[Leu]enkephalin stimulates carbohydrate metabolism in isolated hepatocytes and kidney tubule fragments by interaction with angiotensin II receptors

Sukbinder K. HOTHI, David P. RANDALL and Michael A. TITHERADGE*

Biochemistry Group, School of Biological Sciences, University of Sussex, Brighton BN1 9QG, U.K.

The possibility that the effects of [Leu]enkephalin *in vitro* on hepatic carbohydrate metabolism are mediated by interaction with angiotensin II receptors has been examined. Preincubation of hepatocytes with either the angiotensin II receptor antagonist [Sar¹,Ile⁸]angiotensin II or 10 mM-dithiothreitol abolished the ability of both angiotensin II and [Leu]enkephalin to increase phosphorylase *a* in hepatocytes prepared from fed rats. Dithiothreitol had no effect on the stimulation of phosphorylase in the presence of glucagon or phenylephrine, although it also inhibited the response to vasopressin. [Leu]enkephalin displaced specifically bound ¹²⁵I-labelled angiotensin II from hepatic plasma membranes over a concentration range of 10⁻⁷–10⁻⁵ M. This correlated with the dose-response required to stimulate phosphorylase activity in intact hepatocytes and suggests that the effects of the opioid peptides on carbohydrate metabolism in liver are the result of cross-reactivity of the peptides with angiotensin II receptors. Addition of 10⁻⁵ M-[Leu]enkephalin to isolated kidney tubule fragments stimulated gluconeogenesis from 5 mM-pyruvate, the magnitude of stimulation being comparable to that by either angiotensin II or adrenaline. This effect of the opioid peptide was also abolished by pretreatment of the tubules with [Sar¹,Ile⁸]angiotensin II, suggesting that the ability of [Leu]enkephalin to interact with angiotensin II receptors is not restricted to the liver, but may occur in other tissues where both receptors occur together.

INTRODUCTION

Previous studies have demonstrated that the addition of the opioid peptides, [Leu]enkephalin, [Met]enkephalin, dynorphin-(1–13) and β -endorphin, to isolated hepatocytes results in rapid, dose-dependent stimulation of both glycogenolysis and gluconeogenesis (Allan *et al.*, 1983; Matsumura *et al.*, 1984; Leach *et al.*, 1985; Leach & Titheradge, 1986; Hothi *et al.*, 1988). With the exception of β -endorphin (Matsumura *et al.*, 1984), the response of hepatic carbohydrate metabolism to these peptides is mediated by an increased inositol lipid turnover and a mobilization of intracellular Ca²⁺ (Leach *et al.*, 1986), while cyclic AMP formation and adenylate cyclase activity are simultaneously inhibited (Leach & Titheradge, 1986; Hothi *et al.*, 1988). Specific binding of β -endorphin to receptors has been demonstrated in liver (Dave *et al.*, 1985) and although the ability of the enkephalins and dynorphin to inhibit adenylate cyclase activity is suggestive of the existence of specific opiate receptors similar to those reported in neuronal tissue (Sharma *et al.*, 1975; Blume *et al.*, 1979; Cooper *et al.*, 1982), we have been unable to demonstrate specific binding at opiate receptors for either enkephalins or dynorphin analogues in liver plasma membranes (Khawaja *et al.*, 1987a). *In vivo* experiments designed to delineate a direct role for opiate agonists in the regulation of carbohydrate metabolism have failed to produce consistent alterations in plasma glucose levels (Stubbs *et al.*, 1978; Leslie *et al.*, 1979; Leslie *et al.*, 1982); however, this may reflect the transient parallel increases in both glucose production and utilization reported by Werther *et al.* (1985). Therefore experiments were carried out to

characterize further the response of the liver to opioid peptides to determine whether the *in vitro* response may be explained by binding to sites other than opiate receptors in the liver.

EXPERIMENTAL

Materials

[Leu]enkephalin, des-Tyr-[Leu]enkephalin, angiotensin II, [Sar¹,Ile⁸]angiotensin II and collagenase were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). ¹²⁵I-labelled angiotensin II and [U-¹⁴C]glucose-1-phosphate were obtained from Amersham International (Amersham, Bucks., U.K.). Glucagon was a gift from Novo Laboratories (Basingstoke, Hants., U.K.). All other chemicals were either of AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.) or from Sigma.

Methods

Hepatocytes were prepared from fed male Sprague-Dawley rats weighing 180–240 g, essentially by the method of Berry & Friend (1969) as described previously (Allan *et al.*, 1983). The cells were resuspended in Krebs–Henseleit (1932) bicarbonate buffer, pH 7.4, to a final concentration of approx. 60 mg wet wt./ml and incubated at 37 °C under an atmosphere of O₂/CO₂ (19:1). The cells were routinely preincubated for 30 min before the addition of the hormones or effectors, and samples were removed after a further 3 min of incubation for the determination of phosphorylase *a* activity. Where appropriate, [Sar¹,Ile⁸]angiotensin II was added 5 min prior to the addition of angiotensin II or [Leu]enkephalin.

* To whom correspondence and reprint requests should be addressed.

Phosphorylase *a* activity was determined as described previously (Leach *et al.*, 1985).

Rat kidney tubule fragments were prepared from fed rats essentially as described by MacDonald & Saggerson (1977), except that the animals were not treated with heparin prior to the removal of the kidneys and the cortex was finely chopped and forced through a fine nylon mesh prior to incubation with the collagenase. The tubules were resuspended in Krebs-Henseleit bicarbonate buffer containing 10 mg of fatty acid free albumin/ml to a final concentration of 1 mg wet wt./ml. The rate of gluconeogenesis from 5 mM-pyruvate was determined by removing 200 μ l samples at the appropriate time intervals and immediately deproteinizing with 20 μ l of 2.7 M-perchloric acid. After neutralization, glucose output was determined fluorimetrically using hexokinase (Lowry & Passonneau, 1972). The control rate of glucose production was 117 ± 9 μ mol/h per g dry wt. of tissue ($n = 6$), which is comparable to previously reported values (Guder & Rupprecht, 1975; MacDonald & Saggerson, 1977; Guder, 1979; Saggerson & Carpenter, 1979; Kessar & Saggerson, 1980). All of the preparations tested responded to hormones, although the degree of stimulation was variable and dependent on the preparation.

Rat liver plasma membranes were prepared essentially by the method of Pohl (1976) as modified by Campanile *et al.* (1982). The preparation medium consisted of 10 mM-Tris, pH 7.4, 5 mM- $\text{Na}_2\text{-EDTA}$ and 0.1% β -mercaptoethanol. Binding experiments were carried out in an incubation volume of 100 μ l containing 100 mM-NaCl, 10 mM-MgCl₂, 20 mM-Tris, pH 7.5, 0.2% bovine serum albumin and 0.05 mg of membrane protein. ¹²⁵I-angiotensin II was added to a final concentration of between 0.1–0.3 nM (approx. 50000 c.p.m./tube), along with varying concentrations of unlabelled angiotensin II. Non-specific binding was measured in the presence of an excess of unlabelled angiotensin II (10^{-6} M final concentration) and represented 5–10% of the total binding. Preliminary experiments showed that under our conditions the optimal incubation time was 2 h at 22 °C. Separation of bound and free angiotensin II was achieved by addition of 5 ml of ice-cold incubation medium prepared without the albumin, followed by rapid filtration through Whatman GF/B glass fibre filters. The incubation tube and filter was then washed with two further 5 ml aliquots of the same incubation medium and the radioactivity determined by counting the filters in an LKB 1260 Multigamma II gamma counter. Each determination was carried out in triplicate and the results presented were obtained from experiments using at least three separate membrane preparations. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V) as the standard.

Results are expressed as means $\pm$ S.E.M. with the number of observations given in parentheses. Statistical analysis of the results was performed by using a pooled *t*-test.

RESULTS AND DISCUSSION

Effect of [Sar¹,Ile⁸]angiotensin II on the increase in phosphorylase *a* in isolated hepatocytes following treatment with angiotensin II and [Leu]enkephalin

We have previously demonstrated that the effect of the

Fig. 1. Effect of increasing concentrations of [Sar¹,Ile⁸]-angiotensin II on the activation of phosphorylase by [Leu]enkephalin and angiotensin II in isolated hepatocytes

Isolated hepatocytes were preincubated for 25 min before the addition of increasing concentrations of [Sar¹,Ile⁸]-angiotensin II. At 30 min either 10^{-5} M-[Leu]enkephalin or 10^{-6} M-angiotensin II was added and the incubation continued for a further 3 min before samples were removed for the determination of phosphorylase *a* activity. The results shown are the means $\pm$ S.E.M. for 4 separate hepatocyte preparations. Symbols: ○ control incubation; □, control plus 10^{-5} M-[Sar¹,Ile⁸]angiotensin II; ■, [Leu]enkephalin; ●, angiotensin II.

opioid peptides on hepatic carbohydrate metabolism is not inhibited by naloxone, a relatively non-specific opiate receptor antagonist (Allan *et al.*, 1983). Similarly, experiments to demonstrate specific mu, delta or kappa receptors for opioid peptides in liver membranes have proved negative (Khawaja *et al.*, 1987a). The metabolic response of the liver to [Leu]enkephalin has proved to be very similar to that to the catecholamines or angiotensin II (Leach & Titheradge, 1986; Leach *et al.*, 1986; Hothi *et al.*, 1988), although it is not affected by either α - or β -adrenergic antagonists, precluding the involvement of these receptors (Leach *et al.*, 1985). To examine the possibility of cross-reactivity of opioid peptides with angiotensin II receptors the effect of increasing concentrations of [Sar¹,Ile⁸]angiotensin II, a specific antagonist of angiotensin II receptors (Bumpus, 1977), on the stimulation of phosphorylase *a* activity in hepatocytes by 10^{-5} M-[Leu]enkephalin and 10^{-6} M-angiotensin II was examined (Fig. 1). Addition of [Sar¹,Ile⁸]angiotensin II had no significant effect in control cells, however it resulted in a dose-dependent antagonism of the effect of both angiotensin II and [Leu]enkephalin. The effect of the opioid peptide was slightly more sensitive to the angiotensin II antagonist at low concentrations, suggesting a lower affinity of the receptor for the enkephalin than for angiotensin II or its analogue.

Table 1. Effect of dithiothreitol on the hormonal stimulation of phosphorylase activity in isolated hepatocytes

Isolated hepatocytes were preincubated for 30 min in the presence or absence of 10 mM-dithiothreitol. The hormones were then added to the final concentrations indicated and samples removed for the determination of phosphorylase *a* activity after 3 min. The results shown are means $\pm$ S.E.M. for four separate cell preparations except for the experiments with glucagon and phenylephrine which were for three. * $P < 0.01$, ** $P < 0.001$ for difference from control in the absence of dithiothreitol; † $P < 0.05$ for difference in the appropriate incubation in the presence and absence of dithiothreitol.

Hormone	Phosphorylase <i>a</i> activity (μ mol/min per g dry wt. of cells)	
	– Dithiothreitol	+ Dithiothreitol
Control	43.4 $\pm$ 2.9	46.2 $\pm$ 3.1
Angiotensin II, 10^{-6} M	67.9 $\pm$ 3.8**	46.9 $\pm$ 2.9†
Phenylephrine, 10^{-5} M	58.7 $\pm$ 3.8**	60.7 $\pm$ 3.7**
Glucagon, 10^{-9} M	59.6 $\pm$ 1.1**	56.6 $\pm$ 2.1**
[Leu]enkephalin, 10^{-5} M	68.7 $\pm$ 4.3*	46.6 $\pm$ 2.5†
Vasopressin, 10^{-7} M	65.3 $\pm$ 4.3*	47.2 $\pm$ 3.2†

Effect of dithiothreitol on the activation of phosphorylase by angiotensin II and [Leu]enkephalin

It is evident that liver contains two binding sites for angiotensin II, one high and one low affinity site (Campanile *et al.*, 1982; Gunther, 1984). On the basis that dithiothreitol inhibits both the binding of angiotensin II to the high affinity sites and the activation of phosphorylase in hepatocytes, but not binding to low affinity sites or the inhibition of adenylate cyclase, it has been suggested that the high affinity site is linked to a mobilization of intracellular calcium whereas occupation of the low affinity site is coupled to the inhibition of adenylate cyclase (Gunther, 1984). [Leu]enkephalin displays both of these characteristic responses (Hothi *et al.*, 1988) and therefore it was of interest to determine whether incubation of hepatocytes with 10 mM-dithiothreitol abolished the activation of phosphorylase. Table 1 shows that addition of dithiothreitol completely prevented the increase in phosphorylase *a* in the cells in the presence of angiotensin II, while it had no effect on the response to either glucagon or phenylephrine. This confirms the previous observation of Gunther (1984) and is in agreement with the suggestion that the effect of dithiothreitol is the result of a specific inhibition of angiotensin II binding to its receptor rather than it having an effect on the mobilization of calcium by hormones or the activation of phosphorylase, as the effects of phenylephrine and glucagon were unchanged. Preincubation of the cells with dithiothreitol also abolished the effect of [Leu]enkephalin, which may be a further indication that the opioid peptides exert their response in liver through angiotensin II receptors. However this interpretation is not unequivocal as the increase in phosphorylase *a* in cells incubated with vasopressin was also inhibited by dithiothreitol. Whilst it is possible that the binding of vasopressin to its receptor in liver is also susceptible to inhibition by thiol reagents, it is conceivable that inhibition occurs at another site in the transduction system which is common to these agents but which is not identical to that used by the catecholamines.

Effect of [Leu]enkephalin and its des-Tyr derivative on ^{125} I-angiotensin II binding to hepatic plasma membranes

To determine whether [Leu]enkephalin was specifically

binding to angiotensin II receptors or whether there was a non-specific inhibitory action of [Sar¹,Ile⁸]angiotensin II at opiate receptors, the effect of the enkephalin and its inactive des-Tyr derivative on the binding of ^{125} I-angiotensin II was studied. ^{125} I-angiotensin II showed specific, saturable binding to purified liver plasma membranes in the presence of 100 mM-Na⁺ ions and 10 mM-Mg²⁺ ions. Analysis of the binding parameters by Scatchard analysis (Scatchard, 1949) indicated that the binding data were best fitted to a two-site model with a K_{d1} of 0.11 ± 0.02 nM, $N_1 = 277 \pm 16$ fmol/mg of protein and K_{d2} of 4.7 ± 1.1 nM, $N_2 = 1054 \pm 81$ fmol/mg of protein. These results are in close agreement with those of Campanile *et al.* (1982) and Gunther (1984). Fig. 2 shows a correlation of the ability of [Leu]enkephalin and angiotensin II to displace ^{125} I-angiotensin II bound to liver plasma membranes with the activation of phosphorylase in intact hepatocytes. Using either effector, both parameters showed a similar dose-response relationship. The half-maximal effect on radiolabelled ligand displacement and phosphorylase activation occurred at approximately 10^{-6} M for [Leu]enkephalin and 10^{-9} M for angiotensin II. These results indicate that [Leu]enkephalin does indeed cross-react with angiotensin II receptors in liver. The affinity of the receptor for [Leu]enkephalin is approximately 3 orders of magnitude less than that for angiotensin II, being comparable to the affinity of the receptor for angiotensin I (Campanile *et al.*, 1982). However, at saturating concentrations of the enkephalin a maximal response is produced which is indistinguishable from that of angiotensin II itself (Hothi *et al.*, 1988). Although the des-Tyr derivative of [Leu]enkephalin is inactive in hepatocyte preparations (Leach *et al.*, 1985), at concentrations between 10^{-5} M and 10^{-3} M there was a dose-dependent decrease in ^{125} I-angiotensin II binding. Whether this represents specific binding to the receptor without activation remains to be established; however it may be a non-specific protein effect as addition of vasopressin and glucagon also caused significant displacement of label at these concentrations (results not shown). Addition of the opiate receptor antagonist, naloxone, resulted in no displacement of the ^{125} I-angiotensin II at any concentration tested between 10^{-8} M and 10^{-3} M, thus explaining its inability to antagonize the effects of the opioid peptides in liver.

Fig. 2. Displacement of bound ^{125}I -angiotensin II from purified liver plasma membranes and activation of phosphorylase activity

^{125}I -angiotensin II was incubated with purified liver membranes at a final concentration of between 0.1–0.3 nM (50 000 c.p.m./tube) in the presence of increasing amounts of unlabelled angiotensin II ($\square$), [Leu]enkephalin ($\blacksquare$) or des-Tyr-[Leu]enkephalin ($\bullet$). After incubating for 2 h at 22 °C, the bound and free ligands were separated by rapid filtration. The data have been corrected for non-specific binding and are plotted as a percentage of the control binding measured in the absence of competing peptides (solid lines). Phosphorylase α activity was measured in isolated hepatocytes 3 min after the addition of [Leu]enkephalin ($\blacktriangle$) or angiotensin II ($\blacktriangledown$) as described in the Experimental section (dotted lines). The results shown are the means $\pm$ S.E.M. for 6 separate experiments using 3 different membrane preparations for the binding experiments and 6 different cell preparations for the phosphorylase results.

Effect of [Sar¹,Ile⁸]angiotensin II on the stimulation of gluconeogenesis in isolated kidney tubules by [Leu]enkephalin and angiotensin II

To determine whether the cross-reactivity of opioid peptides with angiotensin II receptors was a property of the liver or whether it may be a more general phenomenon, we investigated the possibility that enkephalins influence carbohydrate metabolism in the kidney cortex tubules, as renal gluconeogenesis is also enhanced by angiotensin II (Guder, 1979; Saggerson & Carpenter, 1980). Fig. 3 shows that addition of 10^{-5} M-[Leu]enkephalin to isolated kidney tubule fragments significantly increased glucose synthesis from 5 mM-pyruvate over the first 45 min of the incubation. The effect of the opioid peptide was comparable to that of either adrenaline or angiotensin II, other agents which are known to stimulate gluconeogenesis in this system (Guder & Rupprecht, 1975; MacDonald & Saggerson, 1977; Guder, 1979; Saggerson & Carpenter, 1979; Saggerson & Carpenter, 1980; Kessar & Saggerson, 1980), the percentage stimulation being 42%, 42% and 35% for [Leu]enkephalin, adrenaline and angiotensin II respectively. In contrast to the effects of the other two agents, the response to the opioid peptide was not apparent over the last 15 min of the incubation. This may reflect the rapid degradation of the opioid peptide, as it is well established that the kidney possesses a large number of enzymes with the capability of degrading enkephalins to inactive breakdown products (Llorens & Schwartz, 1981; Malfroy & Schwartz, 1982; Fulcher *et al.*, 1982; Fulcher & Kenny, 1983). A similar situation

has been demonstrated in hepatocytes, where the effect of the opioid peptides is also of a shorter duration than that of the other effectors (Leach *et al.*, 1985).

The sensitivity of the effect of 10^{-5} M-[Leu]enkephalin and 10^{-6} M-angiotensin II to increasing concentrations of [Sar¹,Ile⁸]angiotensin II is shown in Fig. 4. In agreement with other studies (reviewed in Bumpus, 1977), [Sar¹,Ile⁸]angiotensin II acted as a weak partial agonist in the control incubations of kidney tubules and produced a small but reproducible dose-dependent increase in glucose synthesis. However when added together with angiotensin II or [Leu]enkephalin, there was a concentration-dependent inhibition of the effect of both peptides. In agreement with the hepatocyte studies, the effect of [Leu]enkephalin was more sensitive to the antagonist than that of angiotensin II, and indeed the difference in sensitivity was more marked in the kidney tubules. This may reflect the different responses being measured and the shorter duration for which the enkephalins are in contact with the enzymes involved in enkephalin degradation in the hepatocyte incubations. However it may also be an indication of the difference in the properties of the receptors in the two tissues (reviewed in Howard & Smeby, 1986). In favour of the latter is the finding that there was no evidence for [Sar¹,Ile⁸]angiotensin II acting as a partial agonist in liver.

Conclusions

From the above results it is suggested that the effects of the opioid peptides on carbohydrate metabolism in liver and kidney are mediated through interaction with

Fig. 3. Time course for the effects of [Leu]enkephalin, angiotensin II and adrenaline on glucose synthesis in isolated kidney tubules

Kidney tubules were incubated with 5 mM-pyruvate and either vehicle (●), 10^{-5} M-[Leu]enkephalin (○), 10^{-6} M-angiotensin II (△) or 10^{-6} M-adrenaline (□). At the times indicated samples were removed for the determination of glucose production as described in the Experimental section. The results shown are the means $\pm$ S.E.M. for 6 different tubule preparations. * $P < 0.05$

angiotensin II receptors. Both effectors are small peptides; angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is an octapeptide while [Leu]enkephalin (Tyr-Gly-Gly-Phe-Leu) is a pentapeptide. Conformation studies have indicated that the three aromatic side-chains of Tyr⁴, His⁶ and Phe⁸ are critical for maintaining the biological effects of angiotensin II, substitution of any one markedly reducing the biological potency (reviewed by Howard & Smeby, 1986). [Leu]enkephalin also contains both tyrosine and phenylalanine residues although they are separated by two glycine residues in the opioid peptide in contrast to the three residues in angiotensin II. However, it is suggested that these must be making a major contribution towards binding to and activation of the receptor, as previously we have demonstrated that removal of the *N*-terminal tyrosine residue from [Leu]enkephalin results in complete loss of biological activity (Leach *et al.*, 1985). From the use of structural analogues, it is evident that marked modification of the structure in the side-chains of amino acids in residues 1, 2 and 3 of the angiotensin II molecule can be made without significant loss of activity, and indeed removal of the aspartate in residue 1 only results in a small loss of the potency of the molecule to activate phosphorylase in liver (Campanile *et al.*, 1982). This suggests that the *N*-terminus is of minor importance in binding to the receptor. In favour of this, structural studies have indicated that angiotensin II exhibits stable folding in aqueous solution with the *N*-terminal region folded back upon itself (Howard & Smeby, 1986). As the enkephalin molecule has been proposed to exist in an

Fig. 4. Effect of increasing concentrations of [Sar¹,Ile⁸]-angiotensin II on the stimulation of gluconeogenesis by (a) [Leu]enkephalin and (b) angiotensin II in isolated kidney tubules

The kidney tubules were preincubated with increasing concentrations of [Sar¹,Ile⁸]-angiotensin II for 5 min before the addition of 5 mM-pyruvate and either 10^{-5} M-[Leu]enkephalin (a) or 10^{-6} M-angiotensin II (b). After a further 45 min, samples were removed for the determination of glucose synthesis by the tubules. The results shown are the means $\pm$ S.E.M. for 4 separate kidney tubule preparations. Symbols: ●, control incubation; ■, [Leu]enkephalin (a) or angiotensin II (b).

extended conformation in aqueous solution (Olatunji & Premilat, 1987), the size of the molecule in contact with the receptor may not be grossly different with the two peptides.

From the initial work of Wadja *et al.* (1976), it is evident that enkephalin-like compounds exist in both liver and kidney, as judged by displacement of specifically-bound [³H]dihydromorphine and [³H]-naloxone from brain receptors by tissue extracts. Further studies using liver extracts have confirmed these observations and have demonstrated that the active agents present in liver extracts show equal potency in displacing radiolabelled ligands from mu-, delta- and

kappa-opiate receptors in brain (Khawaja *et al.*, 1987b). At least one component has now been identified by radioimmunoassay as [Met]enkephalin (Khawaja *et al.*, 1987b) suggesting a possible local action of endogenous opioid peptides in the liver. Indeed the work of Werther *et al.* (1985) has suggested that the transient increase in glucose production following administration of [D-Met², Pro⁵]enkephalinamide may be a result of locally released enkephalins. However in view of our inability to demonstrate binding to specific opiate receptors in liver and the finding that the response *in vitro* is the result of cross-reactivity with angiotensin II receptors, it would appear unlikely that the enkephalins are directly involved at the level of the liver to produce the hyperglycaemia which is characteristic of opiate administration *in vivo*.

From these results it is evident that, in at least two tissues, opioid peptides can interact with angiotensin II receptors and mimic the effects of the vasoactive peptide. In order to overcome the rapid degradation of the peptides, enkephalins are frequently employed at concentrations similar to those required to elicit a response in liver and kidney, and in many of these tissues angiotensin II receptors are also present, for example in the kidney, smooth muscle preparations including the guinea pig ileum, and brain homogenates. It is conceivable that some of the reported metabolic responses observed in these tissues may also be the result of cross-reactivity with angiotensin II receptors rather than specific opiate receptors.

This work was supported by grants from the Wellcome Trust and British Diabetic Association.

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