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Aortic wall proteomic analysis in spontaneously hypertensive rats with a blood pressure decrease induced by 6-week load-free swimming

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Abstract. Decreased arterial compliance is one of the earliest detectable manifestations of adverse structural and functional changes within the vessel wall in hypertension. The proteomic approach is a powerful technique to analyze a complex mixture of proteins in various settings. Physical activity level was negatively associated with blood pressure. Sixteen 4-week-old male spontaneously hypertensive rats (SHR) and 16 Wistar-Kyoto (WKY) rats were randomly divided into four groups: i) SHR exercise group, ii) SHR rest group, iii) WKY exercise group and iv) WKY rest group. In the SHR and WKY exercise groups, rats were treated with a 6-week load-free swimming protocol (1 h/day, 5 days/week). The blood pressure of the rats was tested by the CODA^{TM2} single non-invasive blood pressure measurement appliance. After the 6-week swimming protocol, the total aorta excluding abdominal aorta was extracted. The proteins were separated by two-dimensional gel electrophoresis and identified via LC-mass spectrometry (MS)/MS. After 6-week load-free swimming, blood pressure decreased in the SHRs. Compared with sedentary SHRs, 11 spots on the 2D-gel showed a significant difference in exercised SHRs. Nine of these were chosen for further identification. There were 5 upregulated proteins (long-chain specific acyl-CoA dehydrogenase, heat shock protein β-1, isocitrate dehydrogenase subunit α, actin, α cardiac muscle 1 preprotein and calmodulin isoform 2) and 4 downregulated proteins (adipocyte-type fatty acid-binding protein, tubulin β-2C chain, 78 kDa glucose-regulated protein precursor and mimecan). Proteomics is an effective method to identify the target proteins of exercise intervention for hypertension.

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Introduction

Hypertension is the most frequent chronic disease in the developed world. It is a multisystemic disease that affects the heart and kidneys among other organs. The overall functional, structural and biochemical alterations in vasculature have been extensively studied during hypertension, but the molecular mechanisms remain unclear. Decreased arterial compliance is one of the earliest detectable manifestations of adverse structural and functional changes within the vessel wall (1). It has been shown that the proteomic approach is a useful technique to analyze a complex mixture of proteins in various settings, usually by combining two-dimensional electrophoresis and mass spectrometry (MS) (2). Bian *et al* (3) recently analyzed the proteome of aorta from spontaneously hypertensive rats (SHR). They found that SHR showed a significant alteration in the aortic wall protein profile compared with normal rats.

Exercise is a key antihypertensive therapy. It is reported that the physical activity level was negatively associated with blood pressure. In addition, the blood pressure can be decreased with long-term physical activity. Blood pressure of SHRs undergoing the physical activity protocol was lower than that of the normal SHRs. The functional and structural alterations in vasculature occurred in hypertensives following exercise training. Aerobic physical activity may alter the aortic wall remodeling to adapt the artery to a hyperkinetic blood flow resulting in alterations of the extracellular matrix modulation and vascular resistance. Certain data showed that the aorta wall thickness was smaller in the SHRs undergoing an aerobic physical activity protocol for 20 weeks (4). Furthermore, the alteration in the aortic wall protein profile was shown in SHRs with exercise. Kimura *et al* (5) demonstrated that the 4-hydroxynonenal and 3-nitrotyrosine levels in the aorta of running-trained SHR

were significantly lower than those in the non-exercised group. Bobillier *et al* (6) indicated that there was an increase in the aortic metallothionein amounts in swimming-trained SHRs. Swimming-trained SHRs showed an apelin-immunoreactivity level in the aorta (7), but the overall aortic wall protein profile remained unclear.

The present study used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) for protein separation and identified some of the proteins by MS. Nine proteins were identified that had a significant difference between swimming-exercised SHRs and non-exercised SHRs. The molecular mechanism of exercise decreasing the blood pressure is also discussed.

Materials and methods

Animals and research design. Studies were performed with male SHRs and their normotensive counterpart Wistar-Kyoto (WKY) (180-200 g in weight). The animals were housed in double cages in a temperature-controlled room (21-22˚C; 50-60% humidity) with a 12-h reversed light cycle and provided free access to food and tap water. All the experiments were approved by the Institutional Review Board of the Tianjin University of Sport Research Animal Resource Center (Tianjin, China).

Each type of rat was divided into an exercise-trained and sedentary control group. Thus, the rats were randomly allocated into four groups (n=8 each): i) Sedentary WKY (SED-WKY), ii) exercised WKY (EX-WKY), iii) sedentary SHR (SED-SHRs) and iv) exercised SHR (EX-SHRs).

Exercise training. During week 1, the exercise-trained SHR and WKY were acclimated to 15-min load-free swimming in a basin (water depth of 50 cm, water temperature of 36˚C). The duration was progressively increased. At the end of week 1 the rats were able to swim for 60 min. This intensity was maintained during the rest of the training period (5 days/week for 6 weeks). Sedentary rats were kept under the same living conditions as the exercise-trained rats, except for the training.

Measurement of blood pressure. Weekly systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured with the CODA^{TM2} non-invasive single channel BP measuring instrument (Zenda Instrument Co., Ltd., Austin, TX, USA).

Sample preparation, two-dimensional electrophoresis and analysis. Total protein was extracted only from the aorta (abdominal aorta was not included). Briefly, aorta samples were pulverized after being frozen by liquid nitrogen. Pulverized tissue powder was homogenized in lysis buffer (9 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer, 40 mM dithiothreitol and 40 mM Tris-base) and centrifuged at 20,000 x g for 45 min at 4˚C. The supernatant was collected as a protein sample and the concentration was determined by the Bradford protein assay (8).

A sample containing 250 μg of protein was applied to the first-dimensional isoelectric focusing in the ReadyStrip immobilized pH gradient strips (18 cm; pH 3-10 NL). Separation of proteins in the second dimension was achieved by SDS-PAGE (10%). Two-dimensional gels were stained by coomassie brilliant blue and scanned by Imagescanner (GE-Amersham Biosciences Corp., Piscataway, NJ, USA). Digitized images were recorded and imported to ImageMaster 2D Platinum 6.0 software (GE Healthcare Life Sciences, Fairfield, CT, USA). Analysis was performed matching the spots from different gels of different animals for each group.

Trypsin digestion and protein identification by MS. Protein spots of interest were excised manually from the gels and digested with trypsin as described previously (9). The identification of protein was performed by peptide mass fingerprinting using LCQ Deca XP (Thermo Electron Corp, San Jose, CA, USA) mass spectrometers.

Statistical analyses. Results of blood pressure are expressed as means ± standard error of the mean. Comparisons between multiple groups were performed by or two-way analysis of variance. Tests were performed using the SPSS 12.0 software package (SPSS, Inc., Chicago, IL, USA). Proteomic statistical analysis was performed using TurboSEQUEST 3.3 software (Bioworks, Thermo Electron, Marietta, OH, USA), which includes a statistical package. Spots were tested with the t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Blood pressure decrease in SHR induced by 6-week load-free swimming. The blood pressure of 12-week-old SED-SHR was significantly higher than that of the age-matched SED-WKY [SBP, 195.78±11.46 vs. 126.63±11.70 mmHg; DBP, 144.05±21.47 vs. 85.43±9.73 mmHg; mean arterial pressure (MAP), 166.32±11.93 vs. 104.59±16.23 mmHg; $P<0.01$]. Contrasted to that of SED-SHR, the blood pressure of the EX-SHR that underwent 6-week load-free swimming was reduced (SBP, 163.44±12.90 vs. 195.78±11.46 mmHg; DBP, 121.19±12.61 vs. 144.05±21.47 mmHg; MAP, 134.13±18.31 vs. 166.32±11.93 mmHg; $P<0.01$). Six-week load-free swimming did not reduce the blood pressure in exercised WKY rats (SBP, 123.92±12.55 vs. 126.63±11.70 mmHg; DBP, 85.20±12.54 vs. 85.43±9.73 mmHg; MAP, 98.28 ±9.19 vs. 104.59±16.23 mmHg). The results are shown in Table I.

Protein expression profile. Comparison among SED-WKY, EX-WKY, SED-SHR and EX-SHR was performed by the replicate group option and the statistical software package of ImageMaster 2D Platium 6.0. Spot quantification was normalized on the basis of the total staining density of the image to compensate for any variation in protein loading and development level of coomassie brilliant blue staining. The differential expression was calculated for every spot that could be matched in all the samples of ≥1 group.

From all the spots resolved in the pH 4 to 7 range by two-dimensional electrophoresis of aortic wall tissue from WKY and SHR, the same 453 well-resolved spots were focused on in a 12- to 90-kDa molecular weight, as previously analyzed (6). Significant differences were shown in 11 analyzed spots in EX-SHR versus SED-SHR ($P<0.05$); 5 increased and 6 decreased. Ten protein spots were found to

Table I. Effects of 6-week load-free swimming on the blood pressure in SHR and WKY rats.

	SED-WKY (n=8)		EX-WKY (n=8)		SED-SHR (n=8)		EX-SHR (n=8)	
Pressure	0 weeks	6 weeks	0 weeks	6 weeks	0 weeks	6 weeks	0 weeks	6 weeks
SBP	119.62±8.03	126.63±11.70	117.14±12.15	123.92±12.55	153.17±9.91[a]	195.78±11.46[a,b]	153.17±17.08	163.44±12.90[c]
DBP	93.79±6.90	85.43±9.73	87.58±7.40	85.20±12.54	110.69±11.92[a]	144.05±21.47[a,b]	112.74±7.65	121.19±12.61[c]
MAP	81.25±7.31	104.59±16.23	75.17±6.79	98.28±9.19	97.67±11.40[a]	166.32±11.93[a,b]	98.70±6.30	134.13±18.31[c]

[a]P<0.01 for SED-SHR versus SED-WKY; [b]P<0.05 for 6 vs. 0 weeks in SED-SHR; [c]P<0.01 for EX-SHR versus SED-SHR. Data are mean mmHg ± standard deviation. SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto; SED-WKY, sedentary WKY; EX-WKY, exercised WKY; SED-SHR, sedentary SHR; EX-SHR, exercised SHR; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure.

Table II. Identification of the differentially expressed proteins in EX-SHR versus SED-SHR.

Protein number	Ms identification	NCBInr index score	Top score	pI	Mw (kDa)
S2	Adipocyte-type fatty acid-binding protein	gil2494405	155	7.71	14.699
S4	Tubulin β-2C chain	gil5174735	276	4.79	49.799
S5	78 kDa glucose-regulated protein precursor	gil25742763	261	5.07	72.302
S6	Mimecan	gil157824206	133	5.85	34.048
S7	Long-chain specific acyl-CoA dehydrogenase	gil6978431	228	7.63	47.842
S8	Heat shock protein β-1	gil94400790	147	6.12	22.808
S9	NAD subunit α	gil16758446	130	6.47	39.588
S10	Actin, α cardiac muscle 1 preprotein	gil4885049	116	5.23	41.992
S11	Calmodulin isoform 2	gil71664	62	4.09	16.696

EX, exercised; SHR, spontaneously hypertensive rats; SED, sedentary; NAD, isocitrate dehydrogenase.

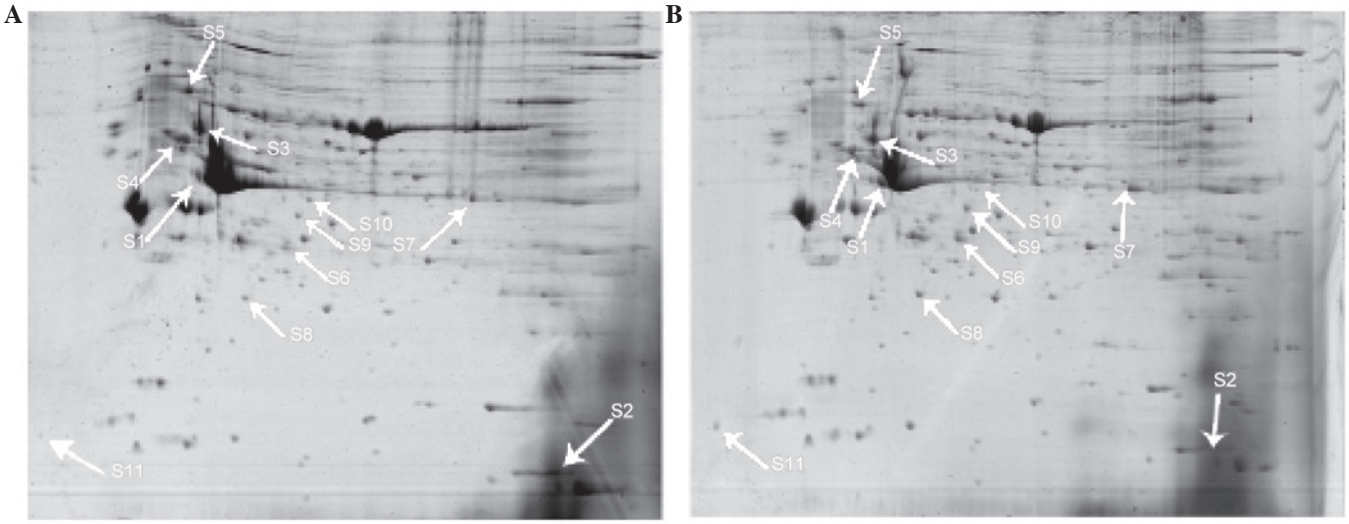

Figure 1. Two-dimensional gel images of (A) sedentary spontaneously hypertensive rats (SED-SHR) and (B) exercised SHR (EX-SHR). Proteins that were significantly differentially expressed >2-fold are indicated with arrows and number. Nine of the proteins were identified using LC-mass spectrometry (MS)/MS.

change significantly in EX-SHR versus EX-WKY (P<0.05); 5 increased and 5 decreased. There were 8 protein spots that showed significant differences in EX-WKY versus SED-WKY (P<0.05); 1 increased and 7 decreased. Thirteen protein spots were found to change significantly in EX-WKY versus SED-WKY (P<0.05); 11 increased and 2 decreased. Accurate protein identification was achieved for 9 spots and failed in 2 in EX-SHR versus SED-SHR. The spots are all indicated with arrows in Fig. 1.

Protein identification. All the 11 spots were excised from the rehydrated gels and subjected to in-gel trypsin digestion for subsequent identification by MS. Nine of them contained peptides and 2 failed identification. Five upregulated spots and

4 downregulated spots were identified finally. The results are shown in Table II.

Discussion

The aim of the present study was to screen and identify proteins secreted by the aortic wall as potential biomarkers of susceptibility to exercise-induced blood pressure decreasing. SHR and WKY rats were chosen for the contrasting susceptibilities of different blood pressure level. The results also showed that the blood pressure is normal in WKY rats while hypertension occurs in SHR. After 6-week load-free swimming, the blood pressure decreased significantly in SHR, which was consistent with other studies (10,11).

Recent research found that aerobic physical activity may alter the aortic wall remodeling to adapt the artery to a hyperkinetic blood flow resulting in alterations of the extracellular matrix modulation and vascular resistance (4). Certain studies identified that the expression of several proteins changed in the aortic wall induced by exercise in SHRs (5,6). However, the overall aortic wall protein profile remains unclear.

In the present study, the proteomics of the aortic wall in SED-SHRs and EX-SHRs were presented to explore the molecular mechanism for exercise decreasing the blood pressure. Differentially expressed proteins were detected in the aortic wall from SED-SHRs compared with EX-SHRs. Five upregulated proteins and 4 downregulated proteins were obtained in the aortic wall of EX-SHRs. An association between these proteins and decreasing blood pressure was suggested.

Spot S2 was identified as the adipocyte-type fatty acid-binding protein (A-FABP). A-FABP was downregulated 12.8-fold in the EX-SHR group compared with the SED-SHR group. FABPs are abundantly expressed 14-15 kDa proteins that reversibly bind hydrophobic ligands (12). A-FABP is highly expressed in adipocytes and macrophages (13). It is involved in insulin resistance, lipid metabolism and atherosclerosis. Expression of A-FABP is highly regulated during differentiation of adipocytes and its mRNA is transcriptionally controlled by fatty acids, PPAR-γ agonists and insulin (14,15).

Spot S4 was identified as tubulin β-2C chain. Tubulin β-2C chain was downregulated 2.3-fold in the EX-SHR group compared with the SED-SHR group. Exercise training decreased β-tubulin protein expression in the kidney of chronic heart failure (CHF) rats, which suggests that exercise training can significantly improve the renal dysfunction in CHF rats (16).

Spot S5 was identified as the 78 kDa glucose-regulated protein precursor. In the present study, glucose-regulated protein 78 precursor was downregulated 2.2-fold in the EX-SHR group compared with the SED-SHR group as determined by 2D-PAGE. Glucose-regulated protein 78 (GRP78) is a well-characterized molecular chaperone that is ubiquitously expressed in mammalian cells. GRP78 is best known for binding to hydrophobic patches on nascent polypeptides within the endoplasmic reticulum and for its role in signaling the unfolded protein response. Studies have shown that GRP78 is expressed on the cell surface in numerous tissue types *in vitro* and *in vivo*. GRP78 disregulation is also indicated in atherosclerotic, thrombotic and auto-immune disease (17). Previous studies showed that the levels of GRP78 were upregulated in the soleus muscle of Wistar rats and the brain of Alzheimer's disease mice adhering to treadmill running for 3 to 4 months duration (18,19). However, investigators found that treadmill running for 5 days did not increase GRP78 expression in cardiac muscle (20).

Spot S6 was identified as mimecan. Mimecan was downregulated 2.1-fold in the EX-SHR group compared with the SED-SHR group as determined by 2D-PAGE. Mimecan (also known as osteoglycin) belongs to the family of small leucine-rich proteoglycans, a group of 11 proteins, characterized by leucine-rich repeats in their central domain. These proteins are located in the extracellular matrix and are important for the regulation of the matrix structure, but also in the regulation of cell cycle and growth factor actions (21). Mimecan in the aorta was mainly produced by VSMCs and perivascular fibroblasts. A downregulation was confirmed following the onset of arteriogenesis (22). Its expression was also regulated during atherosclerosis in patients and animals. Differential expression of mimecan was identified in VSMCs during neointima formation and in atherosclerosis plaques (23,24). The downregulation of osteoglycin expression in the collateral arteries of rabbits subjected to femoral artery occlusion indicated a function of osteoglycin as a negative regulator of the mitotic activity in the wall of collateral arteries (22).

Spot S7 was identified as the long-chain specific acyl-CoA dehydrogenase (LCAD). In the present study, LCAD was upregulated 2.0-fold in the EX-SHR group compared with the SED-SHR group as determined by 2D-PAGE. LCAD catalyzes the α,β-dehydrogenation of acyl-CoAs, the initial step of mitochondrial β-oxidation. LCAD has been shown to be involved in the degradation of branched-chain fatty acids originating from peroxisomal catabolism of phytanic acid, but LCAD is also able to handle straight-chain and certain monounsaturated acyl-CoAs (25). The phenotype of the $LCAD^{-/-}$ mouse is characterized by unprovoked sudden death, fasting and cold intolerance, hypoketotic hypoglycaemia and marked fatty changes in liver and heart (26). A previous study found that the 3-hydroxyacylcarnitines were absent in $LCAD^{-/-}$ tissues and a profound deficiency of acetylcarnitine was observed in $LCAD^{-/-}$ hearts, indicating that the cardiomyopathy in the $LCAD^{-/-}$ mouse is caused primarily by a severe energy deficiency in the heart, stressing the important role of LCAD in cardiac fatty acid metabolism in the mouse (27). LCAD was elevated in SHR cardiac mitochondria (121%) (28).

Spot S8 was identified as heat-shock protein β-1 (HSPB1). HSPB1 was upregulated 1.9-fold in the EX-SHR group compared with the SED-SHR group as determined by 2D-PAGE. HSPB1, also known as HSP27, is a member of the mammalian heat-shock protein family (29). In smooth muscle cells, HSP27 appears to be the link between the signal transduction cascade and the contractile machinery (30). Park *et al* (31) reported lower HSP27 levels in plaques, lower levels of phosphorylation of HSP27 and higher plasma levels of secreted HSP27 in humans with acute coronary syndrome raised, in regards to the contribution of HSP27 in atherogenesis. Expression can increase in response to physical and chemical stressors including heat, mechanical strain, oxidative stress and proinflammatory mediators. Expression of small HSPs in

striated and smooth muscle is frequently constitutive, but can also be modified by chemical or physical stressors (32).

Spot S9 was identified as isocitrate dehydrogenase (NAD) subunit α. NAD subunit α was upregulated 1.9-fold in the EX-SHR group compared with the SED-SHR group, as determined by 2D-PAGE. NAD-dependent isocitrate dehydrogenase, Idh, one of the eight enzymes of the Krebs cycle, is an octamer composed of Idh1p and Idh2p (encoded by IDH1 and IDH2, respectively) (33). In a previous study, the yeast enzyme was shown to be composed of two non-identical subunits, IDH1 and IDH2, with both being equally represented in the holoenzyme (34). These two subunits were shown to be essential for holoenzyme activity, since disruption of either or both genes encoding the subunits results in yeast strains that exhibit no detectable NAD1-specific isocitrate dehydrogenase activity and that are unable to grow with acetate as a carbon source (33). Research demonstrated that the yeast Krebs cycle enzyme IDH binds specifically and with high affinity to the 5'-untranslated leader sequences of mitochondrial mRNAs *in vitro* and have proposed a role for the enzyme in the regulation of mitochondrial translation. Cells disrupted for the IDH genes exhibit a strong increase in mitochondrial translation activity and the newly synthesized products are also more rapidly degraded, which suggested that binding of Idh to mitochondrial mRNAs may suppress inappropriate translation of mitochondrial mRNAs (35). Research found significantly lower protein levels of IDH2 (93%) and depressed total IDH activities (68%) in SHR heart mitochondria. IDH1 appeared to have a higher level in SD rats undergoing 8 weeks of swimming training (36). IDH activity increased 32% in the 6 weeks of treadmill running in trained intermyofibrillar mitochondria (37).

Spot S10 was identified as actin, α cardiac muscle 1 preprotein. This preprotein was upregulated 1.9-fold in the EX-SHR group compared with the SED-SHR group as determined by 2D-PAGE. The highly conserved actins are the major constituents of the thin filaments in the muscle sarcomere. They are involved in force generation within the sarcomere and force transmission from the sarcomere to the surrounding syncytium (38). The α-cardiac actin gene (ACTC) 1 is the major component of the sarcomeric thin filaments and is essential for cardiac muscle contraction. Knockdown of ACTC1 in chicks shows less developed atrial septa supporting a dose-dependent effect of ACTC1 on cardiac development. It has been suggested that the lack of ACTC1 may induce apoptosis leading to disrupted cardiac differentiation (39). ACTC was the first gene identified to harbor HCM and DCM mutations, with 6 mutations leading to HCM and 2 mutations leading to DCM (40).

Spot S11 was identified as calmodulin (CaM) isoform 2. CaM isoform 2 was downregulated by 1.8-fold in the EX-SHR group compared with the SED-SHR group as determined by 2D-PAGE. CaM is a calcium-binding protein which, when complexed with calcium, may mediate numerous calcium-dependent cellular activities (41). CaM is associated with a variety of cell functions including inflammation, apoptosis and muscular contraction. A previous study has shown that in the aorta from SHR, expression levels of several CaM-related proteins, including eukaryotic elongation factor kinase and death-associated protein kinase protein, increased, while Ca(2+)/CaM-dependent protein kinase IIδ and histone deacetylases 4 protein decreased. CaM-related proteins may at least be in part associated with the pathogenesis of hypertensive vascular diseases (42).

In conclusion, proteomic analysis of proteins in the aortic wall from SHRs performing 6-week load-free swimming provided an effective approach for elucidating the molecular mechanisms of the exercise-induced blood pressure decrease. Eleven protein spots with different abundance were found, of which 9 differentially expressed proteins were identified by MALDI-TOF MS. The roles of these identified proteins in the exercise-induced blood pressure decrease were discussed. These findings provide information for understanding the mechanism of exercise decreasing blood pressure in the aortic wall.

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