Role of Inflammatory Pathways, Blood Mononuclear Cells, and Gut-Derived Bacterial Products in Alcohol Dependence

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Background: Inflammation might play a role in the development of several psychiatric diseases. However, the origins of processes that mediate inflammation are unknown. We previously reported increased intestinal permeability, elevated blood lipopolysaccharide levels, and low-grade systemic inflammation associated with psychological symptoms of alcohol dependence in alcohol-dependent subjects. In this study, we tested inflammatory responses of peripheral blood mononuclear cells (PBMCs) to gut-derived bacterial products during detoxification and the relationship to alcohol craving.

Methods: In 63 actively drinking noncirrhotic alcohol-dependent subjects, testing was performed at the beginning (day 2) and end (day 18) of alcohol detoxification and compared with testing in 14 healthy subjects. Activation of various intracellular signaling pathways by gut-derived bacterial products was analyzed by quantitative polymerase chain reaction, Western blotting, and DNA binding assays (for transcription factors). Toll-like receptor activation was assessed by cell cultures.

Results: In addition to lipopolysaccharides, we showed that peptidoglycans may also cross the gut barrier to reach the systemic circulation. Both activate their respective Toll-like receptors in peripheral blood mononuclear cells. Chronic alcohol consumption inhibited the nuclear factor kappa B proinflammatory cytokine pathway but activated the mitogen-activated protein kinase/activator protein 1 pathway, together with the inflammasome complex. This activity resulted in increased messenger RNA and plasma levels of interleukin (IL)-8, IL-1β, and IL-18. Activated proinflammatory pathways, in particular, IL-8 and IL-1β, were positively correlated with alcohol consumption and alcohol-craving scores. Short-term alcohol withdrawal was associated with the recovery of lipopolysaccharide-dependent receptors but not peptidoglycan-dependent receptors.

Conclusions: Lipopolysaccharides and peptidoglycans from the gut microbiota stimulate specific inflammatory pathways in peripheral blood mononuclear cells that are correlated with alcohol craving.

Key Words: Alcohol craving, inflammasome, lipopolysaccharides, MAP kinases, peptidoglycans, proinflammatory cytokines

Studies suggest a role for inflammation in the development of several psychiatric diseases (1), including alcohol dependence (2), a disorder that affects 5%–7% of the population in developed countries (3). Inflammation in alcohol dependence (AD) has been ascribed to a local proinflammatory effect of ethanol, either in the brain or in the liver (2). However, heavy chronic alcohol consumption induces gut mucosal damage, increases intestinal permeability (4–6), induces changes in the composition of the gut microbiota (7,8), and induces bacterial overgrowth in the small intestine (9,10). Gut bacteria are classified into one of two major groups, according the multilayered structure of their cell envelopes. Gram-negative bacteria are surrounded by a thin peptidoglycan (PGN) cell wall, which itself is surrounded by an outer membrane containing lipopolysaccharide (LPS). Gram-positive bacteria lack the LPS-associated outer membrane but are surrounded by layers of PGN many times thicker than is found in gram-negative bacteria (11). The view that systemic inflammation could play a role in alcohol dependence and be induced by increased intestinal permeability and permeation of LPS is supported by more recent data in humans (6). Nevertheless, the possibility of an inflammatory effect of other bacterial components such as PGN has not been tested to date.

Gut-derived bacterial LPS and PGN interact with receptors on lymphocytes and monocytes, the membrane-bound Toll-like receptors (TLR4 and TLR2, respectively), and the cytosolic Nod-like receptors to elicit inflammatory responses (12,13). On receptor activation, a signal transduction cascade converges toward a common set of signaling molecules, leading to the activation of various transcription factors that drive the production of proinflammatory cytokines and type I interferons (14,15).

The Toll-like receptor TLR4 interacts with coreceptors CD14 and MD2 to activate signal transduction pathways through adapter molecules, including myeloid differentiation primary-response protein 88 (MyD88) and TIR domain-containing adapter inducing interferon-β (16–18). The MyD88-dependent pathway, which is common to most Toll-like receptors, leads to the activation of two distinct intracellular pathways: the inhibitor of nuclear factor kappa B (NFκB) kinase pathway, culminating in the activation of the transcription factor NFκB (14), and the mitogen-activated protein kinase (MAPK) pathway inducing another transcription factor, activator protein 1 (AP-1) (19,20). The receptor TLR4 is also able to activate a second MyD88-independent pathway, which results more specifically in the induction of the interferon regulatory factor IRF3, leading to increased production of type I interferons, in particular interferon-β (21). The receptor TLR2 is directly stimulated by PGN, whereas muramyl dipeptide (MDP), which is the minimal bioactive cytosolic structure of PGN, interacts with Nod-like receptor

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proteins NOD2 and NLRP3 (22,23). The receptors TLR2 and NOD2 also activate the MyD88-dependent pathway. The receptor NLRP3 forms the inflammasome, which is a multiprotein complex that also comprises the enzyme caspase-1. This converting enzyme cleaves the precursor forms of interleukin (IL)-1β and IL-18 into mature and active cytokines (24).

The aim of the present study is threefold: 1) to test whether gut-derived bacterial products activate peripheral blood mononuclear cells (PBMCs), which intracellular pathways are involved, and whether they contribute to the systemic proinflammatory cytokine response under natural conditions in noncirrhotic AD subjects; 2) to assess whether activation of specific pathways, and especially the proinflammatory cytokines, are related to the amount of alcohol consumed and to alcohol craving; and 3) to investigate the recovery of pathway activation after 18 days of detoxification.

Methods and Materials

Subjects and Study Design

We recruited 63 actively drinking AD inpatients from the alcohol-detoxification unit of the Departments of Gastroenterology and Psychiatry, Saint-Luc Academic Hospital, Brussels, Belgium. The following minimal eligibility criteria were required: alcohol dependence according to the DSM-IV (25) and alcohol drinking until the day of admission. Exclusion criteria were as follows: the use of antibiotics, probiotics, glucocorticoids, or nonsteroidal anti-inflammatory drugs currently or during the 2 months preceding enrollment and the presence of metabolic disorders such as diabetes and obesity (body mass index >30 kg/m^2), chronic inflammatory diseases (e.g., inflammatory bowel disease or rheumatoid arthritis), cancer, or other severe medical conditions, including cirrhosis or significant liver fibrosis (fibrosis ≥ 2 on transient liver elastography) (Supplement 1). Fasting blood was drawn from the antecubital vein on the day after admission (T1) and at the end of the detoxification program, on day 18 (T2). Among the 63 subjects, 41 participated to both time points (subjects who abandoned their treatment or resumed alcohol consumption were excluded at T2). The AD subjects were compared with 14 control (CT) subjects who were matched for age, gender, and body mass index and who consumed socially low amounts of alcohol (<20 g/day). The study protocol was approved by the ethical committee of the hospital (reference B40320096274), and written informed consent was obtained from all subjects.

Alcohol Consumption

At T1, subjects were asked to self-report the number of drinks that they were having each day before hospitalization. In a subset of 21 subjects, alcohol consumption was evaluated more carefully, with the timeline follow-back approach (26), as detailed in de Timary *et al.* (27).

Isolation of Human PBMCs and RNA

The PBMCs were isolated from blood by centrifugation on a Ficoll-Paque Plus gradient medium (GE Healthcare Biosciences AB, Uppsala, Sweden) (Supplement 1). RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, California).

Reverse Transcription and Real-Time Quantitative Polymerase Chain Reaction

Complementary DNA was synthesized, and quantitative polymerase chain reaction was performed with the Step One Plus device and software (Applied Biosystems, Inc, Carlsbad, California) by using the fluorogenic SYBR Green PCR Master Mix (Applied Biosystems, Inc), as previously described (28). The ΔΔCT method was used for quantification normalized to ribosomal protein L19 RNA (internal standard). Primers (Table S1 in Supplement 1) were designed with Primer Express design software (Applied Biosystems, Inc).

Quantification of Transcription Factor Activation

Activation of transcription factors (p65, c-Fos, phospho-c-Jun) in PBMCs was assessed in whole-cell extracts (Nuclear Extract Kit; Active Motif, la Hulpe, Belgium) by using a sensitive TransAM detection kit (Active Motif) according to the manufacturer's guidelines.

Western Blotting

Western blot analysis was performed on whole-cell extracts according to standard electrophoresis and transfer techniques. Membranes were revealed with the Western Lightning chemiluminescent detection system (PerkinElmer, Waltham, Massachusetts) before quantification of the blots with the Molecular Imager ChemiDoc XRS System (Bio-Rad Laboratories, Nazareth, Belgium). Membranes were stripped (Thermo Fisher Scientific, Erembodegem, Belgium) and reprobed with several antibodies (Table S2 in Supplement 1). β-Actin was used as a loading control.

Plasma PGN and Cytokine Measurements

Plasma was diluted 1:2, and detection of PGN was performed using a PGN enzyme-linked immunosorbent assay kit (CUSABIO Antibodies-online, Aachen, Germany) following the manufacturer's guidelines. Plasma cytokines including tumor necrosis factor (TNF)-α and IL-6, IL-1β, and IL-8 were assayed in duplicate with a multiplex immunoassay (Millipore, Molsheim, France) and Luminex xMap technology (Bio-Rad Laboratories) following the manufacturer's instructions.

Short-Term Cell Culture and In Vitro Stimulation

To test in vitro Toll-like receptor activation on LPS and PGN stimulation, PBMCs from four AD subjects and four healthy CT subjects were cultured (Supplement 1).

Assessment of Alcohol Craving

The Obsessive-Compulsive Drinking Scale (OCDS) questionnaire assesses the cognitive aspects of alcohol craving during the preceding 7 days (29) and provides a total craving score and two subscores: an obsessive and a compulsive subscore (Supplement 1). A validated French version was used in this study (30).

Statistical Analysis

Statistical analyses were performed with SPSS version 20.0 (IBM Corporation, Armonk, New York) after log transformation for non–normally distributed data. Independent *t* tests were performed to compare AD subjects with CT subjects, and paired *t* tests were performed to compare AD subjects at T1 and T2. Correlations were calculated by using the Pearson product-moment correlation coefficient and multiple regression by the stepwise method (Supplement 1). Statistical significance was defined as $p < .05$. Data presented in the graphs are nontransformed means ± SEM.

Results

Demographic Data and Alcohol Consumption

The principal demographic data are summarized in Table 1. The average values of alcohol consumption obtained by using the