Importance of Plasma Angiotensin Concentrations in a Comparative Study of Responses to Angiotensin in the Maturing Newborn Lamb

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SUMMARY Plasma angiotensin concentrations were measured in a longitudinal study of the vascular, renal, and adrenal responses to infusions of angiotensin II (AII) in the maturing newborn lamb. Basal plasma concentrations of angiotensin increased with age and correlated with the rising arterial pressure that occurred with maturation. However, age was a stronger determinant of arterial pressure than was plasma angiotensin concentration. For any given dose of AII per kilogram of body weight, the actual plasma angiotensin concentration achieved increased as the lambs matured and gained weight. Therefore, a comparative study of biologic responses to AII in maturing animals must be based on actual plasma angiotensin concentrations achieved rather than on dose of AII infused per kilogram of body weight. When analyzed on the basis of actual plasma angiotensin concentration, the increase in arterial pressure and the suppression of plasma renin activity in response to increasing plasma angiotensin concentrations did not differ significantly as the lambs matured. However, the increment in plasma aldosterone concentrations in response to increasing plasma angiotensin concentrations was diminished in immature lambs (less than 18 days) when compared to the aldosterone responses in the same lambs at older ages. (Hypertension 3 (suppl II): II-18-II-24, 1981)

KEY WORDS Ontogeny of the renin-angiotensin-aldosterone system • converting enzyme activity • newborn lamb

DATA from a number of studies have suggested that the pressor response to angiotensin II (AII) is diminished in the immature organism compared to the adult organism.1-4 This would be consistent with the low arterial pressure5 and the elevated plasma renin activity (PRA) and plasma angiotensin concentrations reported in immature organisms.6-10 However, these studies have not been longitudinal in design and have not been carried out under conditions of controlled sodium intake.

We recently reported that the pressor response to AII did not change in a longitudinal study of newborn lambs raised on a constant sodium intake from birth to 8 weeks of age.10 We also reported no consistent effect of age on the response of PRA or plasma aldosterone concentration to AII.20 As in previous studies, these conclusions were based on a comparison of responses obtained at different ages during intravenous infusions of AII at doses that were standardized by body weight.

To determine whether comparable plasma concentrations of angiotensin were achieved during the AII infusion at each age as the lambs matured, we assayed plasma angiotensin concentrations. We report here the plasma angiotensin concentrations that resulted from the AII infusions as the lambs matured and an analysis of the pressor, renin, and aldosterone responses based on these angiotensin concentrations.

Materials and Methods

Materials and methods have been described in detail previously.20 In brief, seven newborn female Dorset lambs were weaned from their ewes and fed...
160 cc per kilogram per day of a standard lamb milk replacer (Land o Lakes milk replacer, Webster City, Iowa, sodium content = 54 mEq/liter) in order to insure a constant sodium intake (8.6 mEq/kg/day). This sodium intake is approximately three times that provided by ewe's milk on a calorie for calorie basis (calculations based on information on content of ewe's milk provided by Dr. Robert Jenness, University of Minnesota). Catheters were placed in the carotid artery and jugular vein or in the femoral artery and vein in lambs 3 to 4 days of age under general anesthesia and were filled daily with a solution of heparin (100 U/ml) in saline. In a longitudinal manner at periodic intervals from birth to 8 weeks of age, the lambs were infused with AII amide (Asn¹, Val⁴ angiotensin II, Ciba, M-1229) in cumulative, sequential doses of 0, 2, 4, 10, 20, and 40 pmole/kg/min. via a Harvard infusion pump (Harvard Infusion/Withdrawal Pump #901 Harvard Apparatus Company, Millis, Massachusetts).

During the infusion period, arterial pressure was recorded directly (Gould Recorder 2400, Gould Inc., Cleveland, Ohio). Prior to and at the end of each infusion period, 15 cc of blood was withdrawn from the arterial catheter into a syringe containing 15 mg Na₃EDTA and immediately placed in a chilled plastic tube and centrifuged at 4°C at 16,000 rpm X 20 minutes. Aliquots of plasma were subsequently removed and stored frozen at -76°C for subsequent analysis of PRA and plasma concentration of angiotensin and aldosterone. In addition, 2 cc of blood was withdrawn at the beginning of each study and placed in heparin (20 U/cc). The plasma from this sample was also frozen and later assayed for converting enzyme activity. All withdrawn blood was immediately replaced with an equal volume of warmed sheep blood in citrate-phosphate-dextrose buffer to insure a constant intravascular volume. Prior to each study period, a 24-hour urine sample was collected for determination of electrolyte excretion. Dexamethasone (0.25 mg) was administered intravenously the night before the infusion studies and again on the morning of the infusion to suppress endogenous ACTH secretion.

Assays

Urinary sodium concentration was determined by flame photometry using lithium as the internal standard. PRA was assayed by a modification of the radioimmunoassay method of Sealey and Laragh as previously described, and plasma aldosterone concentration was assayed by the method of Bühler et al.

Plasma angiotensin was assayed by radioimmunoassay using a modification of the method of Freedlender and Goodfriend. Angiotensins were extracted from plasma using 0.7 g AG50W-X₄, hydrogen form (Bio-Rad Laboratories, Richmond, California), per cc of plasma for 60 minutes on a rotator at 20 rpm at 4°C. After the plasma was discarded, the resin was washed successively with 1 cc water and 1 cc methanol:water (1:1, vol:vol) and the supernate discarded. Angiotensins were eluted from the resin with two washes of 2.0 cc ammonia:methanol (4:6, vol:vol) for 15 minutes. The eluates were dried under N₂ at 40°C and radio-immunoassayed. The antiserum used cross-reacted equally with AII and AIII but demonstrated less than 0.1% cross reactivity with other angiotensin degradation products that are known to be devoid of biological activity.

Plasma angiotensin-converting enzyme activity was estimated by radioenzymatic assay with a commercial kit (Angiotensin converting enzyme activity radioassay system, Ventrex, Portland, Maine) which is based on the hydrolysis of [³H] Hip - Gly - Gly to [³H] hippuric acid + Gly - Gly by converting enzyme and the partitioning of [³H] hippuric acid at low pH into ethyl acetate.

Statistics

For the relationships among age, weight, sodium excretion, PRA, and plasma angiotensin concentration, models were hypothesized in which the relationships among age, PRA, and sodium excretion were exogenous to the main model (the direct effects of age, sodium excretion, and PRA on plasma angiotensin concentrations). To accommodate the reality of related independent variables in the main model, a two-stage least squares regression was used to identify and remove exogenous effects (SYSREG Procedure, Statistical Analysis System).

The effects of age or weight and AII dose on plasma angiotensin concentrations were examined using hypothesized models expressed as linear equations (fitted curve of actual data) with age group or weight group and plasma angiotensin concentration as independent variables (GLM procedure Statistical Analysis System, SAS System, Inc., Cary, North Carolina; version 79.3). An initial model was constructed hypothesizing the effects on plasma angiotensin concentration due to: 1) age group or weight group; 2) dose of AII; and 3) a term representing the interaction of age group or weight group and dose of angiotensin II. The terms were entered in the model in the above order to provide: 1) an adjustment of variation from age group to age group in overall plasma angiotensin concentration; 2) a test of the main effects of dose of AII; and 3) a final test (homogeneity of slopes) of the possible interaction of age group or weight group and dose of AII infused.

In a similar fashion, the effects of age group and plasma angiotensin concentration on arterial pressure, PRA, and plasma aldosterone concentration were analyzed. The initial model hypothesized the effects on each dependent variable for 1) age group; 2) plasma concentration of angiotensin; and 3) a term representing the interaction of plasma concentration of angiotensin and age group. These terms were likewise entered in the model in the above order to provide: 1) an adjustment for variation in overall plasma angiotensin concentrations from age group to age...
group; 2) a test of the main effects of plasma angiotensin concentration; and 3) a final test (homogeneity of slopes) of the possible interaction of plasma angiotensin concentration and age groups.

Levels of significance for all analyses were accepted when \( p < 0.05 \).

**Results**

**Urinary Sodium Excretion**

Although 24-hour urinary sodium excretion decreased with age \( (r^2 = 0.210; p = 0.008) \), only three observations accounted for this relationship. If the three observations greater than 12 mEq/kg/day are excluded, urinary sodium excretion can no longer be shown to diminish significantly with age \( (p = 0.110, \text{fig. 1}) \). These three observations were made in two lambs at less than 17 days of age.

**Basal Plasma Angiotensin Concentration**

Basal plasma angiotensin concentration was associated with both age \( (r^2 = 0.289; p = 0.0015) \) and PRA \( (r^2 = 0.157; p = 0.025) \). The relationship between basal angiotensin concentration and age remained apparent even after the data were corrected for variations attributable to sodium excretion and PRA (two-stage least squares, \( p = 0.0023 \)).

**Basal Mean Arterial Pressure**

The increase in basal mean arterial pressure (MAP) was associated with an increase in age \( (r^2 = 0.510; p < 0.0001) \), weight \( (r^2 = 0.420; p < 0.0001) \) and basal plasma angiotensin concentration \( (r^2 = 0.138; p = 0.037) \) and with a decrease in urinary sodium excretion \( (r^2 = 0.212; p = 0.008) \). When the effect of basal plasma angiotensin concentration was accounted for, MAP still correlated with age \( (p < 0.0001) \). However, when the effect of age was removed, basal MAP was no longer significantly associated with basal plasma angiotensin concentration. Similarly, when the effect of sodium excretion was removed, MAP still was associated with age \( (r^2 = 0.533; p < 0.0001) \) whereas when the effect of age was removed, MAP could no longer be shown to correlate with sodium excretion. Thus, age appeared to be the strongest determinant of MAP.

**Angiotensin II Dose and Plasma Angiotensin Concentration**

Plasma angiotensin concentrations increased in response to All infusions in all age groups (fig. 2). However, the magnitude of the induced rise in plasma angiotensin concentrations in the youngest lambs (5-11 days and 12-18 days) was significantly less than that for older lambs. This discrepancy was also evident when the plasma angiotensin concentration achieved during the All infusion was examined as a function of weight (data not shown). Thus, for any specified dose of All infused, the plasma concentration of angiotensin increased as the lambs grew older and gained weight.

**Responses of Arterial Pressure, Plasma Renin Activity, and Aldosterone Concentration to Angiotensin**

Because the concentrations of angiotensin achieved in the plasma in response to the All infusion differed as a function of age, responses of MAP, PRA, and plasma aldosterone concentration were analyzed over the plasma concentrations of angiotensin actually shared in common by all age groups. The MAP increased in response to increasing angiotensin concentrations in all age groups. However, age had no significant effect on the slope of this response \( (p = 0.739; \text{fig. 3}) \). Similarly, PRA decreased in response to increasing plasma angiotensin concentrations in all age groups. The slopes of the fitted response curves for each age group also did not differ significantly \( (p = 0.9533; \text{fig. 4}) \).

In contrast, age did influence the plasma aldosterone response to increasing plasma angiotensin concentrations. The youngest age groups (5-11 days and 12-18 days) had flattened aldosterone response curves when compared with the older three age groups (fig. 5). In fact, the slope of the fitted aldosterone response curves for these two groups could not be shown to be significantly different from zero \( (p = 0.563 \text{ for lambs 5-11 days old and } p = 0.2923 \text{ for lambs 12-18 days old}) \) whereas lambs in the older age groups had significant increases in plasma aldosterone concentration. The aldosterone response curve for lambs 28-31 days of age was steeper than those of the other age groups. When the slope of the aldosterone response curve in this age group was compared to the response curve of the lamb at all older ages, the difference was significant \( (p = 0.0313) \).
Plasma Angiotensin Concentrations In The Lamb/Wilson et al.

**Discussion**

We previously reported that age was a strong determinant of basal MAP in this experimental lamb model of the ontogeny of blood pressure. We now determined that plasma angiotensin concentration also increased with age. Therefore the influence of age on arterial pressure might have been mediated by angiotensin. However, after adjustment of the data for variations attributable to plasma angiotensin concentration, the association between age and basal MAP remained highly significant. Thus, age itself appears to have a strong independent influence on arterial pressure.

**Plasma Converting-Enzyme Activity**

Plasma converting-enzyme activity was not associated with age ($r^2 = 0.0002; p = 0.929$), weight ($r^2 = 0.043; p = 0.270$), or angiotensin concentration ($r^2 = 0.042; p = 0.277$). Mean plasma-converting-enzyme activity for all lambs at all ages was $82.6 \pm 14.7$ nmoles/min/ml.
Our data demonstrate that the plasma concentration of angiotensin achieved at any infused dose of AII per kilogram increased as the lambs matured. At least three possible mechanisms might account for this: 1) a decrease in angiotensinase activity occurring with maturation; 2) a decrease in plasma volume per kilogram of body weight occurring with growth, thereby yielding greater angiotensin concentrations for any given dose of AII per kilogram; and/or 3) an increase in plasma albumin concentration occurring with maturation, thereby leading to greater binding of AII and decreased degradation. A decrease in the activity of plasma angiotensinases seems unlikely in view of the fact that most degradative enzyme systems increase rather than decrease in activity with maturation. Since we did not measure plasma volumes or plasma albumin concentrations in these lambs as they matured, we cannot comment on the influence of plasma volume or protein binding on the plasma angiotensin concentrations that we observed.

Regardless of the mechanism responsible for the greater angiotensin concentrations achieved in the older animals in response to the AII infusion, it is clear that a comparison of biologic responses to angiotensin in the maturing organism must be based on actual angiotensin concentrations. Furthermore, since biologic dose-response curves generally are sigmoid and not linear, a comparison of biologic responses to angiotensin is best carried out over angiotensin concentrations shared by all groups. We therefore analyzed the various responses over the angiotensin concentrations achieved in common by all age groups.

In taking this approach, no difference in the MAP response to angiotensin could be defined among the various age groups. Furthermore, PRA was suppressed in a similar fashion in all age groups. Therefore, no difference in either the pressor response or in the negative feedback inhibition of AII on renin release could be defined among the age groups. Of course, a comparison of PRA responses of this sort is based on the assumption that the half-life for renin does not change appreciably as a function of age. These findings support our previous conclusions which were based on pressor and renin responses as a function of dose of AII per kilogram of body weight, but they differ from the findings of previous investigations. However, previous studies were not controlled for sodium intake, were not longitudinal in design, and were based on vascular responses either to fixed doses of AII or to fixed doses of AII per kilogram of body weight rather than on the actual concentrations of angiotensin achieved in the circulation.

In contrast, the aldosterone responses to angiotensin did depend on age when analyzed as a function of plasma angiotensin concentration. Lambs at the youngest ages (less than 18 days) had significantly diminished aldosterone responses in comparison to the same lambs at older ages (greater than 28 days). Furthermore, the lambs at 28–31 days of age had significantly steeper aldosterone responses to angiotensin. By analyzing the data on the basis of the infused dose of angiotensin per kilogram, we previously observed a steeper response in lambs at 28–31 days of age compared with the other age groups. However, we had not been able to appreciate a diminished aldosterone response in the lambs at a younger age. By comparing aldosterone responses over plasma angiotensin concentrations shared by all age groups, this difference became apparent. The finding of diminished aldosterone responses in the lambs at the youngest ages would be consistent with the finding of Alexander et al. that the zona glomerulosa of fetal lamb adrenals was unresponsive to AII. Our findings do not support the conclusion of Siegel that aldosterone responses to AII are enhanced in immature lambs. However, Siegel's studies were carried out in lambs with diminished sodium intake in comparison to the ewes, and the conclusions were not based on a comparison of plasma angiotensin concentrations achieved. A low sodium intake in itself would lead to higher endogenous concentrations of angiotensin and would be expected to enhance the aldosterone response to exogenous angiotensin.
It is not clear by what mechanisms aldosterone responses to angiotensin might be diminished in the immature lamb. Pernollet et al. reported that AI receptors in the zona glomerulosa of the newborn rabbit increase with maturation. It is possible that a similar mechanism might apply in the lamb and could account for the diminished aldosterone response that we observed in the youngest two age groups.

The negative association between age and sodium excretion was due to high sodium excretion in three 24-hour urine collections in two lambs at less than 17 days of age. Since the lambs were receiving a constant intake of milk replacer, this high sodium excretion may be a reflection of immaturity of the renal tubular capacity to conserve sodium. Such renal immaturity has been described in the kidney of the fetal lamb. When these three observations are excluded, sodium excretion did not change significantly with age. It is unlikely that these two lambs would have influenced our conclusions about the aldosterone response to angiotensin since an increased urinary excretion of sodium in the face of a constant sodium intake would have been expected to enhance the response of the zona glomerulosa to angiotensin.

In contrast, we found a diminished response in the lambs at less than 18 days of age.

We were unable to document a consistent change in plasma converting-enzyme activity occurring with age. Converting-enzyme activity has been reported to increase during gestation in the fetal lamb and rabbit and postnatally in the lamb and in the rat. However, it appears that no major change in plasma converting-enzyme activity occurs postnatally in the lamb. The increase in basal angiotensin concentrations that we observed with age cannot therefore, be explained by an increase in plasma converting-enzyme activity. It is still possible, however, that changes in converting enzyme activity do occur with maturation but are limited to the endothelial cells within the vasculature and therefore are not reflected in plasma converting-enzyme activity.

In conclusion, in the maturing newborn lamb, plasma concentrations of angiotensin achieved in response to doses of AI of ALI that are standardized on the basis of body weight depend on both the age and weight of the animal. Therefore, a comparison of responses to AII must be made on the basis of actual plasma angiotensin concentrations. With this method of comparison, plasma aldosterone responses to angiotensin appear to be diminished in immature lambs. Aldosterone II-mediated pressor responses and negative feedback suppression of renin release do not appear to change with maturation.

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T A Wilson, D L Kaiser, E M Wright, Jr, E M Ortt, A E Freedlender, M J Peach and R M Carey

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