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K. Murali Krishna Rao & Terence Meighan

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EXPOSURE IN VIVO TO SILICA OR LIPOPOLYSACCHARIDE PRODUCES TRANSIENT OR SUSTAINED UPREGULATION, RESPECTIVELY, OF PYPAF7 AND MEFV GENES IN BRONCHOALVEOLAR LAVAGE CELLS IN RATS

K. Murali Krishna Rao, Terence Meighan

Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA

A family of proteins containing PAAD [for PYRIN, AIM (absent in melanoma), apoptosis-associated protein speck-like protein containing a caspase recruitment domain, and death domain] domain was found to be involved in modulating inflammatory responses, by its ability to regulate nuclear factor (NF)-κB and procaspase-1 activation. In this study, intratracheal instillation of silica in rats was found to produce transient upregulation of mRNA levels of the PAAD family of proteins, PYPAF7 (PYRIN containing Apaf1-like protein; Apaf stands for apoptosis activating factor) and MEFV (for Mediterranean fever), in bronchoalveolar lavage (BAL) cells. The levels were markedly elevated at 4 h, returning to basal levels by 24 h. In contrast, intratracheal instillation of LPS produced a sustained upregulation of the two genes in BAL cells. In vitro exposure of BAL cells to silica or lipopolysaccharide (LPS) produced no changes in the expression of these genes, indicating that silica or LPS exposure in vivo induces some factors that are responsible for the upregulation of PYPAF7 and MEFV. The mRNA levels of these two genes in peripheral blood monocytes and PMN following LPS exposure did not change, indicating that AM and peripheral blood cells show similar response to LPS exposure in vitro. This study provides the basis for a physiological model to study the effects of these two genes in modulating the inflammatory response after particle exposure.

A family of genes with nucleotide-binding domains (NBD) and leucine-rich repeat (LRR) domains was shown to play an important role in apoptosis, immune function, and inflammatory response. A subgroup of these genes named PAAD [for PYRIN, AIM (absent in melanoma), apoptosis-associated protein speck-like protein containing a caspase recruitment domain, and death domain] or CATERPILLER (caspase recruitment domain, transcription enhancer, r(purine)-binding, pyrin, lots of leucine repeats) (Harton et al., 2002) was shown to affect inflammatory responses by modulating nuclear factor (NF)-κB and procaspase-1 activation. The first gene belonging to this family was identified by positional cloning from patients suffering from familial Mediterranean fever. The gene was named MEFV, and the predicted protein was called pyrin or marenostrin. It is expressed in early leukocyte development and is regulated in response to

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Address correspondence to K. Murali Krishna Rao, MD, Box 2015, PPRB/HELD/NIOSH, 1095 Willowdale Road, Morgantown, WV 26505, USA. E-mail: mir8@cdc.gov

inflammatory mediators (Centola et al., 2000; Tidow et al., 2000). Mutations in this gene produce a disorder characterized by periodic attacks of fever accompanied by serosal, synovial, or cutaneous inflammation (French FMF Consortium, 1997; International FMF Consortium, 1997).

Another member of this family of proteins, Monarch-1/PYPAF7 (PYRIN containing Apaf1-like protein; Apaf stands for apoptosis activating factor), was shown to be expressed in monocytic–myeloid lineage and controls the expression classical and nonclassical MHC class I genes (Williams et al., 2003). Coexpression of this protein with ASC [apoptotic speck-like protein containing a caspase-recruitement domain (CARD)] resulted in the synergistic activation of NF-κB and caspase-1-dependent cytokine processing (Wang et al., 2002). It was suggested that this protein may play a role in the transduction of proinflammatory signals leading to the activation of NF-κB and procaspase-1.

The focus of our investigation has been the regulation of inflammatory responses in lung following exposure to silica. Silica exposure activates NF-κB in alveolar macrophages (AM) and induces production of a number of cytokines (Rojanasakul et al., 1999; Porter et al., 2002; Kang et al., 2000). Similarly, lipopolysaccharide (LPS) exposure also stimulates AM and induces production of a number of cytokines through NF-κB activation (Guha & Mackman, 2001). However, LPS produces an acute inflammatory reaction, whereas silica produces a chronic inflammatory response. Therefore, it is clear that although both stimuli activate NF-κB, there must be other events that are regulating the final outcome.

A number of mechanisms have been proposed for the deactivation of LPS-induced signaling pathways (Fujihara et al., 2003), but an understanding of the role of pyrin family of proteins in regulating NF-kB activation has come to prominence in a number of recent studies. Pyrin, the product of MEFV gene, inhibits cryopyrin-mediated apoptosis and NF-κB activation by disrupting the cryopyrin–ASC interaction (Dowds et al., 2003). A targeted disruption of pyrin produces heightened sensitivity to endotoxin and a defect in macrophage apoptosis (Chae et al., 2003). Similarly, PYPAF7 interacts with ASC and participates in the regulation of NF-κB- and caspase-1-dependent cytokine processing (Wang et al., 2002).

Most of the studies delineating the role of pyrin family of proteins in regulating the inflammatory process were undertaken in systems in vitro. In this study, the expression of two genes, rat MEFV and rat PYPAF7/Monarch1, was evaluated in AM following in vitro and in vivo exposure to silica particles and LPS. In addition, the responses between AM, blood monocytes, and granulocytes were compared after exposure to LPS in vitro and there was no upregulation of these two genes in the three populations. These studies provide a physiological model to study the role of pyrin family of proteins regulating inflammatory processes.

MATERIALS AND METHODS

Animals

The animals used in these experiments were specific-pathogen-free Sprague-Dawley rats [HLA:(SD)CVF] (Hilltop Laboratories, Scottdale, PA), each weighing about 175 g. The animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited, environmentally controlled facility. The rats were monitored to be free of endogenous viral pathogens, parasites, mycoplasmas, *Helicobacter*, and ciliary-associated respiratory bacillus. Rats were acclimated for at least 5 d before use and were housed in ventilated cages, which were provided with HEPA-filtered air, Alpha-Dri virgin cellulose chips (Shepherd Specialty Papers, Watertown, TN), and hardwood Beta chips (NEPCO, Warrensburg, NY) used as bedding. The rats were maintained on 2018S Teklad Global 18% rodent diet (Harlan Teklad, Madison, WI) and tap water, both of which were provided ad libitum.

Reagents

Lipopolysaccharide B (LPS; from *Escherichia coli* 026:B6) was obtained from Difco Laboratories (Detroit, MI). The culture medium consisted of Dulbecco's modified Eagle's medium (DMEM: Sigma, St. Louis, MO), 1 m*M* glutamine (GIBCO, Life Technologies, Grand Island, NY), 10 m*M* *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES; Sigma, St. Louis, MO), 100 U/ml penicillin–streptomycin (GIBCO), 100 μg/ml kanamycin (GIBCO), and 10% (v/v) heat-inactivated fetal bovine serum (GIBCO). Min-U-Sil 5 (diameter <5 μm) was from U.S. Silica, Berkeley Springs, WV. It was examined by proton-induced x-ray emission (PIXE) spectrometry for inorganic contaminants and for desorbable organic compounds by gas chromatography–mass spectroscopy. The results of these analyses were reported elsewhere (Porter et al., 2001). Silica samples were found to be $>99\%$ pure quartz.

Isolation of Alveolar Macrophages

The animals were anesthetized with pentobarbital sodium (150 mg/kg body weight) and exsanguinated by cutting the abdominal aorta. AM were obtained by bronchoalveolar lavage according to the method of Myrvik et al. (1961). The lungs from each animal were lavaged 8 times with 5 ml phosphate-buffered medium (145 m*M* NaCl, 5 m*M* KCl, 9.4 m*M* Na_2HPO_4, and 1.9 m*M* NaH_2PO_4, pH 7.4). The cells were separated from the lavage fluid by centrifugation at 300 × g for 5 min and then washed 3 times by alternate centrifugation and resuspension in phosphate-buffered medium. The cells were then resuspended in the culture medium for use in all experiments. Cell number was determined by an electronic cell counter (model ZB, Coulter Electronics, Hialeah, FL).

Preparation of AM- and PMN-Enriched Fractions

AM and PMN enriched fractions were obtained from BAL fluid obtained from rats treated with silica in vivo, as described by Huffman et al. (2003).

Briefly, the method consists of layering bronchoalveolar lavage (BAL) cell populations obtained by lavage, onto a Histopaque double-density gradient composed of equal amounts of Histopaque 1083 and Histopaque 1119 (Sigma, St. Louis, MO). The gradients are then centrifuged (400 × g, 30 min, 22 °C). The AM-enriched fraction localizes at the interface between phosphate-buffered saline (PBS) diluent and Histopaque 1083, and the PMN-enriched fraction is located at the bottom as a pellet. This method yields about 60% AM in the AM-enriched fraction and 90% PMN in the PMN-enriched fraction (Huffman et al., 2003).

Isolation of Peripheral Blood Mononuclear Cells and PMN

Peripheral blood from rats was collected in a heparin-containing syringe by cardiac puncture from the same animals that were used for isolation of AM. They were subjected to Histopaque gradient separation as described earlier. The mononuclear cells were obtained from the top layer and the PMN from the top of the red cell pellet. The red cells from the PMN pellet were subjected to hypotonic lysis for 50 s.

Quantitation of mRNAs by Reverse-Transcription Polymerase Chain Reaction (RT/PCR)

Cytokine mRNA levels were measured using a SYBR green polymerase chain reaction (PCR) kit with the ABI 5700 sequence detector (PE Applied Biosystems, Foster City, CA). Total RNA was isolated using RNAqueous-4PCR kits (Ambion, Austin, TX) from AM (~2 million cells). One to 2 μg of the DNAse I-treated RNA was reverse transcribed, using Superscript II (Life Invitrogen, Gaithersburg, MD). The cDNA generated was diluted 1:100 and 15 μl was used to conduct the PCR reaction according to the SYBR green PCR kit instructions. The comparative C_T (threshold cycle) method was used to calculate the relative concentrations (User Bulletin 2, ABI PRISM 7700 sequence detector, PE Applied Biosystems, Foster City, CA). Briefly, the method involves obtaining the C_T values for the cytokine of interest, normalizing to a housekeeping gene (18 S in the present case), and deriving the fold increase compared to the control, unstimulated cells. The primers sets for PYPAF7 (accession no. XM 218181) were: forward, 5′-GGC CTC ACT TCC AAA GCA TGT G, reverse, 5′-GCC TCT TGC ACA GCA GAC GAA C; for MEFV (accession no. AF143410), forward, 5′-TTG CCA CAC CCA AGG AGA ACT G, reverse, 5′-CTT TCC TGC ACG CGA GCT TTG; for 18 S, forward, 5′-GGA CCA GAG CGA AAG CAT TTG C, reverse CGC CAG TCG GCA TCG TTT ATG. In preliminary experiments, the products were analyzed by gel electrophoresis, and a single product was obtained with each primer set. In addition, dissociation curves yielded single peaks.

In Vivo Experiments

Rats were anesthetized with an intraperitoneal injection of 30–40 mg/kg body weight sodium methohexital (Brevital, Eli Lilly and Company, Indianapolis, IN) and were intratracheally instilled using a 20-gauge, 4-inch, ball-tipped animal feeding needle. Silica (Min-U-Sil 5 from U.S. Silica, Berkeley Springs, WV) was suspended in endotoxin- and Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS: BioWhittaker, Walkersville, MD), and rats received 2 mg silica/100 g body weight or 10 μg LPS/100 g body weight or an equivalent volume of PBS.

Statistical Methods

Data are presented as the mean ± SEM of separate experiments as noted in the figure legends. A paired *t*-test was used for evaluating in vitro experiments and two-way analysis of variance for in vivo experiments. The significance was set at $p < .05$.

RESULTS

Effect of Silica and LPS in AM

AM were treated with either silica (200 μg/ml) or LPS (1 μg/ml) for 4 h, and mRNA levels of PYPAF7 and MEFV were measured. There was no significant change in expression with either treatment (Figure 1). In contrast, when silica was instilled intratracheally (2 mg/animal), the mRNAs for the two genes were markedly increased in BAL cells at 4 h, returning to basal levels by 24 h (Figure 2),

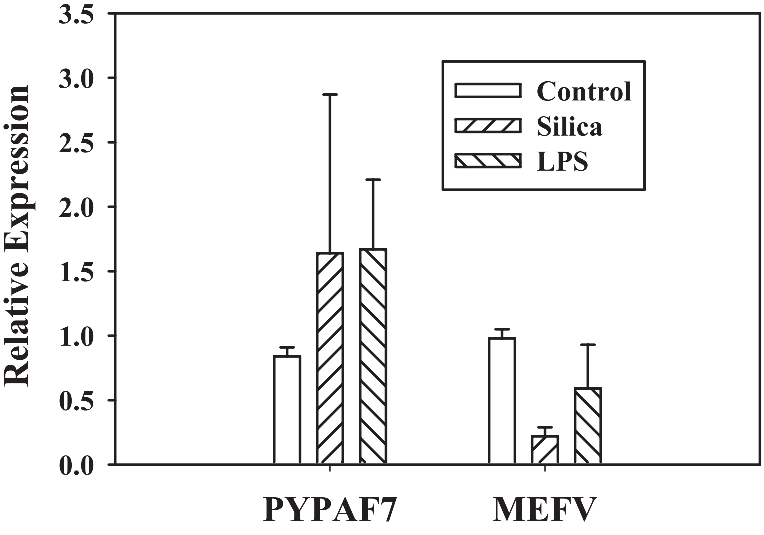

FIGURE 1. Effect of in vitro silica or LPS exposure on the mRNA levels of PYPAF7 and MEFV genes. Control AM (2 × 10^6 cells) in 1 ml culture medium were incubated in 6-well plates for 2 h at 37 °C in a CO_2 incubator for adherence to take place. The nonadherent cells were washed, and the cells were exposed to either 200 μg/ml of silica or 1 μg/ml of LPS for 4 h. Total RNA was isolated, and the message levels were determined by real-time RT/PCR. The bars represent mean ± SEM from four separate experiments run in duplicate.

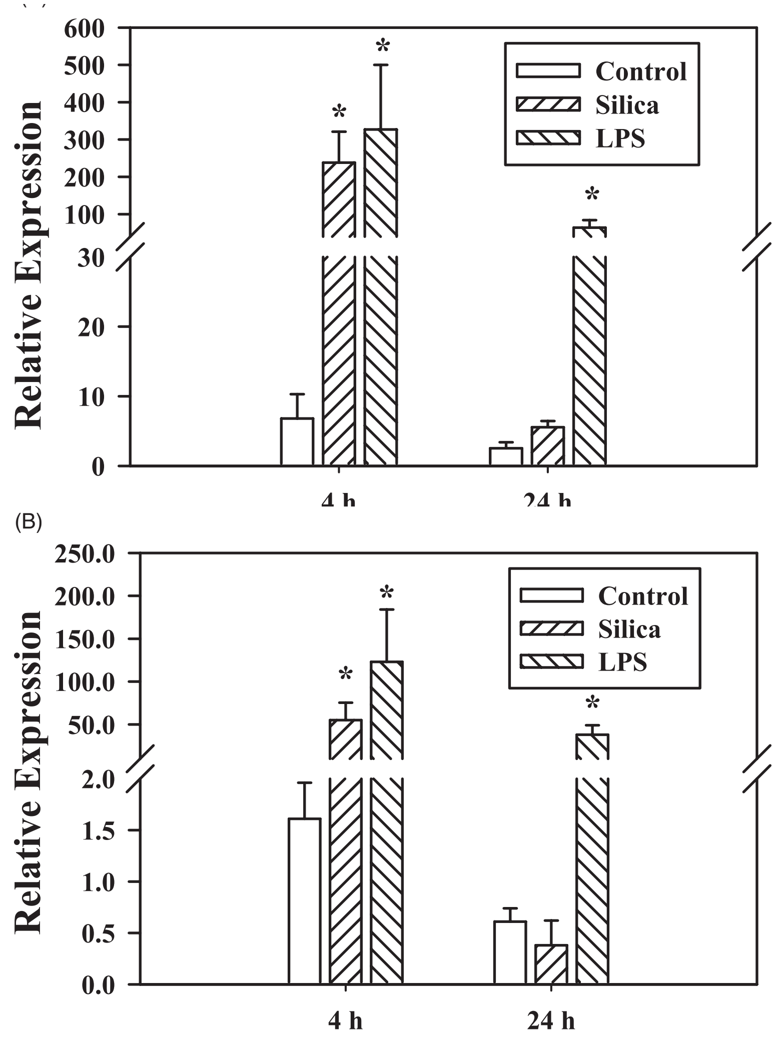

FIGURE 2. Effect of intratracheal instillation of silica or LPS on the mRNA levels of (A) PYPAF7 and (B) MEFV genes run in duplicate from bronchoalveolar lavage cells obtained at 4 and 24 h. The bars represent mean ± SEM from three separate animals. (A) PYPAF7. (B) MEFV. Asterisk indicates significantly greater than control, $p < .05$.

compared to saline-injected controls. However, intratracheal instillation of LPS produced a sustained elevation of both genes.

MEFV and PYPAF7 Expression in PMN and AM

BAL cells obtained from saline injected cells contain mainly AM, whereas BAL cells obtained following silica exposure in vivo contain large number of PMN. In order to determine if the PMN infiltration is responsible for the

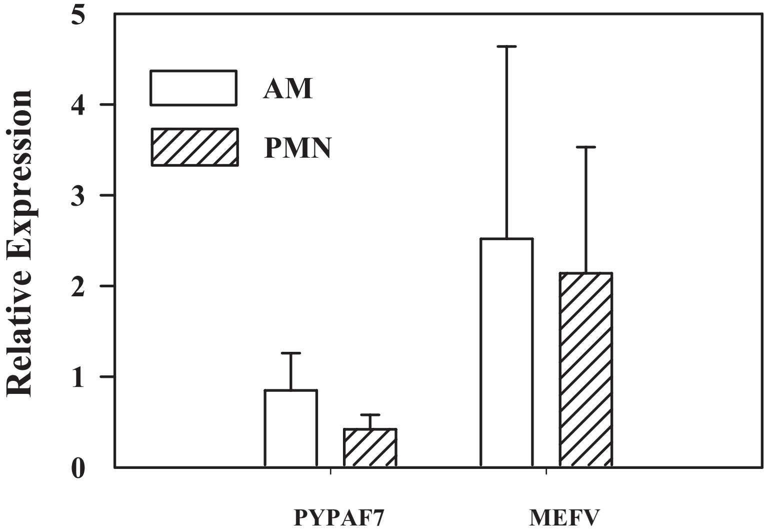

FIGURE 3. PYPAF7 and MEFV mRNA levels in AM and PMN separated from bronchoalveolar lavage cells obtained from rats exposed to silica intratracheally. It should be noted that the expression is relative to AM exposed to silica.

increase seen in mRNA levels of these two genes, AM and PMN were separated and the message levels in the separated populations were measured. As shown in Figure 3, the relative mRNA levels were similar in AM and PMN. It should be noted that these levels were not compared to saline-injected animals, but they were expressed relative to AM in silica-exposed animals.

Effect of LPS on PYPAF7 and MEFV Expression in Peripheral Blood Leukocytes

There was no marked change in mRNA levels in MEFV at 4 h or 24 h following LPS exposure, either in peripheral blood mononuclear cells or PMN (Figure 4). In contrast to MEFV expression, the PYPAF7 expression in peripheral blood mononuclear cells and PMN was extremely variable with no detectable message in some samples. In those samples where the message was detectable, there was no change in mRNA either at 4 h or 24 h following in vitro LPS treatment (data not shown).

DISCUSSION

PYPAF7 and MEFV genes belong to a family of proteins containing a protein domain that is named Pyrin (Martinon et al., 2001; Williams et al., 2003) or PAAD (Pawlowski et al., 2001), a domain that is implicated in apoptosis, cancer, and autoimmune diseases. Recent evidence indicates that a variety of

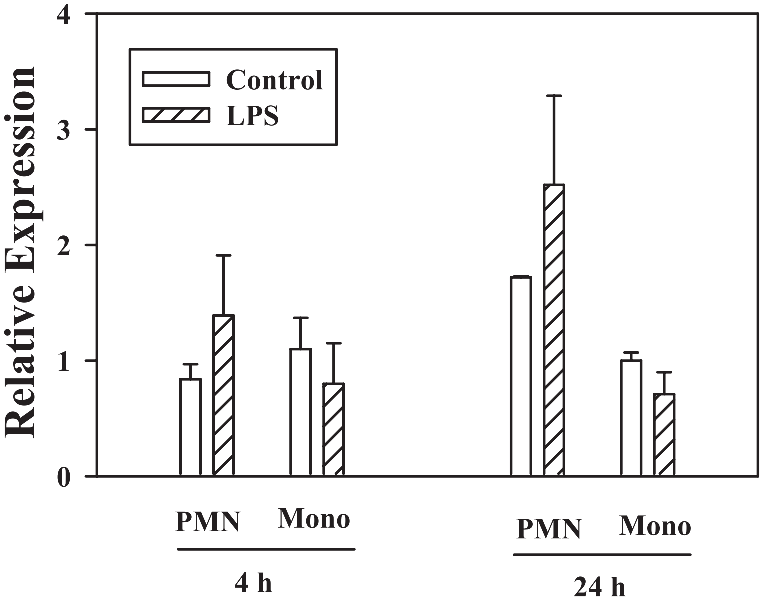

FIGURE 4. Effect of in vitro LPS exposure on the mRNA levels of MEFV gene in peripheral blood mononuclear cells (Mono) and PMN. Peripheral blood cells from control rats were separated into a mononuclear fraction and PMN as described in the methods. The cells were plated (2 × 10^6 cells/well in 1 ml) in 6-well plates in the culture medium and stimulated with LPS (1 μg/ml) for 4 h or 24 h. Total RNA was isolated and mRNA levels were measured by real-time RT/PCR. The mRNA levels were expressed in relation to the levels found in monocytes.

proteins belonging to this group are involved in the regulation of inflammatory responses. These include ASC (Centola et al., 2000) and ASC2/POP1 (Stehlik et al., 2003). Both were found to be regulators of NK-κB activity (Stehlik et al., 2002, 2003) and participate in the processing of IL-1β (Burns et al., 2003; Martinon et al., 2002). Co-expression of PYPAF7 and ASC results in the synergistic activation of caspase-1 and a corresponding increase in the secretion of interleukin-1β (Wang et al., 2002).

The data in this study show that in vivo exposure to silica or LPS upregulates both genes initially in BAL cells, but in vitro exposure does not. This suggests that the contribution of other cell types and microenvironment may play an important role in the expression of these genes.

Most of the studies delineating the role of pyrin family of proteins in regulating the inflammatory process were done in systems in vitro. Studies demonstrating a physiological role for PAAD/CATERPILLER genes have been scarce. The data in this study show that in vivo exposure of AM to silica transiently upregulates PYPAF7 and MEFV genes, but a sustained upregulation occurs with LPS. Both silica exposure and LPS exposure lead to generation of reactive oxygen species (Fubini & Hubbard, 2003; Huffman et al., 1998; Meyer et al., 1991; Zeidler et al., 2004) and activation of NF-κB (Chen & Shi, 2002; Muller et al., 1993), which in turn regulates the production of several cytokines. In the case of LPS, the activation leads to an acute response and if unregulated leads to serious systemic complications such microcirculatory dysfunction, tissue damage, and septic shock with high mortality (Morrison & Ryan, 1987). The sustained expression of MEFV gene may be one mechanism for downregulating LPS response. Indeed, it has been shown that pyrin acts as negative rgulator of cryopryin/PYPAF1 signaling (Dowds et al., 2003). With respect to silica, several explanations may be offered. Studies have shown that AM exposed to silica upregulate message levels but not the protein levels (Rao et al., 2004). The pyrin family of proteins can act as a positive or negative modulator, depending on the context (Stehlik et al., 2002), and may be involved in regulating synthesis of inflammatory mediators. However, further studies are necessary to understand how the fine regulation of inflammatory process is regulated by this family. The observations reported here provide the basis for a physiological model to study the role of PYRIN/PAAD/CATERPILLER family of proteins in modulating the inflammatory response after particle exposure.

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