JPP Journal of Pharmacy and Pharmacology

JPP 2002, 54: 1529–1534

Received February 20, 2002
Accepted July 17, 2002
DOI 10.1211/00223570263
ISSN 0022-3573

Rapid effect of progesterone on the contraction of rat aorta in-vitro

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Abstract

Progesterone induced rapid relaxation of KCl-induced contraction of rat aortic rings. The relaxant effect of progesterone on aortic rings was concentration-dependent (over the range of 10^{-10} to 10^{-5} M) and partially dependent on the endothelium. Application of a nitric oxide (NO) synthase antagonist N^G-monomethyl-L-arginine (L-NMMA, 10^{-5} M) after progesterone treatment partially inhibited the relaxant effects of progesterone. This suggested that part of the effect was through the production of nitric oxide. Washing out the steroid hormone in the bath solutions could quickly reverse the inhibitory effects of progesterone on phasic tension generation in aortic rings. Five minutes after washout, the tension generation in aortic rings was completely restored. Cultured endothelial cells from rat aorta increased release of NO into culture media in response to a 60-min exposure to progesterone. Aldosterone and dexamethasone were also tested, and failed to relax KCl-induced contraction of aortic rings. These data suggest that the vascular effects of progesterone are not mediated by a genomic action of this steroid, and that the vascular effects are mediated partially through endothelial NO production.

Introduction

It has long been known that hormone replacement therapy with estrogen reduces the cardiovascular morbidity and mortality in postmenopausal women, indicating that oestrogen has a protective effect on the cardiovascular system. Studies in humans indicated that hormone replacement is associated with improved arterial endothelial function in healthy postmenopausal women. The benefit was observed in both the estrogen therapy group and the combined hormone (estrogen and progesterone) group (McCrohon et al 1996).

In recent years, many studies suggested that the vasculature may be a direct target of estrogen and progesterone (Stumpf 1990; Orimo et al 1993; Shan et al 1994). 17β-Estradiol and progesterone caused vasorelaxation in isolated human omental arteries (Belfort et al 1996). In-vitro studies have indicated that progesterone caused relaxation in canine and rabbit coronary arteries (Miller & Vanhoutte 1991; Jiang et al 1992). Progesterone itself has been demonstrated to relax rat aortic rings pre-contracted with phenylephrine or KCl (Glusa et al 1997; Mukerji et al 2000).

Regarding the role of the endothelium in the relaxant effect of progesterone, the reports are inconsistent. In some studies, the effects of progesterone on vascular and myometrial contractility were suggested to be mediated through endothelial nitric oxide (NO) production (Belfort et al 1996; Goetz et al 1994; Omar et al 1995). In others, however, the vascular effects of progesterone were not found to be dependent on the endothelium (Jiang et al 1992; Goetz et al 1994). It is possible that the vasorelaxant mechanism is dependent upon the tissue examined, and the integrity of the endothelium.

The aims of this study were: to examine the effects of progesterone on the contractility of rat aortic tissue; to investigate the time course and the reversibility of the effects so that we can determine whether the effects are mediated by a conventional genomic mechanism or by a rapid non-genomic mechanism; and to determine if the effects of progesterone on aorta are dependent upon endothelial NO production.

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Methods

Chemicals

Progesterone, aldosterone, dexamethasone and chemicals for bath solutions were purchased from Sigma Chemical Co. (St Louis, MO). N^G-Monomethyl-L-arginine (L-NMMA) was from Research Biochemicals International (Natick, MA). Progesterone, aldosterone and dexamethasone were each dissolved in dimethyl sulfoxide–ethanol (2:1) for tension studies, and ethanol for the NO measurements. All dilutions were made in this solvent such that a sample of stock solution which was 1000 times the final bath concentration was added to the tissue chamber. This solvent mixture was demonstrated to have no effect on force generation by aortic rings (data not shown). L-NMMA was dissolved in water before addition to the tissue chamber.

Force measurement in rat aortic rings

Isometric contraction was measured in rat aortic rings following the method of Pang et al (1985). Male Sprague-Dawley rats, 250–350 g, were anaesthetized with pentobarbital (65 mg kg^{-1}, i.p.) and the thoracic aortas were removed. The tissue was cleaned of adherent connective tissues and cut into rings, 2–4 mm in length. In some of the rings, the endothelial layer was removed (endothelium-denuded, ED). A long strand of cotton with approximately the same diameter as the aortic ring was inserted into the lumen of the rings. By carefully sliding the aortic rings back and forth along the cotton strand, the endothelial layer was removed and verified as described below. Particular care was taken during this procedure to prevent any damage to the underlying tissue. The aortic rings were then suspended in a Sawyer–Bartlestone tissue bath chamber. The bath solution used was Krebs–Henseleit solution (KHS) with the following composition (mM): 115 NaCl, 5 KCl, 2.1 $CaCl_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 11 glucose, pH 7.4. The tissue inside the bath chamber was attached to an FT.03 Grass force displacement transducer and the force of the tissue was recorded on a Grass 79 D polygraph (Grass Instrument Co., MA) or a Gould polygraph (Gould Inc., Cleveland, OH). The tissue was continuously aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Before experimentation, the tissue was allowed to equilibrate for 60–120 min under a resting force of 1 g. During the equilibration period, tissue in the bath chamber was washed with KHS every 15 min. After equilibration, the tissue was tested for force generation in response to stimulation with 30 mM KCl. The stimulation procedure was repeated twice at 30-min intervals. Only those aortic rings showing reproducible contractions were used for the studies. An additional test was conducted with the denuded aortic rings to determine whether the endothelium had been completely removed. After force generation stimulated by 30 mM KCl reached a stable level, acetylcholine, at a final concentration of 10^{-5} M, was added to the bath. Aortic rings with intact endothelium (EI) exhibited a 40–50% decline in force while aortic rings completely denuded of endothelium (ED) showed no significant change in response to exposure to acetylcholine.

Experimental design

Tonic force was measured in response to 30 mM KCl stimulation. When the force reached a plateau, experiments were carried out to determine the effects of progesterone, L-NMMA or other steroids on the tonic force. In some experiments, progesterone was applied cumulatively (10^{-10} to 10^{-5} M) to obtain a dose–response curve.

Phasic force was generated in aortic rings by 30 mM KCl. When the peak force reached plateau, it was recorded as the control value and the tissue was washed. After a recovery period of 30 min, the tissue was stimulated a second time with the same dose of KCl. Progesterone was applied 15 min before the second KCl stimulus.

To test the reversibility of the effects of progesterone on aortic force generation, the initial phasic force in response to 30 mM KCl was recorded and used as control. Progesterone was added after wash and recovery. Fifteen minutes after incubation with progesterone, 30 mM KCl was applied to some aortic rings for the second time to see the effect of progesterone on the force generation. Other tissues were washed for different periods of time before applying the second KCl stimulation to see the time course of the reversal of progesterone effect. The difference in force generation in the two KCl stimulations was analysed.

Measurement of NO production by cultured endothelial cells

Endothelial cells were prepared as described previously (Wang et al 1999). They were grown in 35-mm^2 dishes. Upon reaching confluence in about 4 days, fresh HBSS with L-arginine (100 μM) and $CaCl_2$ (to 2.5 mM) was added to the dishes. The cells were then equilibrated for 60 min at 37°C. Samples of the supernatant were collected for analysis of nitrite/nitrate by chemiluminescence (considered as 100%). Progesterone (10^{-9} to 10^{-5} M) or vehicle was then added for 60 min to stimulate NO release. The cell supernatants were collected for analysis of nitrite/nitrate as previously described (Wang et al 1999). Samples (100 μL) containing nitrite/nitrate were measured by adding a reducing agent (0.7% VCl_3 in hydrochloric acid) to the purge vessel to convert nitrite/nitrate to NO, which was then carried by a flow of helium to the NO analyser (Model 280, Sievers Research Inc., Boulder, CO). Nitrite/nitrate concentrations were calculated by comparison with standard solution of sodium nitrite. Total protein concentration of each dish was determined spectrophotometrically. The results were normalized to amount (pmol) per mg protein.

Data analysis

The data were presented as mean ± s.e.; n in the force generation figures indicates the number of rings used in the experiments. Aortic tissue from each rat was used to prepare two aortic rings. In the NO studies, n indicates the

number of dishes of cultured cells. The paired or non-paired Student's *t*-test was used for comparisons between two groups. The Newman–Keul's test was applied when the comparison was among multiple groups. *P* values less than 0.05 were considered statistically significant.

Results

The relaxant effects of progesterone on tonic force development in aortic rings

Figure 1 presents the relaxation induced by progesterone on aortic rings with and without endothelium. The tissue was first stimulated with 30 mM KCl to generate a tonic contraction. Progesterone was then administered at final concentrations of 10^{-10} to 10^{-5} M. In EI aortic rings, the relaxant effects were significant when the concentration of progesterone was at or above 10^{-7} M (force decreased by $11.0 \pm 2\%$ at 10^{-7} M and by $55.6 \pm 4\%$ at 10^{-5} M). In the ED aortic rings, 10^{-5} M progesterone significantly decreased force by $27.2 \pm 2\%$. In this study, the highest concentration used was 10^{-5} M. The EI tissue is more sensitive than ED tissue to the relaxant effects of progesterone.

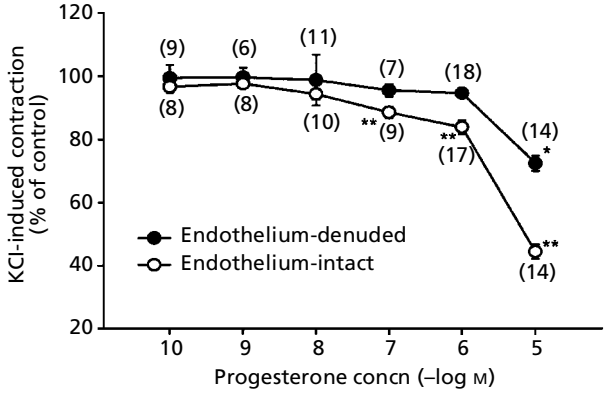

Figure 1 The effect of progesterone on tension in endothelium-intact or -denuded rat aortic rings. Progesterone (10^{-10} to 10^{-5} M) was applied after the tissue was stimulated with KCl (30 mM). Data are presented as percentage changes in tension compared with the tension generated by KCl. Numbers in parenthesis indicate the numbers of samples recorded. $*P < 0.05$, $**P < 0.01$, compared with control values.

Figure 2 shows an original recording of force generation in aortic rings. It is apparent that the effect of progesterone (10^{-5} M) on aortic rings occurred immediately after the application of progesterone in both EI and ED tissues. In this particular case, force was decreased by 52% in EI tissue and 29% in ED tissue.

Endothelium-dependent effects of progesterone on force generation in aortic rings

L-NMMA was used to determine whether NO production was involved in the action of progesterone. L-NMMA was applied once the relaxant action of 10^{-5} M progesterone had been established. Figure 3 presents the summary data indicating that L-NMMA could partially reverse the relaxant effects of progesterone in EI tissue. KCl (30 mM) was first applied to generate a maximal force (control), and then progesterone (10^{-5} M) was applied to relax the force. L-NMMA (10^{-5} M) then partially, but significantly, reversed the effect of progesterone in EI tissue. In ED tissue, however, the relaxation induced by progesterone (10^{-5} M) was not significantly reversed by L-NMMA.

Fast reversal of the effects of progesterone on force generation in aortic rings

The rapid onset of relaxation by progesterone suggested that this action was not mediated via altered gene expression. To further examine this possibility, experiments

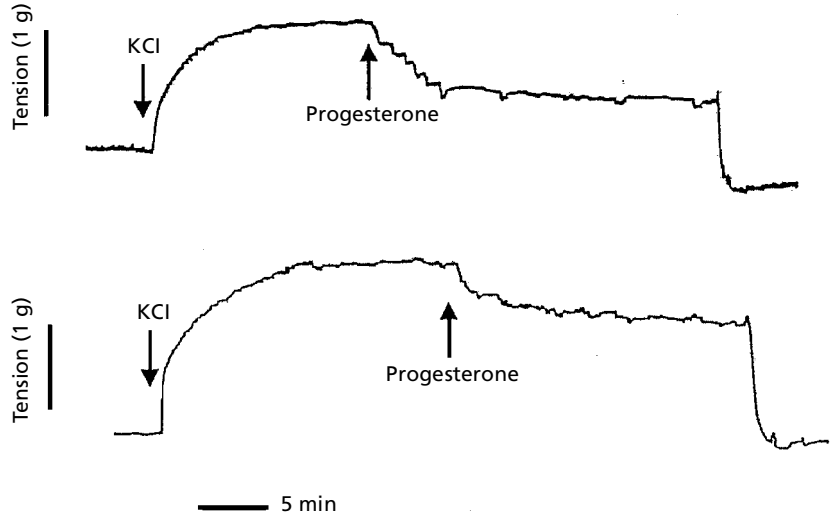

Figure 2 Relaxation effect of progesterone (10^{-5} M) on tonic tension in endothelium-intact (upper panel) or -denuded (lower panel) rat aortic rings. The concentration of KCl used was 30 mM.

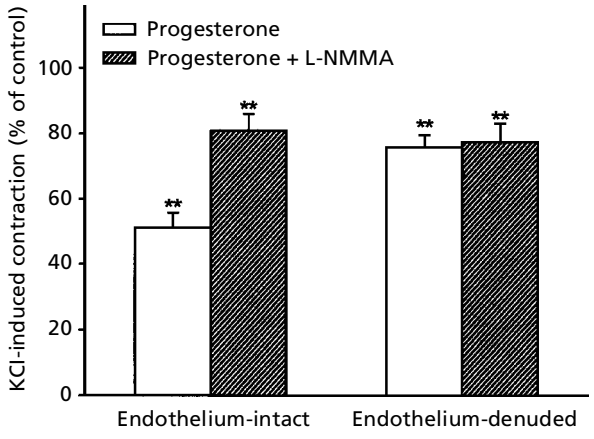

Figure 3 Reversal of the effects of progesterone (10^{-5} M) by L-NMMA (10^{-5} M) on tonic tension in rat aortic rings, showing a partially endothelium-dependent effect. L-NMMA (10^{-5} M) was applied after the effect of progesterone occurred. n = 8, $^{**}P < 0.01$, compared with the control level.

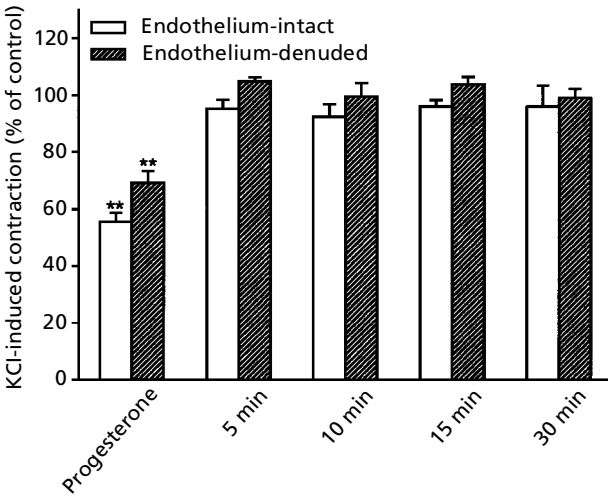

Figure 4 Reversal by washout of the effect of progesterone (10^{-5} M) on phasic tension generation in rat aortic rings. The tissue was stimulated by KCl (30 mM) first as a control; after washout, progesterone was applied and incubated for 15 min before the wash procedure started. After washout for different period of time, a second stimulation by KCl was applied to compare with the control. n = 6–9, $^{**}P < 0.01$, compared with the control level.

that tested the reversibility of progesterone actions were performed. Results are presented in Figure 4. Both EI and ED tissues were stimulated by 30 mM KCl to record a control force. After wash and recovery, the tissues were treated with progesterone (10^{-5} M) for 15 min. Then tissues were washed for 5, 10, 15 or 30 min to remove progesterone. Subsequently, the tissues were subjected to a second KCl challenge. After washout for 5 min or longer, force generation returned to the control level. Progesterone did not have any lingering influence on tissue.

Effects of aldosterone and dexamethasone on force generation in aortic rings

To determine whether the relaxant actions exhibited by progesterone might be non-specific, and related to the steroid structure, two other steroid hormones were tested for their ability to relax aorta. There was no significant change in phasic force generated in response to 30 mM KCl by the subsequent application of 10^{-5} M aldosterone or 10^{-5} M dexamethasone (data not shown).

Effects of progesterone on NO production by cultured aortic endothelial cells

Endothelial cells harvested from rat aorta were grown in culture and acutely exposed to varying concentrations of progesterone. Progesterone may act directly on endothelial cells to stimulate NO production (Figure 5). Furthermore, the short (60 min) exposure to progesterone that causes NO release precludes a genomic action of this steroid.

Discussion

It has been well established that progesterone maintains pregnancy in mammals by inhibiting the contraction of the myometrium. However, contradictory results were obtained in studies conducted on the vascular system. Investigations with human uterine arteries showed that both estrogen and progesterone at a concentration range of 2×10^{-7} to 5×10^{-5} M had inhibitory effects on contractions induced by K^+ depolarisation. At lower doses (2×10^{-8} M), however, the arterial response to vasopressin was enhanced (Kostrzewska et al 1993). Progesterone also

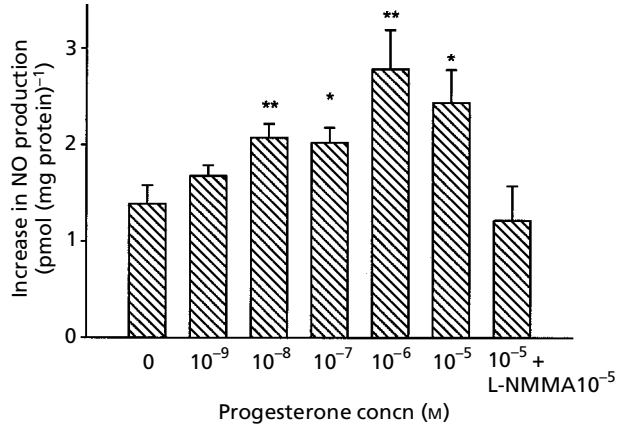

Figure 5 Effect of progesterone (10^{-9} to 10^{-5} M) on NO production by cultured aortic endothelial cells. Endothelial cells isolated from Sprague-Dawley rat thoracic aorta were incubated in HBSS containing Ca^{2+}. Following exposure to progesterone for 60 min, samples of the media were collected for assessing production of NO, as described in Methods. Basal NO content was 15.21 ± 0.80 pmol (mg protein)$^{-1}$. Values are mean$\pm$s.e., n = 5 in each group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control group (0 M progesterone).

inhibited the contraction of rabbit common iliac arterial rings (Hidaka et al 1991) and portal vein (Mukerji et al 2000). In rabbits, progesterone administered with estradiol increased the coronary flow while progesterone alone did not show any significant effect (Gorodeski et al 1998). Other studies with rat aorta demonstrated that KCl-induced contraction was more sensitive to inhibition by progesterone than was phenylephrine-induced contraction (Glusa et al 1997). It should be noted that some inconsistencies might be expected between studies that examine different vascular tissues, and different vasoconstrictors.

Reports on the endothelium dependency of the relaxation effect of progesterone are inconsistent as well. In investigations using human arteries, some results showed that the effect of progesterone was endothelium dependent (Woolfson et al 1992) while others showed that it was endothelium independent (Omar et al 1995). Studies on animals also yielded controversial results. Among the studies, rabbit common iliac arteries (Hidaka et al 1991) and canine coronary arteries (Miller & Vanhoutte 1991) exhibited an endothelium-dependent effect while rabbit coronary artery (Jiang et al 1992) and rat aorta (Glusa et al 1997) exhibited endothelium-independent effects. Experiments with rat aorta showed that estrogen, but not progesterone, increased NO synthase (NOS), which is responsible for the production of NO (Goetz et al 1994). Therefore, the involvement of the endothelium may be dependent on the species and the vascular bed investigated.

In this study, progesterone showed obvious relaxant and inhibitory effects on vasoconstriction stimulated by K^+ depolarisation. These effects were concentration dependent (10^{-10} to 10^{-5} M). These results indicate that progesterone acted directly on the blood vessel wall. When EI and ED aortic rings are compared, the relaxant effects of progesterone were always greater in the EI tissue. L-NMMA, an NO synthase blocker, partially reversed the relaxant effect of progesterone in EI tissue while no reversal was observed in ED tissue. Figure 3 shows that after L-NMMA application, the relaxant effect of progesterone was partially reversed. The force then settled to approximately the same level as in ED tissue. In other words, the effect of L-NMMA in this case appeared to confirm the involvement of NO production. Differences in the extent of the effects between EI and ED tissue indicated that part of the effect was endothelium dependent, and may be mediated through NO produced by the endothelium. A direct action of progesterone on endothelial NO production was demonstrated using cultured aortic endothelial cells. These results suggest that progesterone acted both directly on the smooth muscle and on the endothelium. The ability of L-NMMA to inhibit the action of progesterone in the EI tissue indicates that the relaxant action mediated via endothelial cells may be explained by NO production, while relaxation independent of endothelium may be through a different mechanism.

During the last two decades, numerous studies on the rapid, non-genomic effects of steroids have been published for almost every group of steroid hormones. These effects are presumably non-genomic because they are rapid in onset and not modified by inhibitors of protein synthesis (Schumacher 1990; Cabral et al 1994). Functional non-genomic progesterone receptors have recently been identified in human spermatozoa (Luconi et al 1998).

Studies on rabbit coronary artery indicated that progesterone at micromolar, or higher, concentrations induced relaxation in K^+, prostaglandin or Bay K 8644 pre-contracted arteries (Jiang et al 1992). Progesterone also elicited rapid relaxation of human placental arteries (Omar et al 1995) and rapid relaxation of rat aorta (Glusa et al 1997; Mukerji et al 2000). Rat portal vein was also sensitive to relaxation by progesterone, and the mechanism of action in this tissue was proposed to involve potassium-channel activation (Mukerji et al 2000). Other reports have shown that inhibitory effects of progesterone on myometrium contraction were fast in onset (approx. 10 min) and related to the inhibition of Ca^{2+} influx (Perusqúa et al 1990; Gutierrez et al 1994; Glusa et al 1997). Inhibitors of protein synthesis (such as cycloheximide) or inhibitors of transcription (such as actinomycin) did not modify these effects. Recently, cDNA encoding a progesterone membrane binding protein was isolated from porcine vascular smooth muscle cells (Falkenstein et al 1996), which may account for the fast actions of progesterone.

The circulating progesterone levels in premenopausal women is 10^{-9} to 10^{-8} M. We noted that the effective concentrations of the steroids used in this study were relatively high (10^{-7} to 10^{-5} M). In investigations of the non-genomic effects of steroids, many reports have shown the effective concentrations of steroids to be higher than physiological concentrations. Concentrations of progesterone were 10^{-6} to 10^{-3} M for studies of smooth muscle contractility (Jiang et al 1992; Thomas et al 1995) and $[Ca^{2+}]_i$ mobilization (Blackmore et al 1990; Sanchez-Beuno et al 1991; Föresta et al 1995). In their comprehensive review, Duval et al (1983) discussed the reasons for the requirement of supra-physiological doses in investigations of the non-genomic effects of steroids. They pointed out that circulating hormone concentrations do not accurately reflect those in target tissues. Tissue concentrations may well be higher than plasma concentrations. It is important to note that high levels of steroids could occur under either pathological or physiological conditions. The circulating level of progesterone in pregnancy can reach up to 10^{-6} M. The concentration of free steroids in the blood may also vary as a function of plasma steroid-binding proteins. In these preliminary studies, we were seeking substantial and consistent effects in-vitro. The concentrations needed to produce these effects may be higher than those required for more subtle physiological actions in-vivo.

In summary, this study extends previous findings on the acute effects of progesterone on rat aorta. Progesterone had relaxant effects on force generation in rat aortic rings. The effects of progesterone on rat aortic tissue were likely due to direct action on vascular smooth muscle as well as on endothelium. The action could be reversed by the NOS antagonist L-NMMA in EI aorta, indicating that progesterone acted directly on endothelium to induce NO production. This was supported by the observation that progesterone stimulated NO production by cultured endothelial cells. The vascular effects were fast in onset and

could be quickly reversed by washout. Therefore, they were most likely mediated by a non-genomic mechanism and may occur through a membrane process.

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