Functional role of angiotensin II type 1 and 2 receptors in regulation of uterine blood flow in nonpregnant sheep

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Lambers, Donna S., Suzanne G. Greenberg, and Kenneth E. Clark. Functional role of angiotensin II type 1 and 2 receptors in regulation of uterine blood flow in nonpregnant sheep. Am. J. Physiol. Heart Circ. Physiol. 278: H353–H359, 2000.—The objective was to determine the receptor subtype of angiotensin II (ANG II) that is responsible for vasoconstriction in the nonpregnant ovine uterine and systemic vasculatures. Seven nonpregnant estrogenized ewes with indwelling uterine artery catheters and flow probes received bolus injections (0.1, 0.3 and 1 µg) of ANG II locally into the uterine artery followed by a systemic infusion of ANG II at 100 ng·kg⁻¹·min⁻¹ for 10 min to determine uterine vasoconstrictor responses. Uterine ANG II dose-response curves were repeated following administration of the ANG II type 2 receptor (AT₂) antagonist PD-123319 and then repeated again in the presence of an ANG II type 1 receptor (AT₁) antagonist L-158809. In a second experiment, designed to investigate the mechanism of ANG II potentiation that occurred in the presence of AT₂ blockade, nonestrogenized sheep received a uterine artery infusion of L-158809 (3 mg/min for 5 min) prior to the infusion of 0.03 µg/min of ANG II for 10 min. ANG II produced dose-dependent decreases in uterine blood flow (P<0.03), which were potentiated in the presence of the AT₂ antagonist (P<0.02). Addition of the AT₁ antagonist abolished the uterine vascular responses and blocked ANG II-induced increases in systemic arterial pressure (P<0.01). Significant uterine vasoconstriction (P<0.01) was noted with AT₁ blockade in the second experiment, which was reversed by administration of the AT₂ antagonist or by the nitric oxide synthetase inhibitor N-nitro-L-arginine methyl ester. We conclude that the AT₁ receptors mediate the systemic and uterine vasoconstrictor responses to ANG II in the nonpregnant ewe. AT₂-receptor blockade resulted in a potentiation of the uterine vasoconstrictor response to ANG II, suggesting that the AT₂-receptor subtype may modulate uterine vascular responses to ANG II potentially by release of nitric oxide.

PD-123319; L-158809; pregnancy

DURING PREGNANCY there are dramatic changes in the maternal cardiovascular system that enable the normal growth and development of a fetus. This is an important physiological adaptation to pregnancy and is associated with a substantial increase in cardiac output and uterine blood flow that is essential for fetal growth. Uterine and systemic vasodilation occurs despite an increase in circulating levels of the vasoconstrictor angiotensin II (ANG II). This suggests a refractoriness to ANG II during pregnancy of the systemic and uterine vasculatures that has been demonstrated in human and animal models (7, 14). ANG II appears to play a role in the regulation of uterine and/or umbilical blood flow during pregnancy because the use of angiotensin-converting enzyme (ACE) inhibitors, which inhibit endogenous ANG II production, is associated with fetal growth restriction, fetal hypoxia, and in some cases fetal death (12). These fetal effects of ACE inhibitors may be in part due to reduced uterine and/or placental blood flow (15).

Two main ANG II receptor subtypes have been described in the literature, type 1 (AT₁) and type 2 (AT₂). The AT₁ receptors are predominantly found in the vascular smooth muscle, adrenal cortex, heart, kidney, liver, and lung. The AT₂ receptor is thought to mediate most of the functions that are generally associated with ANG II, including vasoconstriction, uterine contraction, aldosterone and catecholamine release, water intake, and renal salt and water reabsorption (10). The AT₂ receptor is predominantly found in the uterus, brain, adrenal medulla, and heart, and currently its function is not well understood (6, 10, 21).

Cox et al. (3, 5) have reported that the vast majority (85%) of the ANG II receptors in the ovine and human pregnant and nonpregnant uterine vasculature are the AT₂ subtype. Additionally, Cox and co-workers (4) have recently reported that ANG II has no direct vasoconstrictive actions in the uterus but that uterine vasconstriction occurs following release of systemic vasoconstrictors. On the basis of the predominance of the AT₂ subtype in the ovine uterine vasculature and the fact that ANG II is a uterine vasoconstrictor in this species, we hypothesized that in nonpregnant sheep ANG II acts locally in the uterine vasculature to stimulate AT₂ receptors and produce vasoconstriction.

MATERIALS AND METHODS

Seven healthy nonpregnant ewes of mixed breed, weighing between 55 and 85 kg, underwent a thoracotomy and laparotomy on two separate days to catheterize the uterine and femoral vessels and to place transit-time Doppler flow probes (Transonic Systems, Ithaca, NY) for measurement of cardiac output and uterine blood flow. These surgical procedures have been described in detail previously (2). Briefly, nonpregnant ewes were fasted and water was withheld for 24 h prior to surgery. The ewes were sedated with pentobarbital sodium...
(15 mg/kg), intubated endotracheally, connected to a veterinary anesthesia machine, and ventilated with a mixture of 2–3% isoflurane and oxygen. An incision was made through the left, fourth intercostal space, and the pulmonary artery was isolated and fitted with a 24-mm Transonic flow probe for determination of cardiac output and calculation of systemic vascular resistance. A chest tube, connected to a hemovac, was inserted during the thoracotomy and remained in the ewe for 72 h following surgery to drain the chest cavity of excess fluids and air. The flow probe cable was exteriorized via a subcutaneous tunnel and placed in a cloth pouch on the animal’s left shoulder. After the procedure, ewes were placed in portable cages and received water and food ad libitum.

After a 1-wk recovery period, the ewes were again fasted and water was withheld for 24 h prior to the laparotomy. Ewes received pentobarbital sodium sedation and isoflurane anesthesia as described previously. An incision was made in the left groin over the femoral vessels. The femoral artery and vein were cannulated with polyvinyl catheters to the level of the distal aorta and vena cava. A midline, vertical abdominal incision was made to expose the uterus. Both uterine arteries were dissected free and fitted with 2- or 3-mm Transonic Doppler flow probes for continuous uterine blood flow determinations. A lateral branch of each uterine artery was cannulated to infuse the drugs under study directly into the uterine vasculature. A bilateral oophorectomy was then performed to control exposure of the ewe to circulating sex steroids. The abdominal and groin incisions were closed in layers, and the catheters and flow probes were exteriorized via a subcutaneous tunnel and placed in a cloth pouch on the left flank.

Animals were allowed a minimum of 5 days to recover from the second surgery. Ewes received 1 µg/kg of 17β-estradiol intravenously. Within 2 h after the surgery, Ewes received 1 µg/kg of 17β-estradiol intravenously. The purpose of the first experimental protocol was to determine whether ANG II is a direct vasoconstrictor of the ovine uterine vasculature and to evaluate which ANG II receptor subtype (1 or 2) mediates uterine and systemic vasoconstriction in the nonpregnant sheep. Ewes were allowed to acclimate for 30 min prior to receiving 1 µg/kg of 17β-estradiol intravenously. Within 2 h after the estradiol administration, uterine blood flow increased from a baseline of 12 ± 3 to 152 ± 32 ml/min. Once uterine blood flow had increased, it remained stable for approximately 90 min. It was during this steady state of maximal uterine blood flow that the experimental protocol was performed. ANG II was administered into the uterine artery as a series of bolus injections of increasing dose (0.1, 0.3, 1.0 µg) to determine control uterine and systemic vascular responses. Each bolus was administered over a 20-s period. Measurements were obtained at the peak of the response to ANG II (within 60 s of the bolus).

To determine the receptor subtype that is responsible for the observed uterine response, three separate series of the ANG II boluses were given prior to and after each one of the receptor blockades. In the first series, PD-123319 (AT2-receptor antagonist) was infused directly into the uterine artery at the rate of 3 mg/min for 5 min. In the second series, L-158809 (AT1-receptor antagonist) was infused into the uterine artery at the rate of 0.3 mg/min for 5 min after which the ANG II dose-response curve was repeated. Finally, to make sure all AT1 receptors were blocked, L-158809 was infused at 3 mg/min for an additional 5 min and the responses to locally administered ANG II were repeated. The effects of PD-123319 and L-158809 on control responses to ANG II boluses were determined directly for mean arterial pressure and uterine blood flow. The systemic and uterine vascular resistances were calculated by dividing mean arterial pressure by either cardiac output or uterine blood flow, respectively.

Experimental protocol 1. To ascertain the mechanism responsible for the observed uterine response, two separate series of the ANG II boluses were given prior to and after each one of the receptor blockades. In the first series, PD-123319 (AT2-receptor antagonist) was infused directly into the uterine artery at the rate of 0.3 mg/min for 5 min. In the second series, L-158809 (AT1-receptor antagonist) was infused directly into the uterine artery at the rate of 0.3 mg/min for 5 min after which the ANG II dose-response curve was repeated. Finally, to make sure all AT1 receptors were blocked, L-158809 was infused at 3 mg/min for an additional 5 min and the responses to locally administered ANG II were repeated. The effects of PD-123319 and L-158809 on control responses to ANG II boluses were determined directly for mean arterial pressure and uterine blood flow. The systemic and uterine vascular resistances were calculated by dividing mean arterial pressure by either cardiac output or uterine blood flow, respectively.

Experimental protocol 2. To ascertain whether similar uterine responses occur when ANG II is given systemically, ANG II was infused in separate experiments into the femoral vein at the rate of 100 ng·kg⁻¹·min⁻¹ for 10 min. Once control responses to ANG II were obtained, PD-123319 (AT2-blockade) was given (3 mg/min for 5 min). The response to systemic ANG II was reevaluated at its peak response. The effects of AT1-receptor blockade during the systemic ANG II infusion was determined by infusing L-158809 at 0.3 mg/min for 5 min and then repeating the systemic ANG II infusion. Once this was complete, we increased the AT1-receptor blockade dose to 3 mg/min for 5 min (total of 15 mg) and once again repeated the systemic infusion.

Experimental protocol 3. This protocol was designed to investigate the mechanism by which ANG II uterine vascular responses were potentiated in the presence of AT2 blockade (see RESULTS and Fig. 2A and B). We sought to selectively stimulate the AT1 receptor in the presence of AT2-receptor blockade using L-158809. In these studies, ewes did not receive estrogen prior to this protocol to allow a vasodilatory response to be detected. An intrauterine artery infusion of ANG II was given for 10 min at a rate of 0.03 µg/min, which has previously been shown to reduce uterine blood flow by approximately 50%. After the uterine blood flow returned to baseline, the AT1 antagonist L-158809 (3 mg/min for 5 min) was infused into the uterine artery. The infusion of ANG II was then repeated, and increases in uterine blood flow from baseline were observed. To determine whether this vasodilation was mediated by AT1 receptors, PD-123319 (3 mg/min for 5 min) was infused into the uterine artery and then the ANG II infusion was repeated in four ewes. Finally, to determine whether the vasodilation was mediated by nitric oxide or a prostaglandin such as prostacyclin, animals received either N-nitro-L-arginine methyl ester (L-NAME) as a 10-mg bolus into the uterine artery or indomethacin (2 mg/kg) in the femoral vein. Uterine responses were then determined. Sixty minutes were allowed to elapse after the indomethacin was given.

Statistical analysis. Data are reported as means ± SE, either actual or percent change from baseline response. Multiple comparisons were determined by a two-way ANOVA or Student's paired t-test as appropriate. Comparisons within and between groups were made with ANOVA, and mean...
differences were determined with the appropriate post hoc test. P < 0.05 was considered significant.

RESULTS

ANG II as a direct uterine vasoconstrictor. In representative tracings, the mean arterial pressure (Fig. 1A) and uterine blood flows in the experimental and control (contralateral) sides (Fig. 1, B and C) are shown. A significant decrease in uterine blood flow is seen with an intrauterine arterial bolus injection of 1.0 µg of ANG II. This decrease in uterine blood flow is seen prior to any changes noticed in the mean arterial pressure. There are no changes in uterine blood flow in the contralateral uterine artery. This fact supports our hypothesis that ANG II acts as a direct vasoconstrictor in the uterine vasculature of the nonpregnant sheep and that the vasoconstriction seen with its infusion is due to local effects of ANG II and does not reflect changes in the systemic vasculature.

Experimental protocol 1: Intrauterine artery administration of ANG II. ANG II produced dose-related decreases in ipsilateral uterine blood flow in estrogenized ewes. Figure 2A illustrates the percent decrease in uterine blood flow from the estrogenized baseline (152 ± 8 ml/min) in response to bolus injections of 0.1, 0.3 and 1.0 µg of ANG II. Pretreatment of animals with the AT2-receptor antagonist PD-123319 significantly potentiated (P < 0.02) the ANG II vasoconstrictor responses at the two highest doses. Subsequent treatment of the uterine vasculature with the AT1-receptor antagonist L-158809 at 0.3 mg/min significantly inhibited the vasoconstriction seen with ANG II and the 3.0 mg/min dose abolished the vasoconstriction in the uterine vasculature (P < 0.01).

ANG II produced dose-related increases in calculated uterine vascular resistance as represented in Fig. 2B. After AT2 blockade, there was a potentiation of the uterine vascular resistance response by 48% over control at the 0.3 µg dose of ANG II and an increase of 93% at the 1.0 µg dose (P < 0.01). Subsequent infusion with
the AT₁ antagonist, at both doses, ablated this increase in uterine vascular resistance.

Figure 2C depicts the increase in mean arterial pressure, which occurred in response to local intrauterine artery administration of ANG II. Systemic arterial pressure was significantly increased in a dose-dependent fashion, ranging from a 3 ± 2% increase at 0.1 µg of ANG II to a 40 ± 5% increase at 1.0 µg ANG II. In contrast to the vasoconstriction potentiation seen in the uterus, the AT₂ antagonist significantly blunted the arterial pressure responses to ANG II. The AT₂ antagonist at both doses inhibited ANG II-induced increases in systemic arterial pressure (P < 0.001). As evidenced by the increase in MAP and subsequent antagonistic response seen, ANG II and the antagonists did have systemic effects when given locally at these doses.

Experimental protocol 2: Systemic administration of ANG II. Figure 3 shows the time relationship of mean arterial pressure, cardiac output, uterine blood flow, and calculated systemic and uterine vascular resistance during a 5-min infusion of ANG II (100 ng·kg⁻¹·min⁻¹). During the systemic infusion of ANG II, systemic vascular resistance increased, first reaching a peak at 2 min, and this is followed by a peak in uterine vascular resistance at 4 min. Mean arterial pressure reaches a peak at approximately 1 min, but uterine blood flow remains unchanged initially because the increased perfusion pressure is able to overcome the increase in uterine vascular resistance. The rise in uterine and systemic vascular resistance occurs almost simultaneously.

Figure 4, A and B, shows the mean arterial pressure and calculated systemic vascular resistance responses (percent increase from baseline) to the systemic infusion of ANG II (100 ng·kg⁻¹·min⁻¹). After the intrauterine arterial administration of the AT₂ inhibitor PD-123319 (3 mg/min for 5 min), no changes were observed in the ANG II-induced increase in pressure (Fig. 4A) or vascular resistance (Fig. 4B) compared with ANG II alone. As was seen in the uterine vasculature, AT₁ blockade significantly attenuated these systemic pressure responses (P < 0.001). AT₂ blockade had no effect on mean arterial pressure or systemic vascular resistance.

Although systemic administration of ANG II (100 ng·kg⁻¹·min⁻¹) produced only small decreases (4 ± 6%, Fig. 4C) in estrogenized uterine blood flow, marked increases in uterine vascular resistance (90 ± 12%, Fig. 4D) were observed. The magnitude of these responses was significantly (P < 0.05) increased in the presence of AT₂-receptor blockade and decreased following pretreatment with AT₁-receptor blockade (P < 0.001).

Experimental protocol 3. Figure 5 illustrates the uterine blood flow changes from baseline in response in nonestrogenized ewes to local intrauterine arterial administration of angiotensin of 0.03 µg/min before and after AT₁-receptor blockade. ANG II produced a significant decrease in uterine blood flow by 64 ± 3%, from a baseline of 17 ± 4 ml/min. After administration of the AT₁ antagonist into the uterine vasculature, a significant vasodilatory response was seen with ANG II infusion. To determine whether the vasodilation was due to AT₂ stimulation, responses were determined following AT₂-receptor blockade. As shown in Fig. 6, the vasodilatory response was reversed when the ANG II type 2 receptor was blocked (P < 0.05).

To determine whether the AT₂-mediated uterine vasodilation was associated with the local release of nitric oxide or prostaglandins, L-NAME or indomethacin was administered as a bolus after the uterine vasodilation was seen. Figure 7 characterizes the uterine blood flow changes in nonpregnant ewes (n = 4) in response to L-NAME, a nitric oxide synthetase inhibitor. L-NAME effectively reversed the uterine vasodilatory response to ANG II (P < 0.05). The possibility that this vasodilation was mediated by prostacyclin was also investigated in two ewes. Indomethacin, a cyclooxygenase inhibitor, had no effect on the uterine vasodilatory response, thus suggesting that this response is not mediated by prostaglandins (data not shown).

**DISCUSSION**

Our hypothesis for the present research was that uterine vasoconstriction in response to ANG II is mediated by AT₂ receptors because this is the predominant vascular receptor subtype in the pregnant and nonpregnant ovine uterus (3). However, in contrast to our initial hypothesis, the data from the present study indicate that in the ewe ANG II-stimulated uterine vasoconstriction is mediated by the AT₁ receptors. When these receptors were selectively antagonized, both the uterine and systemic vasoconstriction seen with ANG II was completely ablated. This was independent of the route of ANG II administration, local or systemic. These data are consistent with those of other studies, which have demonstrated that in tissues where AT₁ and AT₂ receptors coexist, vasoconstriction that...
occurs with ANG II is mediated entirely by the AT_1 subtype (6, 18, 21).

Although a definite function of the AT_2 receptor was not determined in the present study, a significant potentiation of the ANG II-induced decreases in uterine blood flow and increases in uterine vascular resistance was observed following pretreatment with PD-123319, the AT_2-receptor antagonist. This potentiation within the uterine vasculature was observed following both local and systemic ANG II administration. To our knowledge, this is the first time that potentiation of the vasoconstrictive effects of ANG II have been demonstrated with the inhibition of the AT_2 receptor. Two possible explanations exist. First, stimulation of AT_2 receptors could produce local endothelial or vascular smooth muscle production of a vasodilator such as

Fig. 4. Systemic administration of ANG II. A: percent increases in mean arterial pressure from baseline in response to systemic infusion of ANG II either alone or in presence of AT_1- or AT_2-receptor blockade. **P < 0.01, ***P < 0.001 vs. controls. B: percent increases in calculated systemic vascular resistance from baseline in response to systemic infusion of ANG II either alone or in presence of AT_1- or AT_2-receptor blockade. ***P < 0.001 vs. controls. C: percent decreases in uterine blood flow from baseline in response to systemic infusion of ANG II either alone or in presence of AT_1- or AT_2-receptor blockade. *P < 0.05 vs. controls. D: percent increases in calculated uterine vascular resistance from baseline in response to systemic infusion of ANG II either alone or in presence of AT_1- or AT_2-receptor blockade. **P < 0.01, ***P < 0.001 vs. controls.

Fig. 5. Effect of intrauterine artery ANG II infusion on uterine blood flow changes before and after AT_1 antagonist. *P < 0.05 vs. baseline.

Fig. 6. Effect of intrauterine artery ANG II infusion plus AT_1 blockade on uterine blood flow before and after AT_2 blockade. *P < 0.05 vs. baseline.
nitric oxide or prostacyclin, which in turn could antagonize the vasoconstrictor responses to ANG II, mediated via the AT<sub>1</sub> receptor. One of the novel observations that we made in the present study suggests that nitric oxide is released when ANG II is administered in the presence of AT<sub>1</sub>-receptor blockade. Second, PD-123319 could have some AT<sub>1</sub>-receptor agonistic properties such that the combination of ANG II and PD-123319 would lead to greater vascular responses. This, however, appeared unlikely because administration of PD-123319 directly into the uterine artery at the doses used in the present study had no observable vasoactive properties. The first possibility seems the most logical based on our data as well as on several reports in the literature (8, 9) that describe an increased vasopressor response to ANG II in mice lacking the AT<sub>2</sub> receptor. Also, Magness and co-workers (13) have shown that ANG II stimulates nitric oxide production in the uterine arteries of pregnant and nonpregnant ewes.

Although AT<sub>1</sub>-receptor blockade potentiated uterine vasoconstrictor responses to ANG II, systemic responses did not show similar modulation. In fact, in contrast to the potentiation of ANG II responses observed in the uterine vasculature following uterine artery PD-123319 administration, systemic responses were actually significantly reduced (Fig. 2C). Thus, although the dose of PD-123319 was high enough to have an effect on systemic arterial pressure responses to ANG II, no potentiation was observed. The mechanism by which this occurs is presently unknown.

The ANG II antagonists used in this study, L-158809 and PD-123319, are known to be specific for their respective receptors, AT<sub>1</sub> and AT<sub>2</sub>, in vitro at concentrations of less than 10<sup>-5</sup> M (4, 10, 17). The in vivo concentrations of the antagonists can be estimated from our infusion. By our calculations, the infusion of the antagonists at 3 mg/min for 5 min with an average uterine blood flow of 152 ml/min is approximately 4 x 10<sup>-5</sup> M at the peak. Systemic doses of the antagonists L-158809 and PD-123319 produced similar responses. Local infusions of the antagonists were selected in an attempt to ensure that the receptors in the uterus, and not just the systemic receptors, would be bound.

The renin-angiotensin system has been extensively studied in both the pregnant and the nonpregnant human as well as in animal models. In normal human pregnancy, serum levels of the components of the renin-angiotensin system (including renin, angiotensin, and aldosterone) are elevated compared with the nonpregnant state (16, 20). Yet, normal pregnant women are resistant to the vasoconstrictor effects of infused ANG II (7). This refractoriness may be very important because uterine blood flow steadily increases from 2% of cardiac output in the nonpregnant state up to 20% of cardiac output in late pregnancy, reaching a maximum value of 500 ml/min at term in human (19) and 1,200 ml/min in the ewe (17). Additionally, it is clear that diseases such as preeclampsia, which are associated with increased sensitivity to ANG II, are also associated with reduced uterine blood flow (1). Circulating ANG II levels in the mother and fetus, which are elevated in pregnancy, appear to be important in the regulation of uterine and/or umbilical blood flow because the use of ACE inhibitors in pregnancy is associated with a decrease in placental blood flow and oxygen delivery in the pregnant ewe (12, 15). ACE inhibitors are contraindicated in pregnancy because fetal hypoxia, fetal growth restriction, oligohydramnios, renal dysplasia, and intrauterine fetal death are noted with their use (10a). The fetal effects seen with ACE inhibitors are in part associated with the changes in uteroplacental blood flow and subsequent decreased perfusion of the fetus. Thus it appears that ANG II plays a role in the regulation of uteroplacental and umbilical blood flow dynamics, because inhibition of its actions is deleterious to the fetus. The present study supports the concept that ANG II may be an important regulator of uterine blood flow in the ewe, inasmuch as blockade of both AT<sub>1</sub> and AT<sub>2</sub> receptors modulates the vascular responses to ANG II in the uterus.

In the present study, we have evaluated whether ANG II is a direct-acting vasoconstrictor or acts by releasing a systemic vasoconstrictor as has been suggested by others (4). In the present study, it is clear that when ANG II is administered as an intrauterine artery bolus, local vasoconstriction occurs because the vasoconstriction is only seen in the uterine horn, which receives the ANG II, and not in the contralateral horn. Furthermore, systemic infusion of ANG II, which increases systemic vascular resistance, appears to increase uterine vascular resistance. Within 30 s of beginning the ANG II infusion, mean arterial pressure is increased by 9%, systemic vascular resistance has increased by 43%, and uterine vascular resistance is increased by 28%, whereas uterine blood flow is not changed. Thus, almost immediately, uterine vascular resistance begins to rise, indicating that vasoconstriction is occurring.

In conclusion, although the predominant ANG II receptor in both affinity and number in the ovine uterine vasculature is the AT<sub>2</sub> subtype, the vasoconstriction produced by ANG II appears to be mediated by the AT<sub>1</sub> receptor. Furthermore, blockade of the AT<sub>2</sub> receptor resulted in a potentiation of the uterine vasoconstrictor response to angiotensin, suggesting that the...
AT_2 receptor may serve a vital role in modulating uterine blood flow responses to ANG II. Although possible, it is not currently clear whether selective stimulation of AT_2 receptors will result in direct uterine vasodilation. If this mechanism occurs in pregnancy then this may in part explain why ACE inhibitors, which block ANG II formation, could cause reduced utero-placental blood flow.

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