Effect of chronic alcohol feeding on physiological and molecular parameters of renal thiamin transport

Veedamali S. Subramanian,1,2 Sandeep B. Subramanya,1,2 Hidekazu Tsukamoto,3,4 and Hamid M. Said1,2

1Departments of Medicine, Physiology, and Biophysics, University of California, Irvine; 2Department of Veterans Affairs Medical Center, Long Beach; 3Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles; and 4Department of Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California

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First published April 28, 2010; doi:10.1152/ajprenal.00140.2010—The renal thiamin reabsorption process plays an important role in regulating thiamin body homeostasis and involves both thiamin transporters-1 and -2 (THTR1 and THTR2). Chronic alcohol use is associated with thiamin deficiency. Although a variety of factors contribute to the development of this deficiency, effects of chronic alcohol use on renal thiamin transport have not been thoroughly examined. We addressed this issue by examining the effect of chronic alcohol feeding of rats with liquid diet on physiological and molecular parameters of renal thiamin transport. Chronic alcohol feeding caused a significant inhibition in carrier-mediated thiamin transport across the renal brush-border membrane and was evident as early as 2 wk after initiation of alcohol feeding. Similarly, thiamin transport across the renal basolateral membrane was significantly inhibited by chronic alcohol feeding. The inhibition in renal thiamin transport was associated with a marked decrease in the level of expression of THTR1 and -2 proteins, mRNAs, and heterogeneous nuclear RNAs. Chronic alcohol feeding also caused a significant reduction in the level of expression of thiamin pyrophosphokinase but not that of the mitochondrial thiamin pyrophosphate transporter. These studies show that chronic alcohol feeding inhibits the entry and exit of thiamin in the polarized renal epithelial cells and that the effect is, at least in part, mediated at the transcriptional level. These findings also suggest that chronic alcohol feeding interferes with the normal homeostasis of thiamin in renal epithelial cells.

THIAMIN, A WATER-SOLUBLE vitamin, is essential for cellular function, growth, and development. The vitamin is enzymatically converted into its active form, thiamin pyrophosphate (TPP), in the cytoplasm via the action of thiamin pyrophosphokinase (TPKase), a rate-limiting enzyme that plays an important role in regulating cellular thiamin homeostasis. While all mammalian nucleated cells possess the ability to convert thiamin to TPP, the liver and kidneys are especially active in this regard (10, 22, 36). TPP is then either used in the cytoplasm or is imported in the mitochondria via a carrier-mediated process that involves the mitochondrial TPP transporter (encoded by the SLC25A19 gene; see Ref. 19), for utilization in carbohydrate metabolism and energy production. The importance of thiamin for human health and well-being is evident by the range of clinical abnormalities that result from its deficiency, which include cardiovascular and neurological disorders (4, 31, 35). Beriberi and the Wernicke-Korsakoff syndrome are well-known thiamin deficiency diseases (24, 35).

All mammals cannot synthesize thiamin; thus, they must obtain the vitamin from exogenous sources via intestinal absorption. Circulating thiamin is filtered in the renal glomeruli and then salvaged via reabsorption by renal epithelial cells to prevent its loss in the urine. Thus the kidneys play an important role in maintaining and regulating normal body homeostasis of thiamin. The mechanism(s) involved in the renal thiamin reuptake process has been examined using a variety of human and animal kidney preparations (2, 9, 34). It is known now that the renal thiamin uptake process is carrier-mediated and involves both thiamin transporters-1 and -2 (THTR1 and THTR2, respectively) (2). It is also known that these two thiamin transporters are expressed differentially at the cell membrane of the polarized renal epithelial cells, with expression of THTR2 being restricted to the apical brush-border membrane (BBM) domain only, while that of the THTR1 is expressed at both the BBM and basolateral membrane (BLM) domains of the renal reabsorptive epithelial cells (6). Furthermore, studies from our laboratory have shown that the renal thiamin uptake process is adaptively upregulated in thiamin deficiency via transcriptional regulatory mechanism(s) involving both the hTHTR1 and -2 (2).

Thiamin deficiency is common in chronic alcoholism and may lead to Wernicke-Korsakoff syndrome, a neuropsychiatric condition characterized by ophthalmoplegia, ataxia, and memory loss (17, 24, 30, 32, 33, 35). Although many factors contribute to the development of thiamin deficiency in chronic alcoholism, an inhibition in the renal thiamin uptake process may also be one of the factors. A study in rats has shown that chronic alcohol feeding leads to an increase in urinary loss of thiamin (1). Little, however, is known about the effect of chronic alcohol feeding on physiological and molecular parameters of the thiamin transport process across the polarized renal epithelial cells, i.e., transport across the BBM and the BLM domains. Little is also known about the effect of chronic alcohol use on level of expression of TPKase and the mitochondrial TPP transporter, two important players that are involved in regulating thiamin homeostasis in renal epithelial cells. We addressed these issues using rats that were fed on alcohol liquid diet (ethanol provided 36% of total calories) for different periods and compared the findings from those obtained with control rats that were pair-fed the same liquid diet but without alcohol (maltose-dextrin isocalorically replaced ethanol in the diet). The results showed that chronic alcohol feeding causes a significant inhibition in thiamin transport across the renal BBM and BLM domains of renal epithelial cells. This inhibition was associated with a
marked decrease in levels of expression of THTR1 and -2 at the protein and mRNA levels; it was also associated with a marked decrease in the level of expression of heterogeneous nuclear RNAs (hnRNA) of THTR1 and -2, suggesting possible effects at the transcriptional level. Moreover, chronic alcohol use was found to cause a significant reduction in the level of expression of TPKase but not that of the mitochondrial TPP transporter.

**MATERIALS AND METHODS**

**Materials.** [1H]thiamin (specific activity 20 Ci/mmol; radiochemical purity >99%) was obtained from American Radiolabel (St. Louis, MO). Nitrocellulose filters (0.45-μm pore size) were purchased from Fisher Scientific. Unlabeled thiamin and other chemicals, including molecular biology reagents, were obtained from commercial vendors and were of analytical grade.

**Alcohol feeding of rats.** Male Wister rats (Charles River, Wilmington, MA) weighing ~120 g (~14 wk old) were housed at the Animal Core of the National Institute on Alcohol Abuse and Alcoholism-Funded Southern California Research Center for Alcoholic Liver and Pancreatic Diseases and Cirrhosis at the University of Southern California, Los Angeles, CA. Animal use committees of both the University of Southern California and the Long Beach Veterans Affairs Medical Center approved the experimental protocols. Rats were fed the Lieber-DeCarli alcohol liquid diet (ethanol provided 36% of total ingested calories; 5 g ethanol/dl diet) (18) for 2, 4, or 6 wk. Control rats were pair-fed with the same liquid diet but without alcohol (maltose-dextrin isosorically replaced ethanol). Rats were killed at the time of study, and their kidneys were removed and processed immediately for isolation of renal brush-border membrane vesicles (BBMV) or basolateral membrane vesicles (BLMV). For molecular biological studies (mRNA and hnRNA), part of the fresh kidney tissue from the alcohol-fed rats and their pair-fed controls were removed and stored at ~80°C in Trizol (Invitrogen, Carlsbad, CA) for later use.

**Preparation of rat renal BBMV and BLMV and transport studies.** Purified rat renal BBMV and BLMV were freshly prepared utilizing the divalent (Mg2+) cation chelation method and the Percoll-gradient differential centrifugation method, respectively, as previously described by us and others (5, 11, 12, 27, 28, 37). The final BBMV and BLMV preparations were preloaded with a buffer of 280 mM mannitol and 20 mM Tris-HCl, pH 5.5; incubation was performed in a buffer of 100 mM NaCl, 80 mM mannitol, and 20 mM HEPES, pH 7.4, in the presence of 0.25 μM [1H]thiamin. Transport studies were performed using freshly isolated membrane vesicles at 10 s (initial rate; see Ref. 34) at 37°C using a rapid-filtration method as previously described (13).

**Real-time PCR analysis.** Total RNA (5 μg) was isolated from the kidneys of alcohol-fed rats and their pair-fed controls and was primed with oligo(dT) primers to synthesize first-strand cDNA (Superscript First Strand Synthesis RT-PCR kit; Invitrogen). To amplify the coding region of rat THTR-1, THTR-2, and β-actin, we used gene-specific primers for rat THTR1, THTR2, and β-actin as described in Table 1. Real-time PCR was performed as described previously (23), and data were normalized to β-actin and then quantified using a relative relationship method supplied by the iCycler manufacturer (Bio-Rad) as described before (20, 23).

**Western blot analysis.** Western blot analysis was performed on purified BBM and BLM as well as kidney cortex homogenate prepared from the kidneys of alcohol-fed rats and their pair-fed controls as described earlier (21, 29). BBM, BLM, and homogenate (to detect TPCase protein expression) preparations (30–60 μg) were resolved on 10%–12% Bis-Tris mini-gel (Invitrogen) as described before (29). After electrophoresis, proteins were electroblotted on polyvinylidene difluoride membrane (Bio-Rad) and then blocked with a PBS-Tween 20 solution containing 5% dried milk (Bio-Rad) for 1 h at room temperature. The membranes were then incubated either with rat THTR1 or -2 specific polyclonal antibodies that were raised in rabbits against the KKCRKQEDPPNSQP and EPYLQEPDRVSTKE peptides, which correspond to amino acids 481–494 and 468–481 of the rat THTR1 and -2, respectively, using a commercial vendor (Thermo Fisher Scientific, Huntsville, AL). Specificity of the rat THTR1 and -2 polyclonal antibodies was determined by testing these antibodies with the synthetic antigenic peptides for 1 h at 37°C and then at 4°C overnight before use (21, 29). For rat TPCase detection, the membrane was incubated with TPCase polyclonal antibodies raised in rabbits (Protein Tech Group, Chicago, IL). Immunodetection of the specific bands was performed by incubating the membrane with secondary antibodies [goat anti-rabbit conjugated to horseradish peroxidase (HRP); Santa Cruz Biotechnology, Santa Cruz, CA] and then incubated with bovine anti-goat conjugated HRP secondary antibodies (Santa Cruz). The immunoreactive bands were developed using ECL substrate as described above and then quantitated (as unitless measurements) using the UNSCAN-IT gel automated digitizing system, version 6.1 (Silk Scientific).

**hnRNA analysis.** To examine the effect of chronic alcohol feeding on the level of expression of hnRNA of THTR1, THTR2, and TPCase, total RNA [treated with DNase I (1 μg RNA/unit; Invitrogen) to avoid genomic DNA presence during the amplification process] was isolated from the kidneys of alcohol-fed rats and their pair-fed controls as described previously (3, 8, 14). DNase I-treated RNA was then reverse transcribed with the random hexomer as described above (Invitrogen). To ensure the amplification of hnRNA, the forward and reverse primers were designed in exon and intron, respectively. Semiquantitative RT-PCR was performed using the rat THTR1, THTR2, TPCase, and β-actin gene-specific primers as described in Table 1. The semiquantitative PCR conditions consisted of a 3-min 95°C melt followed by 33 cycles of 95°C melt for 30 s, 58°C annealing for 30 s, 72°C extension for 1 min, and final extension at 72°C for 8 min. The amplified products were run in 1% agarose gel, then image was captured using Gel-doc (Bio-Rad), and specific bands were quantitated (as unitless measurement) using UNSCAN-IT gel automated digitizing system, version 6.1 (Silk Scientific).

**Table 1. Combination of primers used to amplify open reading frame of the respective genes by real-time and semiquantitative PCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward and Reverse Primers (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>THTR1</td>
<td>GCTGCTGCTTCACTCAATGCGG; GATGTACACTGAGCCAGCGAATC</td>
</tr>
<tr>
<td>THTR2</td>
<td>GGTGATCTGCTGCTTGGCGG; GGTAAAGATCTGACGCAACAGG</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GTCAGGTCTGCTATGCGGC; CATGATGCGACAGGTTC</td>
</tr>
<tr>
<td>hnRNA primers</td>
<td></td>
</tr>
<tr>
<td>THTR1</td>
<td>CACCTTTGACTTGGTCTGCTG; GATAGCAGCGAGTTGTAAGC</td>
</tr>
<tr>
<td>THTR2</td>
<td>CTACGTTAAGACAGACAGG; TGATGAAATAGGACCTAC</td>
</tr>
<tr>
<td>TPCase</td>
<td>GCCCTTGCTTCTTGGATAGGAG; GAGACCCAGGCCCTAGTGC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CTGGCTCTTCCAGATTGAG; CTCATAGTGCGACAGAGT</td>
</tr>
<tr>
<td>Semi-quantitative RT-PCR primers for thiamin pyrophosphokinase and slc25a19</td>
<td></td>
</tr>
<tr>
<td>TPCase</td>
<td>GACGAAGAGCCAGCTAGC; GTGTGAGAGCTAGTGAG</td>
</tr>
<tr>
<td>slc25a19</td>
<td>GGCCTATCCACAGCAT; GGCGTTGCATGATGAC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>GTCAGGTCTGCTATGCGGC; CATGATGCGACAGGTTC</td>
</tr>
</tbody>
</table>

r, Rat; THTR, thiamin transporter; TPCase, thiamin pyrophosphokinase; hnRNA, heterogeneous nuclear RNA.
formed using gene-specific primers (Table 1) designed from the open reading frame (ORF) of rat TPKase, mitochondrial thiamin pyrophosphate transporter (slc25a19), and /H9252-actin. Semiquantitative RT-PCR conditions were followed as described above with minor modifications. The amplified products were analyzed on 2% agarose gel, then the image was captured, and bands were quantified as described above.

Statistical analysis. Uptake data presented in this paper are the results of three separate experiments and are expressed as means ± SE in femicoles per milligram protein per 10 s. Differences between the means of alcohol-fed and their pair-fed control rats were tested for a significance level at \( P < 0.05 \) using the Student’s \( t \)-test analysis. Uptake of 0.25 M \([3H]\)thiamin by the carrier-mediated process was determined by subtracting uptake in the presence of a high pharmacological concentration of unlabeled thiamin (1 mM) from uptake in its absence. Western blot, real-time PCR, hnRNA analysis, and semiquantitative RT-PCR were performed on at least three separate occasions.

RESULTS

Thiamin uptake by freshly isolated rat renal BBMV and BLMV. The mechanism of thiamin transport across the apical membrane of renal epithelial cells has been investigated at the functional (2, 6, 9) and molecular (2, 6) levels. The mechanism of exit of thiamin out of the renal epithelial cells, i.e., transport across the BLM, however, is less studied, although findings in our laboratory have shown expression of the THTR1 at this membrane domain (6). We confirmed the existence of a carrier-mediated uptake process for thiamin in rat renal BBMV by demonstrating a significant (\( P < 0.01 \)) inhibition in the uptake of 0.25 μM \([3H]\)thiamin by 1 mM unlabeled thiamin (Fig. 1). We also functionally showed, for the first time, the existence of a carrier-mediated process for thiamin in renal BLMV by demonstrating a significant (\( P < 0.01 \)) inhibition in \([3H]\)thiamin (0.25 μM) by unlabeled thiamin (1 mM) (Fig. 1).

Effect of chronic alcohol feeding on carrier-mediated thiamin uptake by rat renal BBMV and BLMV. In this study, we used the Western blotting technique to investigate the effect of chronic ethanol feeding on the level of expression of the rat THTR1 and -2 proteins at the renal BBM and BLM domains. Rat THTR1 and -2 polyclonal antibodies were used pared with their pair-fed controls (Fig. 2). This inhibition in thiamin uptake was observed as early as 2 wk after the start of alcohol feeding. We used a 4-wk alcohol feeding regimen as the standard feeding period in all of our subsequent investigations.

We also examined the effect of chronic alcohol feeding (for 4 wk) on carrier-mediated thiamin (0.25 μM) uptake by purified renal BLMV. The results showed a significant (\( P < 0.01 \)) inhibition in thiamin uptake by renal BLMV from alcohol-fed rats compared with those isolated from their pair-fed controls (Fig. 3).

Effect of chronic alcohol feeding on level of expression of THTR1 and -2 proteins in rat renal BBM and BLM. In this study, we used the Western blotting technique to examine the level of expression of the rat THTR1 and -2 proteins at the renal BBM and BLM domains. Rat THTR1 and -2 polyclonal antibodies were used...
in this study as detailed in MATERIALS AND METHODS. The results showed a significant ($P < 0.05$) reduction in the level of expression of THTR1 and -2 proteins in BBM preparations isolated from alcohol-fed rats compared with those isolated from their pair-fed controls (Fig. 4, A and B, for THTR1 and Fig. 4, E and F, for THTR2). Similarly, a significant ($P < 0.05$) reduction was observed in the level of expression of THTR1 protein in renal BLM preparations isolated from alcohol-fed rats compared with those isolated from their pair-fed controls (Fig. 4, C and D) [as seen before (6), no expression for THTR2 protein was detected in renal BLM preparations; data not shown]. Specificity of the rat anti-THTR1 and -2 polyclonal antibodies used in the above experiments was confirmed by demonstrating blocking of the specific bands upon pretreatment of the antibodies with their respective antigenic peptides (Fig. 4, A and E, right).

**Effect of chronic alcohol feeding on level of expression of THTR1 and -2 mRNA in rat kidneys.** The effect of chronic alcohol feeding on the steady-state mRNA level of expression of THTR1 and -2 in rat kidney cortex was examined. A real-time PCR using gene-specific primers (Table 1) designed from the ORF of rat THTR1 and -2 was performed on mRNA isolated from alcohol-fed rats and their pair-fed controls (MATERIALS AND METHODS). Data were normalized relative to the rat housekeeping gene $\beta$-actin. The results showed a significant ($P < 0.01$) decrease in the level of expression of THTR1 and...
CHRONIC ALCOHOL CONSUMPTION INHIBITS RENAL THIAMIN UPTAKE

Effect of chronic alcohol feeding on level of expression of THTR1 and -2 hnRNA levels. The steady-state level of an hnRNA of a given gene is widely believed to reflect the transcription rate of that gene and is often used as a surrogate for the nuclear run-on assay (3, 7, 8, 14, 26). In this study, we examined the effect of chronic alcohol feeding on the level of expression of THTR1 (Slc19a2) and THTR2 (Slc19a3) hnRNA in rat kidneys. hnRNA was isolated from kidney cortex of alcohol-fed rats and their pair-fed controls, and the level of expression of THTR1 and -2 was determined as described in MATERIALS AND METHODS. The results showed a significant (P < 0.05) decrease in the level of expression of THTR1 and -2 in the kidneys of alcohol-fed rats compared with their pair-fed controls (Fig. 6, A and B).

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Feeding on the level of expression of hnRNA of from at least 3 sets of rats. *

Chronic alcohol feeding on physiological and molecular parameters of thiamin transport in and out of the polarized renal epithelial cells. We were also interested in examining the effect of chronic alcohol feeding on specific aspects of intracellular thiamin handling that are important for renal thiamin nutrition, namely the level of expression of TPKase and mitochondrial TPP transporter, which play important roles in regulating cellular thiamin metabolism and compartmentalization. Our interest in addressing these issues stems from the fact that, while chronic alcohol feeding affects normal thiamin body homeostasis and physiology, its effect on renal transport is less well studied. Also, chronic alcohol feeding appears to affect thiamin metabolism in a tissue-dependent manner (25). We used a well-established pair-feeding regimen of rats with alcohol (and control) liquid diets. Our results showed thiamin transport across the renal BBM (studied using purified renal BBMV preparations) to be significantly reduced in alcohol-fed rats compared with pair-fed controls. This inhibition was evident as early as 2 wk after the initiation of alcohol feeding. Similarly, transport of thiamin across renal BLM (studied using purified renal BLMV) was significantly reduced in alcohol-fed rats compared with their pair-fed controls. The inhibition of thiamin transport across renal BBM and BLM domains was associated with a significant inhibition in the level of expression of both THTR1 and -2 proteins in rat renal epithelial cells as indicated by the data of the Western blot analysis. As shown before (6), THTR1 protein was found to be expressed at both the BBM and BLM of the rat renal epithelial cells while that of THTR-2 was expressed only at the BBM.

The above-described changes in the level of expression of THTR1 and -2 proteins was associated with a parallel reduction in the level of expression of mRNA of both transporters. This finding is of interest when compared with our recent findings in the intestine (unpublished observations) where chronic alcohol feeding was found to inhibit thiamin uptake via inhibiting the level of expression of THTR1 without affecting the level of expression of THTR2. The observed inhibition in the level of expression of THTR1 and -2 mRNA in the kidneys of alcohol-fed rats suggests that the inhibition may be mediated, at least in part, via transcriptional mechanism(s). This possibility was tested by determining the level of expression of hnRNA for THTR1 and -2 in kidney cortex of alcohol-fed rats and their pair-fed controls. Changes in hnRNA expression have been used as an indicator for the changes in transcription rate for the involved genes (3, 7, 8, 14, 26). Our results showed a significant reduction in the level of THTR1 and -2 hnRNA in alcohol-fed rats compared with pair-fed controls. These findings raise the possibility that the inhibitory effect of chronic alcohol feeding on thiamin uptake may be, at least in part, mediated via a transcriptionally mediated mechanism(s). Further studies are needed to confirm this suggestion and also to examine if other mechanisms (e.g., changes in RNA stability) are involved in causing the decrease in the level of expression of THTR1 and -2 mRNA.

Our studies also showed chronic alcohol feeding to cause a reduction in the level of expression of TPKase in renal epithelial cells, a finding that is in line with previous functional data showing a decrease in the activity of this enzyme in kidneys of alcohol-fed rats compared with controls (16). The effect, however, does not appear to be transcriptionally mediated, since no change in the level of expression of TPKase in the two rat groups was observed. This suggests that other mechanism(s) (i.e., changes in RNA and/or protein stability) may be in-

**DISCUSSION**

Our aim in this study was to investigate the effect(s) of chronic alcohol feeding on physiological and molecular parameters of thiamin transport in and out of the polarized renal epithelial cells. We were also interested in examining the effect of chronic alcohol feeding on specific aspects of intracellular thiamin handling that are important for renal thiamin nutrition, namely the level of expression of TPKase and mitochondrial TPP transporter, which play important roles in regulating cellular thiamin metabolism and compartmentalization. Our interest in addressing these issues stems from the fact that, while chronic alcohol feeding affects normal thiamin body homeostasis and physiology, its effect on renal transport is less well studied. Also, chronic alcohol feeding appears to affect thiamin metabolism in a tissue-dependent manner (25). We used a well-established pair-feeding regimen of rats with alcohol (and control) liquid diets. Our results showed thiamin transport across the renal BBM (studied using purified renal BBMV preparations) to be significantly reduced in alcohol-fed rats compared with pair-fed controls. This inhibition was evident as early as 2 wk after the initiation of alcohol feeding. Similarly, transport of thiamin across renal BLM (studied using purified renal BLMV) was significantly reduced in alcohol-fed rats compared with their pair-fed controls. The inhibition in thiamin transport across renal BBM and BLM domains was associated with a significant inhibition in the level of expression of both THTR1 and -2 proteins in rat renal epithelial cells as indicated by the data of the Western blot analysis. As shown before (6), THTR1 protein was found to be expressed at both the BBM and BLM of the rat renal epithelial cells while that of THTR-2 was expressed only at the BBM.

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volved. Further studies are required to address these issues. In contrast to the effect of chronic alcohol feeding in the level of expression for THTR1, THTR2, and TPKase, alcohol feeding did not affect the level of expression of the mitochondrial TPP transporter. This finding suggests that not all genes are similarly affected by chronic alcohol feeding, a conclusion that is also supported by a recent observation that chronic alcohol feeding differentially influences the pattern of gene expression (15).

In summary, results of these studies show chronic alcohol feeding leads to an inhibition in thiamine transport across rat renal BBM and BLM and that the effect is exerted, at least in part, at the transcriptional level. In addition, chronic alcohol feeding appears to negatively affect renal thiamin metabolism.

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DISCLOSURES
No conflicts of interest are declared by the authors.

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