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# Effects of Intracerebroventricular Losartan on Angiotensin II-Mediated Pressor Responses and *c-fos* Expression in Near-Term Ovine Fetus

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ABSTRACT

The renin-angiotensin system plays an important role in cardiovascular control. Intracerebroventricular (i.c.v.) angiotensin (ANG) II causes a reliable pressor response in the fetus at 90% gestation. To determine the roles of brain $AT_1$ and $AT_2$ receptors in this response, the effects of the central $AT_1$ and $AT_2$ receptor antagonists losartan and PD123319 were investigated in chronically prepared near-term ovine fetuses. Losartan at 0.5 mg/kg (i.c.v.) abolished central ANG II-induced pressor responses. High-dose losartan (5 mg/kg, i.c.v.) showed a potentiation of the pressor response to i.c.v. ANG II, accompanied by bradycardia. Associated with the pressor responses, *c-fos* expression in the cardiovascular controlling areas was significantly different between the low and high doses of losartan. These areas included the subfornical organ, median preoptic nucleus, organum vasculosum of the lamina terminalis, and paraventricular nuclei in the forebrain, and the tractus solitarius nuclei, lateral parabrachial nuclei in the hindbrain. Low-dose losartan markedly reduced c-fos in these areas after i.c.v. ANG II, while the high-dose losartan together with ANG II elicited a much stronger FOS-immunoreactivity in these areas than that induced by i.c.v. ANG II alone. This is a novel finding, that *c-fos* expression in the brain can be both activated and inhibited under the same condition. Central ANG II-induced fetal pressor responses were not altered by PD123319 (0.8 mg/kg). These results indicate that i.c.v. losartan at a high and a low dose has strikingly different effects on central ANG II-induced pressor responses in fetuses at late gestation, and that the $AT_1$ mechanism plays an important role in fetal cardiovascular regulation. J. Comp. Neurol. 493:571–579, 2005. 

**Indexing terms: losartan; fetal development; brain; c-fos; blood pressure**

Angiotensin (ANG) II is a biologically active product of the renin-angiotensin system (RAS) and plays a major role in the regulation of blood pressure (BP) and body fluid homeostasis. It is also an important etiological factor in the development of some clinical and experimental cardiovascular disorders such as hypertension (Dudley et al., 1990; Timmermans et al., 1993; de Gasparo et al., 2000). ANG receptors have been classified into $AT_1$ and $AT_2$ receptor subtypes, based on the differential binding of several nonpeptide antagonists in a variety of tissues, including the brain (Timmermans et al., 1991; Steckelings et al., 1992). $AT_1$ receptors are defined by their sensitivity to losartan (DuP753) and its active metabolite, EXP3174 (Wong et al., 1990), whereas $AT_2$ receptors are those that are sensitive to PD123319 and related analogs (Bumpus et

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al., 1991). Losartan is a potent ANG receptor antagonist that specially and selectively blocks $AT_1$ receptors (Timmermans et al., 1993). Although the brain contains both $AT_1$ and $AT_2$ receptors, it is generally believed that the central effects of ANG II on cardiovascular homeostasis are mediated by the $AT_1$ subtype (Steckelings et al., 1992).

It is suggested that $AT_2$ receptors are highly expressed in fetuses, predominant, and downregulated over time (Cook et al., 1991.). Recent findings from our laboratory have demonstrated that intracerebroventricular (i.c.v.) ANG II caused an increase of fetal arterial pressure in the near-term ovine fetus, which was associated with an elevation of c-fos expression in brain cardiovascular-regulated areas (Xu et al., 2003). In an attempt to define the role of $AT_1$ and $AT_2$ receptors in fetal cardiovascular control, the effects of losartan and PD123319 were investigated in the ovine fetus at 90% gestation. We found that intravenous (i.v.) losartan could produce an immediate reduction in fetal arterial pressure at near-term, while i.c.v. injection of the same $AT_1$ receptor antagonist had different effects on fetal blood pressure in utero (Shi et al., 2004a). In the present study we sought to determine the effects of pretreatment with different doses of losartan on the central ANG II-mediated pressor responses in the near-term ovine fetus. We also determined the effects of PD123319, a specific antagonist of the $AT_2$ receptors, on ANG II-induced cardiovascular responses. In addition, central neuronal activity marked by c-fos expression was assessed after i.c.v. losartan followed by ANG II. The information gained provides important clues concerning the functional development of central ANG receptors in cardiovascular homeostasis before birth.

## MATERIALS AND METHODS

### Animals and surgical preparations

Studies were performed in conscious, chronically instrumented fetal sheep at a late gestational age (131 ± 3 days; term 145 days). All surgical and experimental procedures were approved and governed by the Institute Animal Care Committee. Animals were housed indoors in individual study cages and acclimated to a lighting regimen of 12:12-hour light/dark before and after surgery. Except for the withholding of food during the 24 hours immediately preceding surgery, food (alfalfa pellets) and water were provided ad libitum.

Following injection of ketamine hydrochloride (20 mg/kg intramuscular, i.m.), general anesthesia (3% isoflurane and 1 L/min oxygen) was maintained using positive-pressure ventilation. Polyethylene catheters (inner diameter 1.8 mm, outer diameter 2.3 mm) were inserted into a maternal femoral vein and artery and advanced into the inferior vena cava and abdominal aorta, respectively. A lower abdominal midline incision was made and the gravid uterus was exposed. Fetal instrumentation was achieved in two stages. The first uterine incision exposed the fetal hindlimbs, and catheters (inner diameter 1.0 mm, outer diameter 1.8 mm) were inserted into a femoral vein and artery. An intrauterine (i.u.) catheter was inserted for measuring amniotic fluid pressure. The second uterine incision exposed the fetal head. A midline incision was made to expose the skull and an intracranial catheter was placed in the fetal lateral ventricle and held in place with dental cement (Xu et al., 2001). The coordinates were: anterior-posterior: + 0.1 cm in front of the bregma; medial-lateral: 0.8 cm from the middle line; and ventral: 1.8 cm below the dura. The placement of cannulae was verified with histological analysis after sacrificing the animals. The uterine incisions were closed in layers. Catheters were filled with heparinzed saline (80 i.u. heparin/ml in 0.9% NaCl), and all catheters were passed through a subcutaneous tunnel and exteriorized through a small incision on the ewe's flank. Catheters were kept in a cloth pouch attached to the ewe's flank. After surgery, pregnant ewes were returned to individual pens and allowed free access to water and food. They generally resumed normal feeding patterns within 24 hours after surgery. Immediately preoperatively and twice daily during the initial 2 days of recovery, gentamicin (8 mg) and oxacillin (33 mg) were administrated i.v. to the fetus, and gentamicin (72 mg), oxacillin (1 g), and chloramphenicol (1 g) were injected i.v. into the ewe.

### Experimental protocol

All experiments were performed on unanesthetized ewes standing in the cages and on fetuses in utero. Fetal arterial blood pH was assessed and studies undertaken only if the fetal arterial pH was 7.3 (Shi et al., 2003). Fetal body weight was estimated according to the following formula: fetal weight (kg) = 0.0961 × gestation age (days) – 9.228 (Robillard et al., 1979). On the test day, studies began with a baseline period (from −60 minutes to 0 minutes) followed by study period (0–120 minutes). To study the effects of losartan in the fetal brain on fetal cardiovascular responses induced by i.c.v. ANG II, sheep fetuses were injected i.c.v. losartan (Merck, Darmstadt, Germany) (0.5, or 5 mg/kg, n = 5, respectively, in 1 ml saline) or vehicle (0.9% NaCl, n = 5, 1 ml) at 0 minutes. Twenty minutes later, another bolus of losartan (0.5, or 5 mg/kg) or vehicle together with ANG II (1.5 μg/kg) injected i.c.v. in the fetus. In the $AT_2$ antagonist study the protocol was similar to that described above. The ovine fetuses were injected i.c.v. PD123319 (Sigma, St. Louis, MO) (0.8 mg/kg, n = 5) or saline vehicle (n = 5) at 0 minutes followed by a bolus of PD123319 (0.8 mg/kg) or vehicle together with ANG II (1.5 μg/kg). The doses of $AT_1$ and $AT_2$ receptor antagonist were chosen based on previous reports that showed efficiency of blocking effects (Weisinger et al., 1997; McKinley et al., 2001). Blood samples were withdrawn from the fetal and maternal arterial catheters for the measurement of $PO_2$, $PCO_2$, hemoglobin, and pH using a Radiometer (BM 33 MK2-PHM 72 MKS acid-base analyzer system, Copenhagen) adjusted to the internal temperature of sheep. Blood hematocrit was measured with a microcapillary reader. Plasma osmolality was measured using freezing point depression on an Advanced Digimatic osmometer (model 3MO, Advanced Instruments, Needham Heights, MA). Plasma electrolyte concentrations were determined by a Nova 5 electrolyte analyzer (Nova Biomedical, Waltham, MA). The withdrawn fetal blood was replaced with an equivalent volume of heparinzed maternal blood withdrawn before the study. Throughout the studies, maternal and fetal systolic pressure (SP), diastolic pressure (DP), heart rate, and amniotic fluid pressure were recorded continuously. The fetal mean arterial pressure (MAP) was corrected against amniotic fluid pressure as adjusted MAP (A-MAP). Fetal and maternal BP were measured with a Beckman R612 (Beckman Instruments, Fullerton, CA) physiological recorder

with Statham (Garret, Oxnard, CA) P23 transducers. BP and heart rate were determined by computer analysis of waveforms utilizing a customized pattern recognition algorithm.

### *C-fos* experiments

Following the $AT_1$ antagonist study, fetal brains were used for c-fos studies after i.c.v. injection of either ANG II (n = 5) or losartan (0.5 or 5 mg/kg) together with ANG II (n = 5, respectively). Two animals were injected i.c.v. with the high dose losartan alone. C-fos experiments were performed in different animals. At the conclusion of the study the animals were anesthetized and ventilated with a mixture of isoflurane and oxygen as described. A middle abdominal incision was made and the fetal head and neck were exposed. An 18-gauge needle was inserted into one side of fetal carotid artery for perfusion. The fetuses were perfused with 0.01 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M PB. Postfixation was performed in the PFA solution for 12 hours, after which the brain was placed in 20% sucrose in 0.01 M PB (pH 7.3–7.4) overnight. Twenty-μm coronal sections were cut through the fetal forebrain and hindbrain on a cryostat. All sections of the subfornical organ (SFO), median preoptic nucleus (MnPO), and every other section of the organum vasculosum of the lamina terminalis (OVLT), paraventricular nuclei (PVN), supraoptic nuclei (SON), the periventricular hypothalamic nuclei (PE), the tractus solitarius nuclei (NTS), lateral parabrachial nuclei (LPBN), area postrema (AP), and ventrolateral medulla (VLM) were used for *c-fos* immunoreactivity (FOS-ir) staining using the avidin-biotin-peroxidase (ABC; Vector Laboratories, Burlingame, CA) technique. The tissue sections were incubated on a gentle shaker overnight at 4°C in the primary antibody (1:15,000; Santa Cruz Biotechnology, Santa Cruz, CA). The antibody was from rabbit against the first 16 amino acids on the N-terminal sequence of the fos protein. This antibody has been used repeatedly and reported in other studies before. Control staining (without the primary antibody while other procedures were the same) showed nonspecific effects. The sections were further incubated in a goat anti-rabbit serum (1:200) for 1 hour and then processed using the Vectastain ABC kit for 1 hour (Vector) at room temperature. The sections were then treated with 1 mg/ml diaminobenzidine tetrahydrochloride (Sigma) (0.02% hydrogen peroxide). All sections were mounted on slides, dehydrated in alcohol, and then coverslipped.

### Data analysis

All analog signals for fetal cardiovascular data were recorded continuously throughout the study, then digitized on a computer with Win-DAQ acquisition software (Data Q Instruments, Akron, OH). The number of FOS-ir positive cells in the brain was evaluated in a qualitative and blinded manner as reported previously (Xu et al., 2001). A repeated-measure analysis of variance (ANOVA) was used to determine differences over time and effects of the treatments. Comparison before and after the treatments was determined with one-way ANOVA followed by the Tukey post-hoc test. Student's *t*-test was used to analyze the differences between the i.c.v. ANG II and i.c.v. losartan plus ANG II. All data are expressed as mean ± SEM. Adobe PhotoShop (San Jose, CA) software was used for contrast of the photos.

## RESULTS

### Blood values

Intracerebroventricular losartan, PD123319, or vehicle had no effect on maternal or fetal plasma osmolality, $Na^+$, $K^+$, and $Cl^-$ concentrations, or arterial blood pH, $PO_2$, $PCO_2$, hemoglobin, and hematocrit (all $P > 0.05$). Intracerebroventricular losartan, PD123319, or vehicle plus ANG II were also without effect on maternal or fetal blood values (all $P > 0.05$). All arterial values were within normal ranges and were not different between the control and experimental groups.

### Cardiovascular responses

***Effects of intracerebroventricular losartan on ANG II-induced pressor responses.*** Histological analysis confirmed that all i.c.v. cannulae were inserted into the fetal lateral ventricle. There was no significant difference between the control and the experimental groups for maternal SP, DP, and MAP (all $P > 0.05$). In the control animals, i.c.v. injection of vehicle had no effect on fetal SP, DP, or A-MAP (all $P > 0.05$). However, after i.c.v. injection of vehicle + ANG II, fetal SP, DP, and A-MAP were increased significantly. A-MAP was elevated 26.0% from baseline (43.6 ± 2.3 mmHg) to a peak (54.9 ± 2.7 mmHg) at 15 minutes (time = 35 minutes) after i.c.v. vehicle + ANG II ($P < 0.01$) (Fig. 1). A-MAP was maintained at the high level for 60 minutes. In the experimental group, i.c.v. injection of low-dose losartan (0.5 mg/kg) had no effect on fetal basal SP, DP, and A-MAP (all $P > 0.05$). After i.c.v. losartan (0.5 mg/kg) + ANG II, fetal SP, DP, and A-MAP showed no significant difference when compared to baseline levels (all $P > 0.05$). However, there was a significant difference in fetal A-MAP between the low-dose losartan-treated group and the control group (vehicle + ANG II) ($F_{8,1} = 24.63$, $P < 0.01$) (Fig. 1A).

As shown in Figure 1B, following i.c.v. injection of losartan (5 mg/kg), fetal A-MAP increased immediately by 15.6% and 26.9% at 2 minutes and 5 minutes from baseline, respectively (both $P < 0.01$). A-MAP reached a peak value at 7 minutes (56.9 ± 2.5 mmHg, $P < 0.01$, compared with the baseline level) after high-dose losartan. The increased fetal A-MAP recovered at 20 minutes i.c.v. injections. As shown in Figure 1B, fetal A-MAP after injection of losartan (5 mg/kg) + ANG II was even higher than that in response to i.c.v. vehicle + ANG II of the control group. After i.c.v. losartan (5 mg/kg) + ANG II at 2 and 5 minutes, fetal A-MAP increased 27.1% and 51.0% (54.9 ± 2.9 mmHg, and 65.3 ± 3.2 mmHg, both $P < 0.01$), respectively, compared to the baseline level (43.3 ± 2.0 mmHg). It reached a peak value (66.7 ± 3.0 mmHg, $P < 0.01$) 10 minutes after i.c.v. losartan + ANG II and increased 54.1% compared to baseline. The increased fetal A-MAP lasted for a longer period (100 minutes after the i.c.v. losartan + ANG II).

In both control and losartan (either 0.5 mg/kg or 5 mg/kg)-treated groups, maternal heart rates were not changed before and after the i.c.v. injection. Fetal heart rate was not changed significantly in the control and low-dose losartan-treated group ($P > 0.05$, Fig. 2A). In response to i.c.v. high-dose losartan (5 mg/kg), fetal heart rate was increased, although there was no statistical difference compared to the baseline level. However, after losartan (5 mg/kg) + ANG II, fetal heart rate decreased significantly (Fig. 2B).

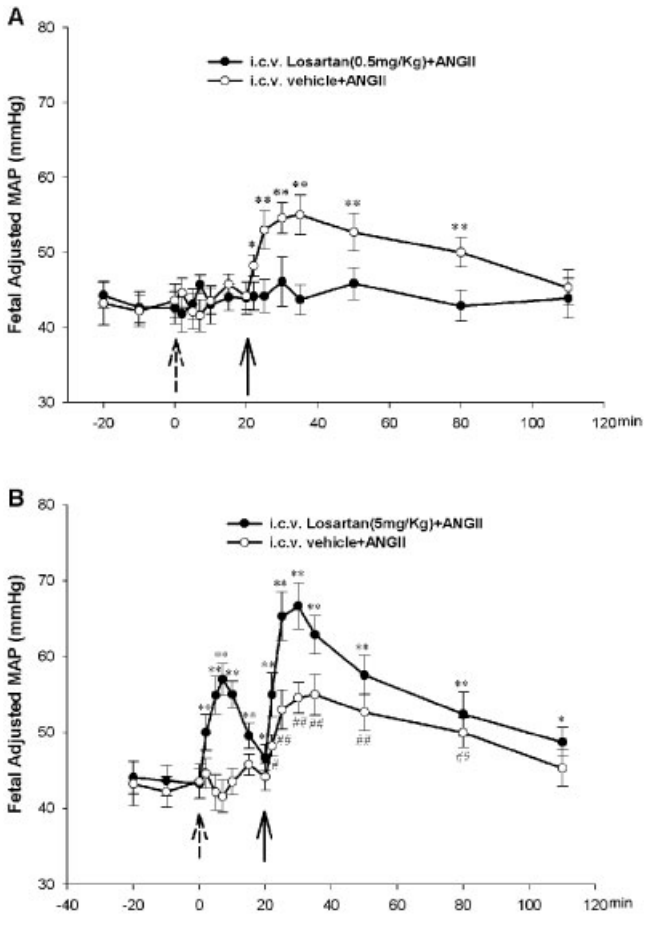


Fig. 1. The effect of low-dose i.c.v. injection of losartan (**A**) or high-dose i.c.v. injection of losartan (**B**) on fetal A-MAP. Low-dose losartan: 0.5 mg/kg; high-dose losartan: 5 mg/kg. ** or ##: $P < 0.01$; * or #: $P < 0.05$, compared to the baseline level (vehicle injections where appropriate). Dotted arrow: time of i.c.v. losartan; solid arrow: time of i.c.v. bolus losartan + ANG II.

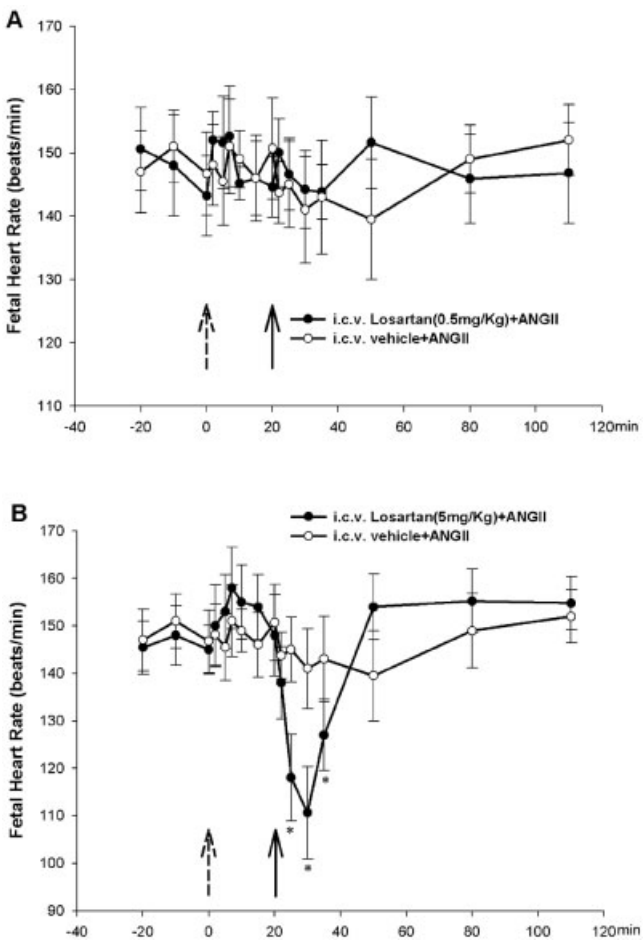


Fig. 2. The effect of i.c.v. injection of low-dose losartan (**A**) or high-dose losartan (**B**) on fetal heart rate. Low-dose losartan: 0.5 mg/kg; high-dose losartan: 5 mg/kg. *$P < 0.01$, compared to the baseline level. Dotted arrow: time of i.c.v. losartan; solid arrow: time of i.c.v. bolus losartan + ANG II.

***Intracerebroventricular PD123319 on cardiovascular responses.*** In the $AT_2$ antagonist study, there were no significant differences between the control and the experimental groups for maternal SP, DP, MAP, and heart rate (all $P > 0.05$). Central administration of PD123319 had no effect on fetal SP, DP, A-MAP, and heart rate (all $P > 0.05$, Fig. 3). After i.c.v. PD + ANG II, fetal A-MAP still increased significantly, as it did in the control group. There was no significant difference between the experimental group and the control group for fetal A-MAP ($F_{8,1} = 0.22$, $P > 0.05$).

## FOS-immunoreactivity

In the present study, in response to the i.c.v. ANG II, *c-fos* expression was increased significantly in many $AT_1$ receptor-abundant areas, including SFO, MnPO, OVLT, PVN, SON in the forebrain, and NTS, LPBN, AP in the hindbrain (Figs. 4–7), which was consistent with our previous studies (Xu et al., 2001, 2003). However, after i.c.v. low-dose losartan (0.5 mg/kg), *c-fos* expression was reduced significantly compared to the control group (i.c.v. vehicle + ANG II) (Figs. 4, 7, 8). Analysis showed that there was a significant difference in FOS-ir between the control and low-dose losartan (0.5 mg/kg)-treated group in the SFO, dorsal and ventral MnPO, OVLT, SON, magnocellular and parvicellular PVN in the forebrain (all $P < 0.01$), and NTS, LPBN, AP, VLM in the hindbrain (all $P < 0.01$) (Figs. 4, 7, 8). On the other hand, in response to high-dose losartan (5 mg/kg) + ANG II, FOS-ir detected in these areas was much higher compared to those induced by i.c.v. ANG II alone (all $P < 0.01$, Figs. 5–8). In the fetal hypothalamus it was noted that i.c.v. high-dose losartan + ANG II increased c-fos not only in the PVN and SON, but also in most parts of the hypothalamus except the areas surrounding the third ventricle, including parts of the ventricle PVN region near the third ventricle and the PE where *c-fos* was totally abolished (Fig. 6). In the hindbrain, i.c.v. ANG-II-induced *c-fos* was also completely abolished in the area surrounding the central canal, while the nearby region, including the commissural part and intermediate part of NTS, were overexpressed with FOS-ir after treatment with the high-dose losartan + ANG II (Fig. 8). In the two animals injected with the i.c.v. high-dose losartan, c-fos in the fetal brain was greater

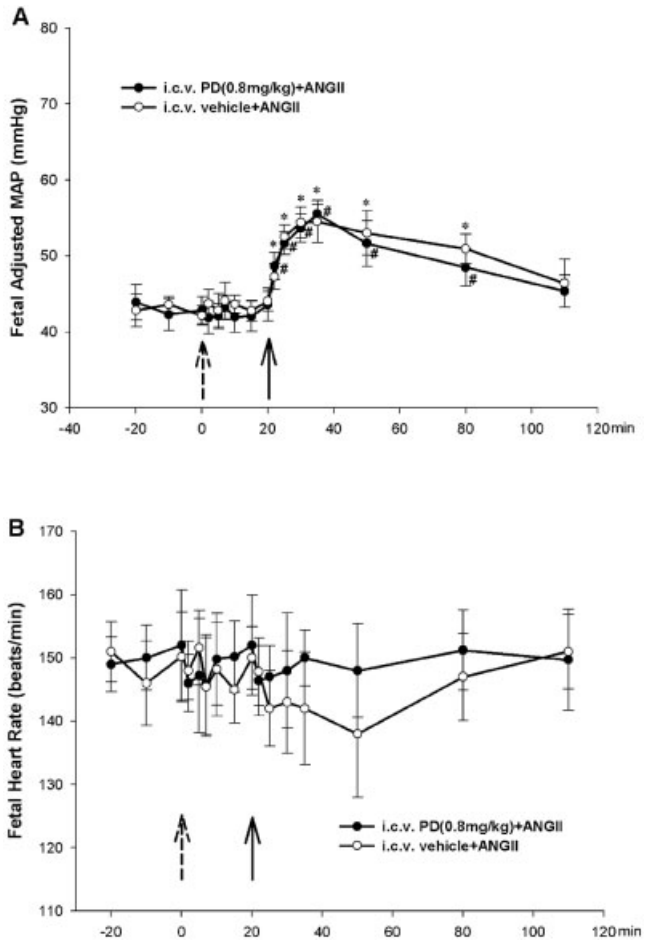


Fig. 3. The effect of i.c.v. administration of PD123319 (0.8 mg/kg) on fetal A-MAP (**A**) and heart rate (**B**). PD: PD123319. ** or ##: $P < 0.01$; * or #: $P < 0.05$, compared to the baseline level. Dotted arrow: time of i.c.v. PD123319; solid arrow: time of i.c.v. bolus PD123319 + ANG II.

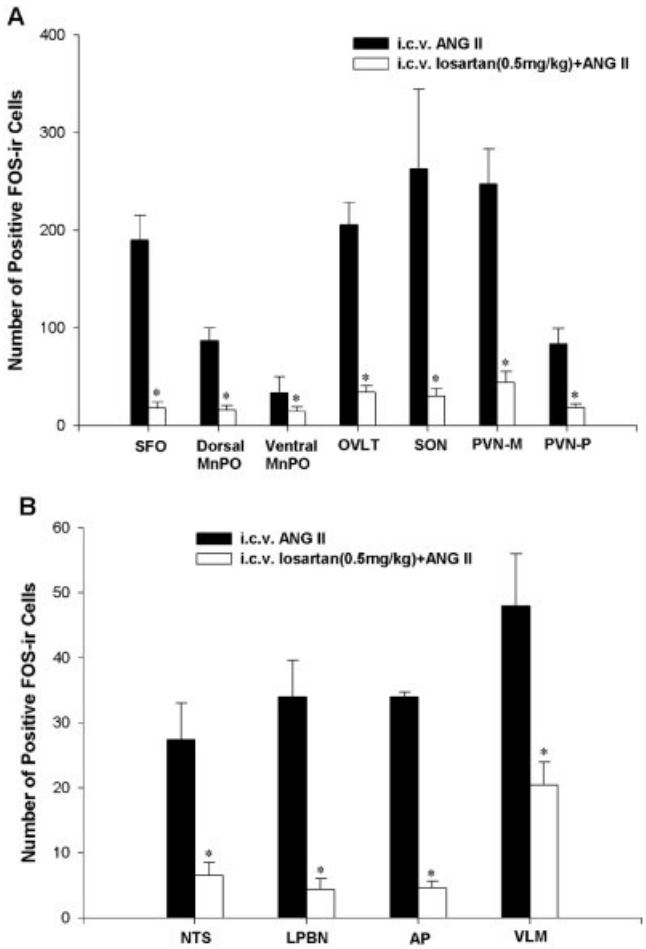


Fig. 4. The effect of low-dose i.c.v. losartan (0.5 mg/kg) on FOS-ir in the fetal forebrain (**A**) and hindbrain (**B**). *$P < 0.01$, compared to the control level. SFO, subfornical organ; MnPO, median preoptic nucleus; OVLT, organum vasculosum of the lamina terminalis; SON, supraoptic nuclei; PVN, paraventricular nuclei; NTS, tractus solitarius nuclei; LPBN, lateral parabrachial nuclei; AP, area postrema; VLM, ventrolateral medullar.

than that induced by low-dose losartan, but less than that caused by high-dose losartan together with ANG II (due to small sample size, no statistical analysis).

## DISCUSSION

In this study we demonstrated that in the fetus at 70–90% gestation, where central ANG II has been shown to produce a pressor response (Xu et al., 2003, 2004, 2005; Shi et al., 2004a) a low dose of losartan administered before and with ANG II displayed no effect on fetal BP but abolished the central ANG II-induced pressor responses. In contrast, i.c.v. PD123319 had no influence on the increased fetal BP by ANG II. This is the first study to demonstrate that losartan administrated centrally at certain doses can effectively block i.c.v. ANG II-produced fetal pressor responses. Combined with the negative results of the $AT_2$ antagonist, the data suggest that i.c.v. ANG II-induced pressor responses are mediated via $AT_1$, not $AT_2$ receptors at near-term. Moreover, endogenous brain ANG II appears not to contribute significantly to the maintenance of basal arterial pressure in fetuses, as has been shown in adults (Rademaker et al., 1995).

The high-dose losartan (5 mg/kg) caused a transient increase of fetal BP by itself, and enhanced pressor responses induced by central ANG II. Central ANG II alone produced an increase of fetal A-MAP as reported (Xu et al., 2003). A-MAP reached the peak at 15 minutes with an increase of 26.2% after i.c.v. ANG II. However, a bolus losartan plus ANG II caused a much greater increase (about 54.1%) in fetal A-MAP than that in response to i.c.v. ANG II or losartan alone. To our knowledge, this phenomenon has not been revealed before in either adults or fetuses.

The majority of the previous literature has reported that central losartan effectively inhibits ANG II-induced cardiovascular responses (Bunting and Widdop, 1995; Lark et al., 1995; Mathai et al., 1998). There is increasing evidence that central losartan could also produces pressor responses in adults. Injection of losartan into either the anterior ventricles or fourth ventricle increases arterial

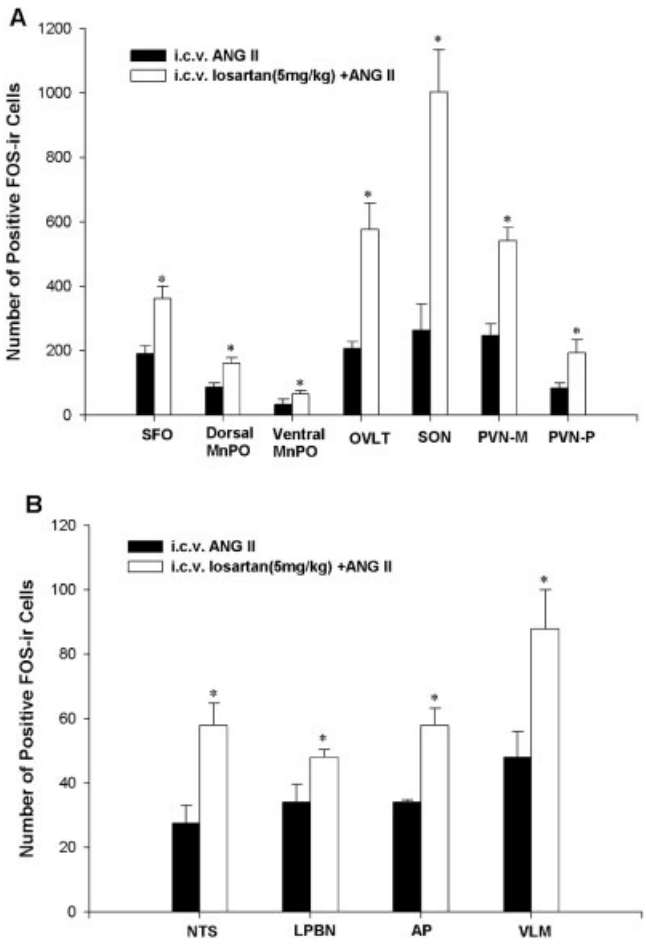


Fig. 5. The effect of i.c.v. losartan (5 mg/kg) and ANG II on FOS-ir in the fetal forebrain (**A**) and hindbrain (**B**). *$P < 0.01$, compared to the control level. SFO, subfornical organ; MnPO, median preoptic nucleus; OVLT, organum vasculosum of the lamina terminalis; SON, supraoptic nuclei; PVN, paraventricular nuclei; PE, periventricular hypothalamic nuclei; NTS, tractus solitarius nuclei; LPBN, lateral parabrachial nuclei; AP, area postrema; VLM, ventrolateral medullar.

pressure (De Luca et al., 1994, 1996, 2000). Activation of ANG receptors is traditionally associated with an increase in arterial pressure (Phillips, 1987; Wright and Harding, 1992), but there are also reports of a decrease in arterial pressure induced by ANG II (De Luca et al., 2000). Thus, the hypertensive effect of central losartan in adults has been speculated to be related, at least in part, to its action on the central depressor ANG receptors (Sugawara et al., 2002). In the present study, the effects of central losartan on ANG II-induced pressor responses depended on the doses. Both depressor and pressor actions of central losartan were demonstrated in the near-term fetus.

ANG II injected into the lateral ventricle, the caudal ventrolateral medulla (CVLM), and the medial NTS causes transient hypotension in rats (Nicolaidis et al., 1983; Muratani et al., 1993; Fow et al., 1994). $AT_1$ antagonists produce sympathoactivation and an increase in arterial pressure when injected into the rostral and caudal ventrolateral medulla (Gaudet et al., 1988; Muratani et al., 1993; Fontes et al., 1997). It is believed generally that the CVLM acts by inhibiting a more rostral excitatory region of the ventrolateral medulla, the RVLM, resulting in a widespread inhibition of the sympathetic vasomotor activity (Feldberg and Guertzenstein, 1976; Blessing and Reis, 1982). Several studies have demonstrated that ANG II can influence these regions, increasing (RVLM) or decreasing (CVLM) sympathetic activity (Andreatta et al., 1988; Muratani et al., 1991; Sesoko et al., 1995; Averill and Diz, 2000). Why did the high-dose losartan cause an additional increase in fetal BP after i.c.v. ANG II? One explanation is that higher doses of losartan in the ventricle system could have a good chance to reach fetal hindbrain structures and thus act on the CVLM and other depressor regions. Another interpretation is that central losartan at certain high doses may behave like an agonist. It has been suggested that ANG receptor antagonists possess agonistic effects (Streeten and Anderson, 1984). For example, saralasin caused a dose-dependent, transient increase in BP (Wong et al., 1991). Similar to ANG II, iontophoretically applied losartan was shown to activate neurons in the medial septum-median preoptic region (Mousseau et al., 1996). More studies are therefore needed to clarify the mechanisms involved.

Although there are several reports regarding central losartan-induced pressor responses, a common limitation is noted in these studies. This is a lack of direct evidence of central neuronal activity in pressor responses after central administration of losartan. Detection of the transcription factor FOS has proven to be a useful marker of activation in central regions (Rowland et al., 1994; Rowland, 1998). In adults, ANG II-induced FOS-ir has been demonstrated in cardiovascular-regulated areas with abundant $AT_1$ receptors, including the SFO, OVLT, MnPO, PVN, SON, NTS, LPBN, and AP (Fitzsimons, 1998). We have reported recently similar c-fos expression by i.c.v. ANG II in the fetus (Xu et al., 2001, 2003). Following the low dose of losartan, ANG II-induced FOS-ir in these areas was inhibited. This suggests that the low-dose losartan could block the effect of central ANG II. However, the high dose of losartan stimulated intense FOS-ir in the same areas as that of i.c.v. ANG II. High-dose losartan induced more c-fos in the fetal brain than that by low-dose losartan, but less than that caused by high-dose losartan together with ANG II. Analysis revealed an interesting FOS-ir phenomenon caused by the combination of the high doses of losartan and ANG II. This phenomenon consists of two distinctive characters: an extremely high expression of *c-fos* in most regions detected, and a complete inhibition of *c-fos* in certain other areas. Increased *c-fos* was observed in the SFO, MnPO, SON, PVN, AP, and NTS. The number of FOS-ir in those areas was overwhelmingly higher than that induced by i.c.v. ANG II alone. At the same time, an abolishment of *c-fos* indicated by a FOS-ir-free band with a clear-cut border in the region surrounding the third ventricle and the central canal was very impressive. Notably, the PE and parts of the ventral PVN were in those "FOS-free bands." The pattern of inhibition of *c-fos* in the PE and overexpression in the nearby PVN/SON under the same condition has not been reported in either adults or fetuses. This means that both inhibition and induction of c-fos by the same treatments are possible, and adds new knowledge of using *c-fos* in neural activation. The PE is rich in $AT_1$ receptors, like its neighbor, the PVN (Lenkei et al., 1998). Central ANG II induced *c-fos* in both PVN and PE. However, the high dose of losartan with

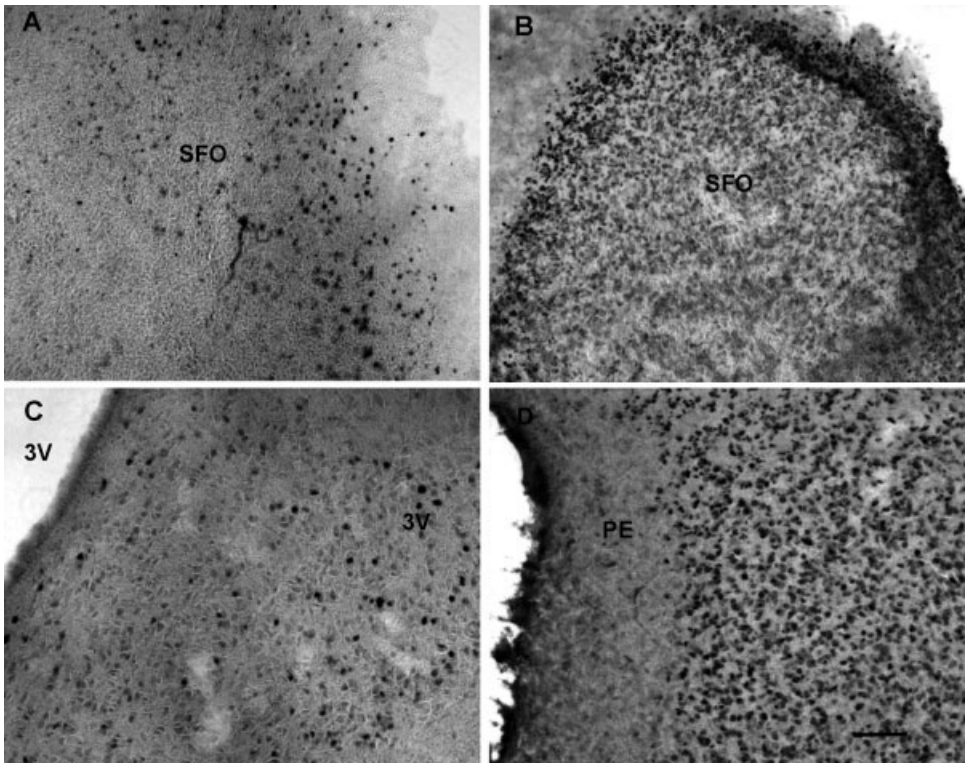


Fig. 6. FOS-ir induced by i.c.v. ANG II or i.c.v. high-dose losartan + ANG II in the fetal SFO (**A,B**) and PVN (**C,D**). A,C: i.c.v. ANG II (1.5 μg/kg). B,D: i.c.v. losartan (5 mg/kg) + ANG II (1.5 μg/kg). SFO, subfornical organ; PVN, paraventricular nuclei. AC, anterior commissure. 3V, the third ventricle. Note: a complete inhibition in the PE and over expression by c-fos in the nearby region. Scale bar = 50 μm in D (applies to A–D).

ANG II totally suppressed *c-fos* in the PE while, overexpressing the same gene in the PVN. Injection of losartan into the PE has been shown to significantly reduce osmotic-induced AVP release (Qadri et al., 1998). Stimulation of periventricular ANG receptors induced a dose-dependent increase in noradrenaline release in the PVN (Stadler et al., 1992) and SON (Qadri et al., 1993). In the present study, i.c.v. ANG II-induced *c-fos* in the PE was completely abolished by losartan, while *c-fos* in the PVN and SON were overexpressed under the same condition. It appears that there may exist interactions between the PE and the PVN/SON. This finding also raises other questions. Why did high-dose losartan inhibit neural activation only in the areas like the PE? Was a complete inhibition of c-fos in one region the cause of an overexpression of cellular activation in the other areas? Or the inverse? In connection with the enhanced pressor responses, several questions are also pertinent: Does an inhibition of the "FOS-free band" play a part in abolishing inhibitory mechanisms in pressor responses; or, would overexpression of c-fos be a predominant factor for augments of fetal BP increase? In addition, whether c-fos expression may also be a consequence of the increase in blood pressure? Further studies are needed.

It is noted that high-dose losartan together with ANG II induced tremendous c-fos expression in some areas in the fetal brain. It appears that c-fos was expressed not only in neurons, but also in glial cells. Previous reports showed that c-fos can be induced in both neurons and glial cells following specific stimulation (Segura et al., 2005). Several studies have shown that $AT_1$ receptors in the brain are located on both neurons and glial cells (Thomas et al., 2004). When the high dose of losartan induced intensive c-fos in the fetal brain, it is possible that the drug acts on $AT_1$ receptors located on neurons and glial cells as a specific effect. Other evidence supporting this argument is that there were notable "FOS-free areas" next to intensively c-fos-expressed regions. If it was an artifact, there should not be "FOS-free areas" nearby. However, further study of double-labeling c-fos and neurons or glial cells would be best for clarification.

Changes of osmolality may induce c-fos (Sharp et al., 1991). However, fetal osmolality, sodium concentrations, and other blood values remained unchanged. This excludes possibilities of osmotic and other factors that may influence FOS-ir in the fetal brain.

The combination of the high dose of losartan and ANG II significantly decreased fetal heart rate. Notably, central ANG II-increased fetal BP was further enhanced after high-dose losartan. Therefore, the fetal bradycardia appears to be a baroreflex response subsequent to acute increase of arterial pressure. In a link to the neural activation after administration of high-dose losartan and ANG II, FOS-ir was utterly abolished in the "FOS-free band." These areas are critical in cardiovascular regulation (Reid, 1992). Therefore, the abolishment of *c-fos* in these areas may be connected to the cardiovascular responses observed.

In summary, the present study is the first to report that i.c.v. losartan at a low dose can inhibit central ANG II-induced pressor responses and neural activity in the fetus at the last third of gestation, while high doses of losartan enhances i.c.v. ANG II-increased fetal BP and produces a subsequent baroreflex. Both inhibitory and excitatory *c-fos* expression in the fetal brain were shown under the condition of high-dose losartan followed by

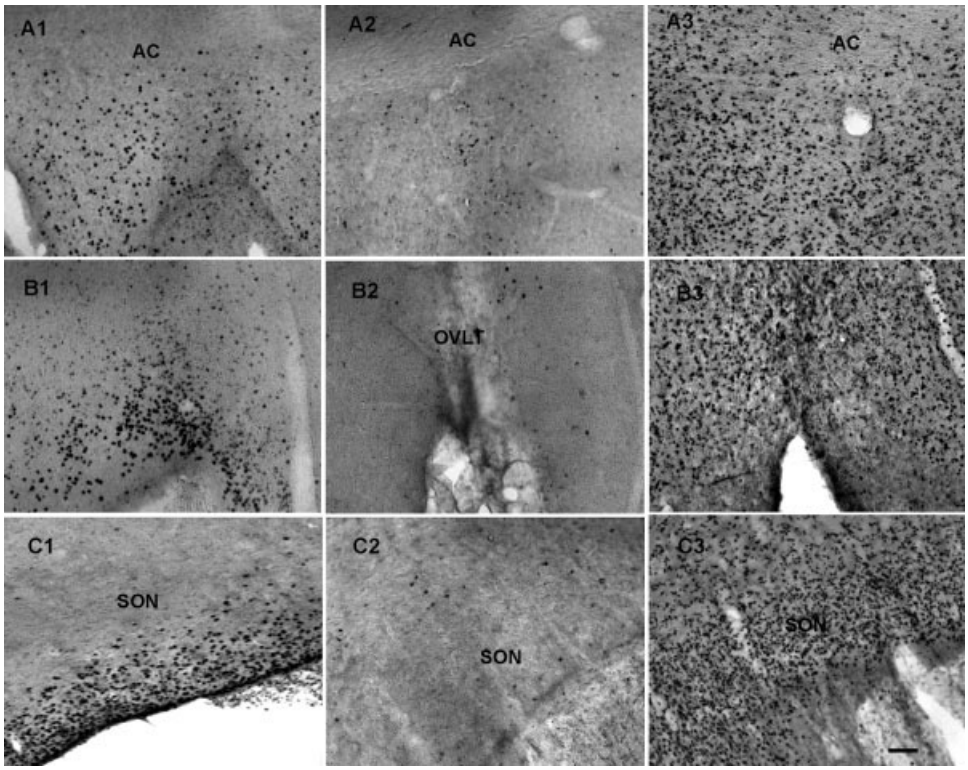


Fig. 7. FOS-ir induced by i.c.v. ANG II or i.c.v. losartan + ANG II in the fetal MnPO (**A**), OVLT (**B**), and SON (**C**). A1: i.c.v. ANG II (1.5 μg/kg). A2: i.c.v. losartan (0.5 mg/kg) + ANG II (1.5 μg/kg). A3: i.c.v. losartan (5 mg/kg) + ANG II (1.5 μg/kg). MnPO, median preoptic nucleus; OVLT, organum vasculosum of the lamina terminalis. SON, supraoptic nuclei. Scale bar = 30 μm in C3 (applies to A1–C3).

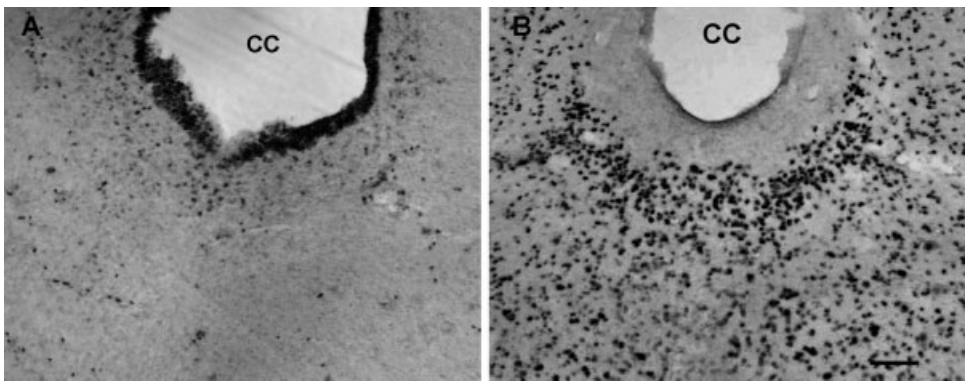


Fig. 8. FOS-ir induced by i.c.v. losartan + ANG II in the fetal NTS. **A:** Losartan (0.5 mg/kg) + ANG II (1.5 μg/kg). **B:** i.c.v. losartan (5 mg/kg) + ANG II (1.5 μg/kg). NTS, tractus solitarius nuclei; CC, central canal. Scale bar = 30 μm in B (applies to A,B).

central ANG II. This is a novel demonstration that *c-fos* expression in the brain could be both abolished and produced under the same conditions. These data have opened up intriguing possibilities for future studies of ANG, its antagonists, its receptors, and central pathways.

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