field has been to determine definitively whether cardiotonic steroids identified in mammals are derived from external sources or are the product of mammalian biosynthesis.

Although Y-1 cells express an incomplete complement of enzymes that convert pregnenolone into steroid hormones, Y-1 AC cell cultures have been a useful tool for the investigation of AC steroidogenesis and its control. Our previous evidence has implicated the adrenal cortex in the production of materials with cardiotonic steroid properties. Therefore, 2 important goals of the present study have been (1) to determine whether the adrenal cortex is the source of an endogenous mammalian cardiotonic steroid with properties similar to BDs and (2) to determine whether material present in human plasma shares properties with material synthesized by and released from Y-1 cultures grown under defined conditions. In the present study, we show that MBG-IR material that was elevated in preeclampsia and partly purified from human preeclamptic plasma has important similarities with MBG-IR material purified from Y-1 murine AC cell culture medium. These include similar migration in thin-layer chromatography, in normal phase silica chromatography, and in reversed-phase HPLC and possession of MBG immunoreactivity.

This material is produced in Y-1 cells grown in the absence of exogenous cholesterol by a pathway that can be inhibited by the HMG-CoA reductase inhibitor mevastatin (Figure 10). Therefore, MBG-IR material is likely to be a product of the same pathway by which cholesterol neogenesis occurs in these steroidogenic cells. Furthermore, we have shown in these studies that AC cells can use cholesterol from the LDL fraction for the production of MBG-IR material as well as synthesize this material de novo in defined serum-free me-
dium. It has been previously reported that Y-1 cells have receptors for LDL and can use cholesterol acquired from LDL in steroidogenesis.35 Hence, we conclude that MBG is the product of biosynthesis of AC cells and is produced in a pathway leading from acetate through mevalonate to cholesterol, which at least partially overlaps the pathway by which all steroid hormones are synthesized in the absence of exogenous cholesterol.

The availability of genetically modified Y-1 cell lines in which the steroidogenic capacity has been severely curtailed by the expression of transfected genes encoding human DAX-1 or RIAB36 provides an opportunity to further examine the dependence of MBG-IR production on cholesterol side-chain cleavage. MBG is synthesized by transfected cell lines (Y-1/DAX and Y-1/RIAB) that do not express STAR and by cells (Y-1/RIAB) that are unable to activate the PKA-dependent pathway by which steroidogenesis proceeds through cholesterol side-chain cleavage. MBG production was not increased after stimulation of the cAMP/PKA-dependent signaling pathway by forskolin (Figure 9). This evidence shows that MBG biosynthesis is independent of STAR-mediated transfer of cholesterol to the inner mitochondrial membrane, and neither cAMP nor PKA appears to be a critical component of the signaling pathway by which MBG production is regulated.

The P450scc expression in Y-1/DAX and Y-1/RIAB cell lines is markedly reduced.36 This results in blockade of the conversion of cholesterol into pregnenolone, the initial step in all known steroidogenic pathways. The fact that all the transfected cell lines produce MBG at a comparable level may be evidence that cholesterol side-chain cleavage is not involved in the MBG biosynthetic pathway in the mammalian adrenal cortex and that pregnenolone is therefore not a precursor of MBG. These results support our previous observation that pharmacological inhibition of side-chain cleavage and of the further metabolism of pregnenolone did not reduce the production of AC cardiotonic steroid.38,46

Studies of BDs in amphibians yield an incomplete view of the biosynthetic pathway. Pregnenolone, the immediate product of side-chain cleavage, is not incorporated into amphibian BDs. Thus, pregnenolone is not a precursor to the production of MBG in Bufo arenarum,47 Bufo arenarum,49 and Bufo marinus.50 Cholesterol may be a precursor of BD biosynthesis in amphibians, and the conversion of cholesterol stably labeled in the sterol nucleus to BDs has been shown.48,50 It is not clear whether cholesterol is a normal and obligatory constituent in the amphibian biosynthetic pathway, because no experiments interfering with the production of cholesterol and its effect on BD production have been reported. Lichtstein et al47 have recently reported studies examining BDs in homogenates of mammalian AC cells. These studies used techniques that cannot be compared directly with our own (eg, the study of intracellular content rather than secreted material and the use of tritiated labels and monitoring of radioactivity rather than analytical techniques targeted toward specific steroids) and suggested that cholesterol side-chain cleavage may be a step in the biosynthesis of mammalian BD. However, the present study is consistent with earlier amphibian evidence indicating that BDs can be synthesized from steroids that may originate from cholesterol but are not in the side-chain cleavage pathway.

The present study has demonstrated that plasma MBG shows important similarities (eg, chromatography, UV absorbance, and MBG antibody cross-reactivity) with material added to defined cell culture medium by Y-1 AC cells and the material present in normal rat adrenal glands. These observations suggest that plasma MBG material may be of AC origin. We show that the AC material is produced, in the absence of exogenous cholesterol, by a pathway inhibited by the HMG-CoA reductase inhibitor mevastatin, a drug that inhibits cholesterol neogenesis. LDL cholesterol appears to increase production when cholesterol neogenesis is inhibited, but modified cells in which cholesterol side-chain cleavage is severely curtailed do not show reduced MBG production. These observations suggest that AC bufadienolide is a steroid whose biosynthesis is independent of cholesterol side-chain cleavage.

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