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Original articles – Fetus

Cholinergic signal activated renin angiotensin system associated with cardiovascular changes in the ovine fetus

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Abstract

Aim: Cholinergic regulation is important in the control of cardiovascular and endocrine responses. The mechanisms behind cardiovascular responses induced by cholinergic activation are explored by studying hormonal systems, including renin-angiotensin and vasopressin (VP).
Results: In chronically prepared fetal sheep, intravenous infusion of the cholinergic agonist carbachol increased fetal systolic, diastolic, and mean arterial pressure accompanied with bradycardia at near-term. Although intravenous administration of carbachol had no effect on plasma VP concentrations, this agonist increased angiotensin I and angiotensin II levels in fetal plasma. Fetal blood values, including sodium, osmolality, nitric oxide, hemoglobin, and hematocrit were unchanged by intravenous carbachol.
Conclusion: Cholinergic activation by carbachol controls fetal blood pressure and heart rate *in utero*. An over-activated fetal renin-angiotensin-system (RAS) is associated with changes in vascular pressure following intravenous administration of carbachol, indicating that the cholinergic stimulation-mediated hormonal mechanism in the fetus might play a critical role in the regulation of cardiovascular homeostasis.

Keywords: Cholinergic activation; fetal RAS; osmoregulation; vasopressin.

Introduction

Nicotine, a cholinergic agonist, is one of major chemicals affecting fetuses exposed to smoking during pregnancy. Cholinergic mechanisms play an important role in the control of cardiovascular, body fluids, and endocrine systems in the body [4, 6, 11, 21]. Numerous studies have demonstrated that administration of the cholinergic agonist carbachol can produce changes in blood pressure in adults [9, 19]. Recently, it was shown that alteration of fetal development by environmental insults may impact on postnatal health and increased risks of diseases during adult life, including hypertension and metabolic illness [9, 19]. Therefore, it is important to study the functional development of the fetal cardiovascular and hormonal systems in response to cholinergic signals *in utero*.

Although the peripheral carbachol-mediated cardiovascular regulation in fetuses is still largely unknown, previous studies has shown that central muscarinic modulation by administration of pilocarpine could cause a very short period of hypotension subsequently followed by a significant increase in systolic, diastolic and pulse pressures in the ovine fetus [20]. One recent report demonstrated that central injection of carbachol in the fetus can cause an increase of mean arterial pressure [18]. In the present study, we determined the influence of carbachol in the peripheral circulation on fetal cardiovascular and hormonal responses in the fetus at near-term.

Our study of hormonal responses induced by cholinergic activation focused in the present study on the renin-angiotensin-system (RAS) and on vasopressin (VP). There are functional relations between these hormones and cholinergic activation, and both angiotensin and VP peptides contribute to the control of cardiovascular systems [14, 17]. For example, renin activity was significantly increased by cholinergic mechanisms and RAS was shown to mediate carbachol produced pressor responses in adult animals [14]. However, whether and to which extent that peripheral cholinergic stimulation may affect expression of angiotensin I, angiotensin II, and VP in the fetus is still unknown. Thus, the experiments in the present study were designed to determine fetal RAS and VP activity associated with cardiovascular responses. Information gained might be important not only to further understanding the fetal functional development, but also to adding knowledge for disease development and prevention related to alterations of cholinergic activation *in utero* during pregnancy.

Materials and methods

Animals

Time-dated pregnant ewes with fetuses (127±3 days of gestation on the study day; term: 145 days) were used. Animals were housed

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in individual study cages and in a light-controlled room (12:12-h light-dark cycle) with food and water provided *ad libitum*. All surgical and experimental procedures had been approved by the Institute's Animal Care Committee.

Surgical preparation

Anesthesia was injected with ketamine hydrochloride (20 mg/kg IM), and general anesthesia was maintained with 3% isoflurane and 1 L/min oxygen. The uterus was exposed by midline abdominal incision, and a small hysterotomy was performed to provide access to a fetal hind limb, as reported previously [24, 25]. Polyethylene catheters (1.0 mm ID, 1.8 mm OD for the fetus) were placed in the maternal and fetal femoral vein and artery and advanced to the inferior vena cava and abdominal aorta, respectively. The fetus was then returned into the uterus, and the hysterotomy was closed in two layers. All catheters were externalized to the maternal flank and placed in a cloth pouch. Immediately preoperatively and twice daily during the initial two days of recovery, gentamicin (8 mg) and oxacillin (30 mg) were administered intravenously to the fetus, and gentamicin (70 mg) and oxacillin (1 g) were injected intravenously into the ewe. Animals recovered for four days after surgery.

Experiments for blood values

Animals were divided into control (n = 5) and experimental (n = 5) groups with computer-randomized selection. On the testing day, sheep were allowed a period of 60–100 min to be acclimatized to the testing room. When animal heart rates and arterial pressures appeared to be stable, a 60-min baseline was followed by 15 min of intravenous infusion and an additional 120 min period. Maternal and fetal blood samples were collected at −30 and −5 min before the intravenous infusion of carbachol, and at 5, 10, 30, 90 min after infusion of carbachol (5 μg/kg) or vehicle (0.9% NaCl solution). The dose was selected according to previous reports [16, 22] and our preliminary testing. All fetal blood samples (3 ml/sample) were replaced with equivalent volumes of heparinized maternal blood withdrawn before the study, and all maternal blood samples were replaced with equivalent volumes of isotonic saline. Blood samples were withdrawn from the fetal and maternal arterial catheters for measurements of blood PO_2, PCO_2, hemoglobin (Hb), pH, electrolyte concentrations by a Nova eleven-electrode analyzer (Nova Biomedical, Waltham, MA). Plasma osmolality was measured by using freezing point depression on an advanced digimatic osmometer (Model 3MO, Advanced Instruments, Needham Heights, MA).

Cardiovascular experiments

Beginning at time 0 (the beginning of intravenous infusions), carbachol (5 μg/kg, in 10 mL 0.9 NaCl; Sigma, St. Louis, MO) was infused intravenously to the experiment fetus over 15 min. For the control animals, the same volume of isotonic saline was infused intravenously. Maternal and fetal blood pressures were monitored during the testing period by means of a Power-Lab Physiological Recorder (AD Instruments, Australia). Systolic, diastolic, and mean arterial pressure, as well as heart rate was determined by computer analysis of waveforms by utilizing the Chart 5 software (AD Instruments, Australia).

Endocrine experiments

Maternal and fetal blood samples were collected into ice-cold tubes containing lithium heparin during the baseline and study periods. Blood samples for hormone assays were centrifuged immediately. Samples were then stored at −20°C before assays. All hormones and biochemicals were measured by radioimmunoassay or biochemical assay (Hua Ying Bio-tech Co, Beijing). The assay experiments and data were handled in a blind manner.

Data analysis

Statistical analysis was preformed with repeated-measures ANOVA (MANOVA). Comparisons before and after treatment were determined with Tukey post-hoc test. All data are expressed as means ± SEM, and statistical significance was set at $P < 0.05$.

Table 1 Fetal arterial values before and after intravenous infusion of vehicle or carbachol into the fetus.

		Time after carbachol infusion			
		Baseline	5 min	30 min	90 min
Hct (%)	(1)	28.01 ± 1.15	28.05 ± 1.65	25.67 ± 2.62	27.17 ± 1.83
	(2)	27.33 ± 0.33	26.67 ± 0.88	27.33 ± 0.33	26.00 ± 1.15
Hb (g/dL)	(1)	8.47 ± 0.14	8.37 ± 0.33	8.08 ± 0.45	8.03 ± 0.34
	(2)	8.47 ± 0.21	8.33 ± 0.09	8.43 ± 0.12	8.00 ± 0.15
pH	(1)	7.40 ± 0.01	7.38 ± 0.01	7.38 ± 0.01	7.38 ± 0.01
	(2)	7.40 ± 0.01	7.40 ± 0.00	7.41 ± 0.01	7.41 ± 0.01
PCO_2 (mm Hg)	(1)	21.21 ± 0.59	48.43 ± 1.03	48.50 ± 1.47	48.37 ± 2.30
	(2)	48.09 ± 0.71	49.63 ± 1.64	47.20 ± 0.74	47.31 ± 0.59
PO_2 (mm Hg)	(1)	21.21 ± 0.59	20.70 ± 0.83	20.81 ± 0.87	21.43 ± 0.75
	(2)	21.82 ± 1.66	21.26 ± 1.01	22.97 ± 1.96	21.87 ± 0.91
Osmolality (mosmol/kg)	(1)	301.00 ± 13.58	306.60 ± 5.40	301.80 ± 4.37	300.25 ± 12.63
	(2)	300.33 ± 1.20	304.33 ± 4.91	302.00 ± 1.53	305.33 ± 1.45
Na^+ (meq/L)	(1)	136.68 ± 0.86	135.73 ± 1.26	137.05 ± 1.30	136.17 ± 1.05
	(2)	138.20 ± 1.35	138.00 ± 1.55	138.17 ± 1.68	138.70 ± 1.62
K^+ (meq/L)	(1)	3.97 ± 0.23	3.93 ± 0.24	3.60 ± 0.24	3.95 ± 0.13
	(2)	4.08 ± 0.80	3.86 ± 0.59	3.94 ± 0.74	3.76 ± 0.52

Values are means ± SEM. (1), iv infusion of carbachol (5 μg/kg); (2), iv infusion of vehicle (0.9% NaCl).
Hct = hematocrit, Hb = hemoglobin.

Table 2 Material arterial values before and after intravenous infusion of vehicle or carbachol into the fetus.

		Time after infusion carbachol			
		Baseline	5 min	30 min	90 min
pH	(1)	7.46±0.01	7.44±0.02	7.47±0.01	7.45±0.01
	(2)	7.47±0.01	7.47±0.01	7.47±0.01	7.48±0.03
PCO_2 (mm Hg)	(1)	24.33±0.98	24.97±1.23	25.93±1.27	24.87±1.30
	(2)	24.01±1.51	26.61±0.21	23.91±1.15	25.72±1.25
PO_2 (mm Hg)	(1)	116.45±2.34	117.35±2.23	115.90±2.71	115.15±2.23
	(2)	116.38±1.53	113.02±2.40	117.76±6.57	115.13±1.23
Hct (%)	(1)	30.60±0.81	30.60±1.21	28.40±2.23	30.60±1.60
	(2)	28.00±0.58	29.00±1.00	28.67±1.20	28.33±1.33
SO_2 (%)	(1)	98.46±0.23	98.34±0.36	97.42±0.75	98.12±0.41
	(2)	98.53±0.32	98.05±0.45	98.73±0.15	98.47±0.26
Hb (g/dL)	(1)	10.18±0.32	10.20±0.40	9.42±0.75	10.20±0.54
	(2)	9.97±0.19	10.20±0.20	9.87±0.32	9.73±0.37
Na^+ (mmol/L)	(1)	142.66±0.45	144.28±0.60	144.46±0.70	144.20±0.67
	(2)	145.20±1.01	144.65±1.15	145.03±1.75	146.47±1.19
K^+ (mmol/L)	(1)	3.87±0.03	3.90±0.10	3.57±0.28	3.99±0.06
	(2)	3.99±0.07	4.04±0.03	3.80±0.13	4.01±0.14
Glu (mmol/L)	(1)	4.49±0.08	3.39±0.09	3.06±0.26	3.00±0.48
	(2)	4.38±0.19	4.63±0.43	4.45±0.49	4.47±0.54
Osmolality (mosmol/kg)	(1)	301.20±2.75	302.00±3.79	308.00±2.77	302.80±2.22
	(2)	301.67±3.18	302.33±1.85	303.00±2.08	307.50±0.50

Values are means±SEM. (1), iv infusion of carbachol (5 μg/kg); (2), iv infusion of vehicle (0.9% NaCl).
Hct=hematocrit, Hb=hemoglobin, Glu=glucose.

Results

Blood values

There was no significant difference in fetal arterial blood pH, PO_2, PCO_2, Hb, and hematocrit (Hct) before or after intravenous infusion of carbachol (Table 1). For both the control and the experiment fetuses, intravenous infusion of carbachol or vehicle had no effect on plasma osmolality levels ($P>0.05$) in the fetuses. Fetal blood K^+ and Na^+ concentrations were not changed between the control and the experimental groups. All arterial values were within normal ranges and did not vary significantly between the control and experimental groups (all $P>0.05$). In the ewes, intravenous infusion of carbachol or vehicle into the fetuses had no effect on plasma osmolality ($P>0.05$). Maternal blood Na^+ and K^+ concentrations, blood pH, PO_2, and PCO_2 were not significantly changed between the control and experimental groups (all $P>0.05$, Table 2).

Cardiovascular responses

There was no significant difference in maternal systolic, diastolic, mean arterial pressure, and heart rate between the control and experimental groups ($P>0.05$, Table 3). Fetal systolic, diastolic, and mean arterial pressure were first decreased for a short time, and then increased following intravenous administration of carbachol (Figure 1). The increased fetal blood pressure returned to baseline within 30 min after infusion. Fetal heart rate was significantly decreased ($P<0.01$) after administration of carbachol (Figure 2).

Table 3 Maternal blood pressure and heart rate before and after intravenous of carbachol into the fetus.

		Time after carbachol infusion				
		Baseline	5 min	15 min	30 min	90 min
SP (mm Hg)	(1)	114.73±3.69	116.79±4.23	115.49±4.29	115.52±4.88	118.79±4.79
	(2)	115.15±2.58	116.23±1.56	116.25±2.35	115.68±4.33	116.26±4.72
DP (mm Hg)	(1)	82.75±3.10	83.21±2.91	82.09±4.07	84.31±4.27	82.96±3.25
	(2)	82.76±3.25	83.20±2.81	82.50±3.58	82.41±4.03	82.33±3.36
MAP (mm Hg)	(1)	95.92±2.62	96.46±2.77	95.71±3.74	98.17±3.96	97.52±3.08
	(2)	95.08±2.36	95.47±3.12	95.26±3.15	97.02±3.36	96.25±2.63
HR (bpm)	(1)	121.75±7.73	125.11±8.77	123.79±7.84	124.43±7.43	125.78±7.73
	(2)	120.39±6.58	125.08±7.33	125.79±6.86	124.53±7.53	126.13±8.06

Values are means±SEM. (1), iv infusion of carbachol (5 μg/kg); (2), iv infusion of vehicle (0.9% NaCl).
SP=systolic pressure, DP=diastolic pressure, MAP=mean arterial pressure.

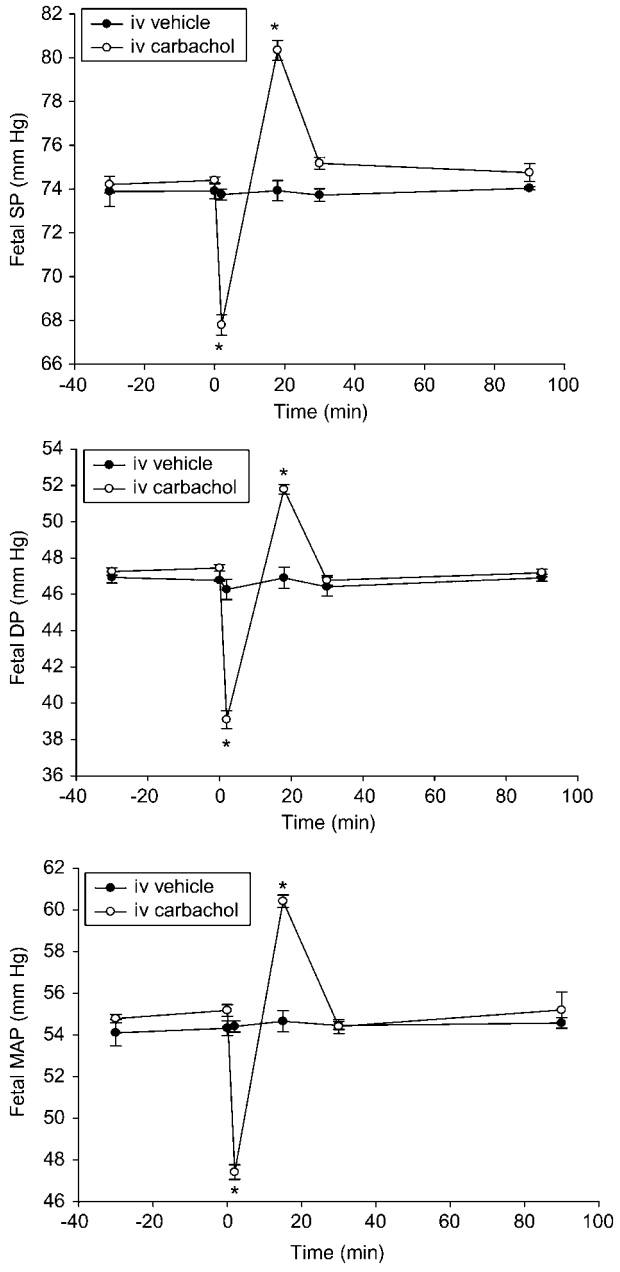

Figure 1 Effect of intravenous injection of vehicle or carbachol on the preterm fetal systolic pressure (SP, mm Hg), diastolic pressure (DP, mm Hg), and mean arterial pressure (MAP, mm Hg). 0 min, time of intravenous injection. *P < 0.05 (statistical significant) compared with the baseline level (n = 5/each group).

Fetal plasma hormones

Although there was no difference in fetal plasma VP concentrations before and after intravenous infusion of carbachol into the fetus and between the control and experimental groups (Table 4), fetal plasma Ang I and Ang II levels were significantly increased (from 1.75 ± 0.23 and 108.61 ± 3.15 to 2.72 ± 0.31 ng/mL and 127.98 ± 7.20 pg/mL, respectively) within 5 min after intravenous carbachol (Figure 3).

Discussion

Although cholinergic systems are important in the control of cardiovascular homeostasis [1–3, 5], limited data existed on the functional development of cholinergic mechanisms in regulation of fetal vascular and endocrine systems. In the present study, we found that cholinergic activation by intravenous carbachol affected fetal blood pressure in association with an increase of fetal RAS activity.

In the chronically prepared ovine fetuses, intravenous administration of the cholinergic agonist induced changes of fetal blood pressure at near-term in the present study. The involvement of the autonomic nervous system in the control of cardiovascular responses following administration of cholinergic agents has been investigated in adult animals [15, 23]. Carbachol could increase sympathetic nervous system activity, which increases blood pressure. The increased blood pressure activates a baroreflex-mediated bradycardia by increasing vagal tone [12]. We observed a short period of decrease of fetal blood pressure accompanied by bradycardia immediately after intravenous infusion of carbachol in the present study. Although a previous study found fetal cardiovascular responses caused by the muscarinic agonist pilocarpine [20], the present study was the first to demonstrate that carbachol could induce a short decrease of fetal blood pressure (this could be due to immediate decrease of fetal heart rate), followed by an increase of fetal systolic, diastolic, and mean arterial pressure at near-term pregnancies. A question raised immediately was what were the mechanisms for the cholinergic stimulation induced fetal blood pressure responses?

Notably, the main trend in the change of fetal blood pressure by carbachol in the present study was an increase of arterial pressure. Many factors may contribute to cholinergic stimulation-induced cardiovascular responses [8, 13]. Besides autonomic control mechanisms, endocrine contribution also has an important role. For example, RAS medi-

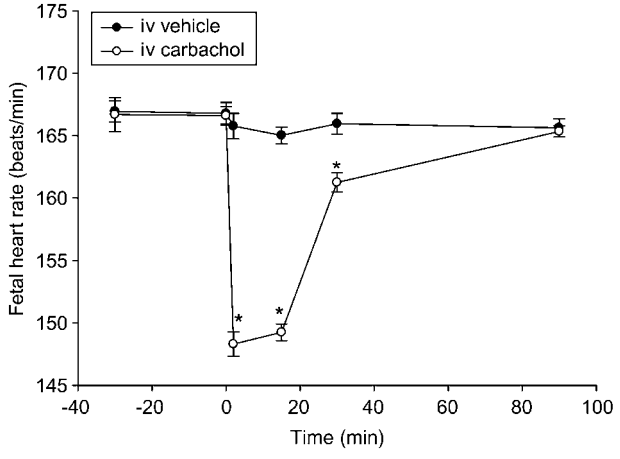

Figure 2 Effect of intravenous injection of vehicle or carbachol on the preterm fetal heart rate (in beats/min). 0 min, time of intravenous injection. *P < 0.05 (statistical significant) compared with the baseline level (n = 5/each group).

Table 4 Fetal plasma VP values before and after intravenous infusion of carbachol into the fetus.

		Time after carbachol infusion			
		Baseline	5 min	30 min	90 min
AVP (pg/mL)	(1)	10.91 ± 0.89	11.10 ± 0.89	9.91 ± 2.20	10.71 ± 0.65
	(2)	9.65 ± 0.55	9.35 ± 0.65	9.36 ± 1.00	9.49 ± 0.37

Values are means ± SEM. (1), iv infusion of carbachol (5 μg/kg); (2), iv infusion of vehicle (0.9% NaCl).
VP = vasopressin.

ated carbachol produced pressor responses [24]. Renin release could be regulated by cholinergic mechanisms [14]. In the present study, angiotensin I and II concentrations were significantly increased in the fetus following infusion of intravenous carbachol suggesting that cholinergic signal-increased higher blood pressure may partially be mediated by angiotensin mechanisms. Further pharmacological studies using angiotensin II antagonists might yield additional information as to whether direct or indirect mechanisms increased RAS activity in the circulation. To the best of our knowledge, this study was the first to provide evidence in the fetus that cholinergic stimulation can activate RAS that may contribute to carbachol-induced pressor responses also. It also demonstrates a link between cholinergic activation and angiotensinergic systems that developed and became functional *in utero* near-term.

In addition to angiotensin II, the cholinergic agonist carbachol is also well known in body fluid balance [10, 13]. Injection of carbachol in conscious animals can increase water intake and stimulate release of hypothalamic VP [7], resulting in changes of vascular volume and/or vessel tone. Previous studies have shown that an increase of VP levels in the circulation is a potent stimulus for pressor responses [19]. However, in the present study, we did not observe any change of plasma VP concentrations in the fetus treated with carbachol. Therefore, we can exclude the possibility of VP mechanisms that may be involved in carbachol-increased fetal blood pressure.

As mentioned above, administration of carbachol into animals can induce drinking [10, 13] and may influence osmoregulation, which might cause changes of body fluids and vascular volume. In the present study, we monitored blood electrolytes levels and plasma osmolality in both maternal and fetal sheep during the testing periods. There was no change of blood sodium concentrations and plasma osmolality in the mother and fetus following administration of carbachol. In addition, fetal Hct levels were unchanged by intravenous infusion of carbachol. Together, the data did not support the possibility that carbachol may cause change of fetal body fluids in contribution to the increased blood pressure.

Conclusion

The finding showed that cholinergic activation by peripheral carbachol was involved in the control of fetal blood pressure and heart rate. Interestingly, in investigation of hormonal and chemical mechanisms that contribute to cholinergic signal-stimulated cardiovascular responses, we found an over-activated fetal RAS associated with changes of vascular pressure following intravenous administration of carbachol. This provides new evidence that both cholinergic and angiotensinergic systems have functionally developed *in utero* at near-term, and offers new insight that the cholinergic stimulation-mediated hormonal mechanism may also play a role in the regulation of fetal cardiovascular homeostasis. Considering that many environmental factors such as smoking during pregnancy may affect cholinergic activation *in utero*,

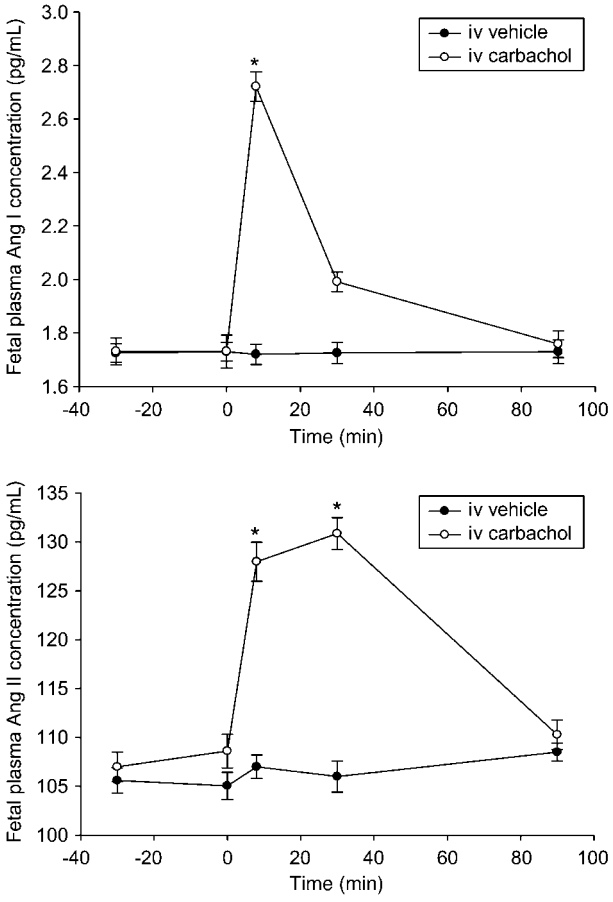

Figure 3 Effect of intravenous (iv) infusion of vehicle or carbachol on fetal plasma angiotensin I, angiotensin II levels. *$P < 0.05$ (statistical significant) compared with baseline level (n = 5/each group).

the findings in the present study are important to both prenatal and postnatal health.

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