Background: Carrageenin (CAR) injection into the pleural cavity causes local inflammation called carrageenin-induced pleurisy (CAR-IP). Inflammation onset is characterized by an activation of pro-inflammatory NFκB, RelA-p50, while inflammation resolution is characterized by an activation of an anti-inflammatory NFκB, p50-p50, that re-establishes homeostasis, an essential process for an organism’s survival. Although chronic alcohol intake disrupts inflammation, the mechanism behind the development of inflammatory disorder in alcoholics is not yet known. Therefore, the aim of this investigation was to study the effects of ethanol intake on CAR-IP and NFκB activation in pleural fluid neutrophils in P rats.

Methods: Alcohol-preferring, P rats were given free choice of alcohol (15% ethanol) and water or water alone (for control) for 15 days. Then, each rat was injected with 0.2 ml of 2% CAR into the pleural cavity under light ether anesthesia. At different time intervals after the CAR injection, rats were anesthetized and their blood and pleural fluid samples were collected. Pleural fluid inflammatory cells were identified with Turk’s or Wright-Giemsa staining. Different cell types were sorted using a fluorescence-activated cell sorter. Pleural fluid neutrophils were examined for apoptosis and activation of the two NFκB subspecies.

Results: In control rats, fluid began to accumulate in the pleural cavity 0.5 h after, which peaked 24 h after, CAR injection. Then, the values declined gradually. The increase in pleural fluid correlated with RelA-p50 activation, while the decline in pleural fluid correlated with p50-p50 activation and apoptosis in neutrophils. In alcohol-drinking rats, pleural fluid remained elevated for up to 6 days after CAR injection. Neutrophils from alcohol-drinking rats exhibited suppressed apoptosis, augmented RelA-p50 activation, and suppressed p50-p50 activation.

Conclusions: Alcohol intake prolonged inflammation in P rats. An alcohol-induced upregulation of RelA-p50 activation and downregulation of p50-p50 activation may be causally related to the alcohol-induced inflammation dysregulation.

Key words: Alcohol, Carrageenin, NFκB, RelA-p50, p50-p50, Apoptosis, Neutrophils

Introduction

Carrageenin (CAR), when injected into the pleural cavity, causes tissue damage and local inflammation called carrageenin-induced pleurisy (CAR-IP). Plasma exudation is followed by neutrophil, monocyte and lymphocyte infiltration in the pleural cavity after CAR injection in rats. The exudate cells are programmed to undergo apoptosis that limits inflammatory response and re-establishes homeostasis. NFκB, a family of dimeric transcription factors consisting of p50, p65 (Rel A), Rel B, c-Rel, p105 and p52/p100 monomers, plays a key role in both the evolution and resolution of inflammation. In resting cells NFκB exists as an inactive NFκB, 1xk complex, where 1xk comprises 1kBα, 1kBβ, 1kBγ/p105, 1kBβ/p100, or 1kBε. The first step in the activation process of NFκB is phosphorylation of 1kB by 1kB kinase complex (IKKα, IKKβ, IKKγ IKKi and IKKε) that phosphorylates 1kB and releases active NFκB, which enters the nucleus and induces transcription of a number of genes. Phosphorylated 1kBα and its degradation by 26S proteosome precedes NFκB nuclear translocation. Onset of inflammation is characterized by an activation of IKKα/IKKB and degradation of 1kBα, leading to
activation of pro-inflammatory NFκB:RelA-p50.13 However, during resolution of inflammation, another NFκB signaling pathway is involved that activates NFκB:p50-p50 homodimer, which induces transcription of anti-inflammatory genes.14,15 Since NFκB:p50-p50 activation occurs when IkB2 is present, it is proposed that IKKz/IκKB is not involved in this process.16 In the nucleus, NFκB:p50-p50 remains bound to a nuclear IkB protein, IκBz.17 Phosphorylation of IκBz activates p50-p50 that, by directly associating with transcriptional co-activators of the C/EBP family or Bcl2, induces gene transcription and apoptosis, respectively.18,19 An interferon-inducible p202 family protein, p202a, suppresses the binding of DNA to RelA-p50, but augments the binding of DNA to p50-p50, leading to an increase in inflammation cell apoptosis.20 Establishment of homeostasis is essential for an organism’s survival. A defect in the resolution of inflammation has been implicated in the development of many diseases including chronic cholecystitis, rheumatoid arthritis, Hashimoto’s thyroiditis, inflammatory bowel disease (ulcerative colitis and Crohn’s disease), celiacis and other pneumoconiosis.21,22 Although chronic alcohol intake has also been shown to disrupt inflammation, immunity and liver function,23,24 the mechanism behind the development of inflammatory disorder in alcoholics is not yet known. A recent study has shown that Kupffer cells isolated from rats after chronic ethanol feeding exhibited upregulated lipopolysaccharide-induced tumor necrosis factor expression and extra-cellular receptor-activated kinases 1/2 (ERK1/2) activity, but repressed nuclear DNA binding of NFκB.25 Contrarily, other studies have shown that chronic ethanol increased NFκB activation, DNA binding of NFκB in liver and other tissues, and susceptibility of rats to endotoxin-induced liver injury.22,26 Because of these conflicting observations regarding the effects of chronic alcohol intake on NFκB activation, further investigation examining NFκB activation in ethanol-dependent rats need to be carried out. We propose that chronic ethanol intake dysregulates CAR-IP by upregulating pro-inflammatory NFκB:RelA-p50 signaling, but suppressing the anti-inflammatory NFκB:p50-p50 signaling in rats. The chronic ethanol-induced dysregulation of inflammation may be critical for the progression of ethanol-induced peripheral toxicity such as liver damage, oxidative stress, and compromised immunity.

Materials and methods

Animal handling

Alcohol-preferring rats (180–200 g, University of Indiana, Bloomington, IN, USA) were kept in a control environment with light from 8:00 p.m. to 8 a.m. Food and water were given ad libitum. A group of rats were given free choice of ethanol (15%) and pure water for 15 days as described previously.27,28 The other group received pure water. Then, pleurisy was induced by injecting each rat with 0.2 ml of 2% CAR into the pleural cavity under light ether anesthesia. At different time intervals (1, 6 and 12 h and 1, 2, 3, and 6 days; n = 4 for each time interval, except 1 h and 2 day groups n = 12) after CAR injection, rats were anesthetized with ether, blood samples were collected from the abdominal aorta, and the pleural cavity was opened. The cavity was rinsed with 1 ml of phosphate-buffered saline and total fluids were collected in a tube containing 1 ml of 77 mM ethylendiamine tetraacetic acid (EDTA). For the 1 h and 2 day samples, pleural exudates from three rats were pooled for n = 4.

Pleural fluid

The exudate volume was determined by subtracting the rinsing volume (1 ml) from the total fluid volume. Then, for each time-line, pleural exudates from three rats were pooled to achieve n = 4. A 100 µl aliquot of pleural fluid was used for cell counting after staining with Turk’s solution. For cell classification, a 200 µl aliquot of pleural fluid was spun on a glass slide coated with poly-L-Lys (Sigma Chemicals Co., St Louis, MO, USA) by centrifugation for 5 min. Then the slide was dried and fixed with 100% methanol. Samples were stained with Wright–Giemsa solution and examined under a light microscope. Different cell types were sorted using a fluorescence-activated cell sorter and analyzed on a Becton Dickinson FACScalibur. The remaining pleural fluid was centrifuged at 800 × g for 15 min and supernatant was collected in a test-tube and stored frozen for analysis of total NO2 and different cytokines. The pellets were subjected to neutrophil extraction as follows.

Processing of exudate for neutrophil isolation

The method described by Bustos et al.29 was modified for isolation of neutrophils from exudates. The anti-coagulated exudate was spun in a narrow test tube. The supernatant was carefully removed as much as possible without disturbing the interface coat. Buffered 2% glutaraldehyde was then very gently layered on top and the tube left to stand in the ice-bath for about a couple of hours. The interface layer was washed in RPMI buffer and then layered onto Ficoll (25 ml of buffer per 12.5 ml of Ficoll) and centrifuged at 950 × g for 7 min. The Ficoll and supernatant interface was collected, mixed with 45 ml of RPMI buffer and centrifuged at 350 × g for 7 min. The cells suspension containing 150–200 × 106 cells were mixed with hyper-osmotic
Percoll solution (48.5 ml of Percoll in 45.5 ml of water and 10 ml of 1.6 M NaCl) in a proportion of 3 ml of cell suspension in 10 ml of hyper-osmotic Percoll. The samples were centrifuged at 1500 × g for 15 min. The pellets were collected and suspended in RPMI buffer. The suspension was centrifuged at 350 × g for 7 min. The supernatant was discarded and the pellets were collected and overlayed onto isotonic Percoll solution (3 ml of cell suspension and 10 ml of iso-osmotic Percoll). Cells were centrifuged at 350 × g for 15 min and the supernatant discarded. The pellets containing neutrophils were suspended in RPMI to achieve approximately 150 × 10⁶ cells/ml.

Assessment of apoptosis in neutrophils

Apoptosis in neutrophils was determined by double staining with annexin-V and propidium iodide. Cells were washed twice with cold phosphate-buffered saline and then resuspended in binding buffer (20 mM Hepes/NaOH, pH 7.4, 132 mM NaCl, 6 mM KCl, 2.5 mM CaCl₂, 1 mM MgSO₄, 1.2 mM KH₂PO₄, 5 mM glucose, and 0.5% bovine serum albumin) at a concentration of ~1 × 10⁶ cells/ml. A 100 µl sample of the solution (~1 × 10⁵ cells/ml) was transferred to a 5 ml culture tube, and annexin-V (25 ng/ml) and propidium iodide (10 ng/ml) were added. Cells were mixed gently and incubated for 15 min at room temperature in the dark. Then 400 µl of binding buffer was added to each tube. The samples were analyzed within 1 h, with 100 µl of the sample viewed under the fluorescence microscope. Green annexin-V fluorescence indicated initiation of apoptosis and red propidium iodide fluorescence indicated late apoptosis.

Preparation of nuclear extract

Neutrophils were suspended in ice-cold HEPES buffer consisting of 10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM phenylmethylsulphonylfluoride, 1.5 µg/ml of soybean trypsin inhibitor, 7 µg/ml of pepstatin A, 5 µg/ml of leupeptin, 0.1 mM of benzamidine and 0.5 mM of dithiothreitol. The samples were homogenized briefly. The samples (2 ml) were mixed with 20 µl of 10% Nonidet P-40 and centrifuged at 1500 × g for 15 min. The supernatant containing the cytosolic fraction was separated and stored at −70°C. The pellets were resuspended in 700 µl of high-salt extraction buffer (20 mM HEPES, pH 9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% v/v glycerol, phenylmethylsulphonylfluoride, 1.5 µg/ml of soybean trypsin inhibitor, 7 µg/ml of pepstatin A, 5 µg/ml of leupeptin, 0.1 mM benzamidine and 0.5 mM dithiothreitol) and incubated by shaking at 4°C for 30 min. The nuclear extract was centrifuged at 13,000 × g for 15 min. The supernatant was collected and stored at −70°C for further analysis.

Electrophoretic mobility shift assay

Nuclear extracts from 1 h and 48 h rats were prepared as described previously. The electrophoretic mobility shift assay was performed using the [³²P]-labeled probes presented in Table 1.

For p202a super-shift assay, the nuclear mixture was incubated with p202a protein (Santa Cruz, Santa Cruz, CA, USA) at room temperature for 2 h and then incubated with p202a antibodies for another 12 h at 4°C. Then the mixture was incubated with the labeled probes and electrophoresis was performed.

A double-stranded probe containing the NFκB recognition sequences was end-labeled with [³²P]-adenosine triphosphate. The reaction mixture (20 µl) included 200 ng of nuclear extract protein, 40 fmol of labeled probe (5 × 10⁴ cpm), 10 mM Hepes (pH 7.9), 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.2–1 µg of poly(dI-dC), and 5% glycerol. After 2 h incubation at room temperature, the samples were loaded onto a 5% non-denaturing polyacrylamide gel electrophoresis gel at 200 V for 2 h. The separated proteins were visualized by autoradiography. For super-shifts, the samples were incubated overnight at 4°C with specific antibodies against p65 (RelA) and p50 proteins prior to adding the probe.

A rationale for using multiple probes is as follows. Activation of NFκB:RelA-p50 and NFκB:p50-p50 is commonly studied by measuring binding of a double-stranded NFκB probe to the two proteins (electrophoretic mobility shift assay) and then causing super-shift with specific antibodies against the dimers. However, the disadvantage of using a single

Table 1. [³²P]-labeled probes used in the electrophoretic mobility shift assay

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
<th>Reference</th>
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<tbody>
<tr>
<td>RelA-p50 probe (P1)</td>
<td>5'-AGT TGA GGG GAC TTT CCC AGG CAA-3'</td>
<td>Reference 15</td>
</tr>
<tr>
<td></td>
<td>3'-TCA ACT CCC CTG AAA GGG TCC GTT-5'</td>
<td>Reference 15</td>
</tr>
<tr>
<td>RelA-p50 probe (P2)</td>
<td>5'-GTT CCG TGA ATT CCC AGG GC-3'</td>
<td>Reference 15</td>
</tr>
<tr>
<td></td>
<td>3'-CCA GGC ACT TAA GGG TCC CG-5'</td>
<td>Reference 15</td>
</tr>
<tr>
<td>RelA-p50/p50 probe (P3)</td>
<td>5'-AAC AGG GGG CCT TCC TCC TCC T-3'</td>
<td>Reference 32</td>
</tr>
<tr>
<td></td>
<td>3'-TTC TCC CCC AAA GGG AGG A-5'</td>
<td>Reference 33</td>
</tr>
<tr>
<td>RelA-p50 probe (P4)</td>
<td>5'-GAT CCG GAG GAT TTC TTG ATG G-3'</td>
<td>Reference 32</td>
</tr>
<tr>
<td></td>
<td>3'-CTA GGC CTC CTA AGG AAG TAG C-5'</td>
<td>Reference 33</td>
</tr>
<tr>
<td>p50-p50 probe (P5)</td>
<td>5'-TCA ACA AGG GGG CCT CCC CTC-3'</td>
<td>Reference 32</td>
</tr>
<tr>
<td></td>
<td>3'-AAT TGT TGG CCC GGC GAG G-5'</td>
<td>Reference 33</td>
</tr>
</tbody>
</table>
probe is that the probe may exhibit high-affinity binding to one protein and low-affinity binding to the other. This may yield erroneous results. The conventional NFkB probe containing a GGGACTTTCC motif exhibited high affinity for RelA-p50 but almost 15-fold lower affinity for p50-p50. Earlier studies have shown that a probe containing the GGGGCTTCC motif exhibited high affinity for NFkB:p50-p50.34 Also, Bohuslav et al.15 designed probes that exhibited differential binding to the two proteins. In this study, we tested six probes for their differential binding to different NFkB proteins.

Results

Pleural exudate and inflammation cells

In control rats, the pleural exudate volume began to rise at 0.5 h after, and peaked at 2 days after, CAR injection. Then the volume declined gradually (Fig. 1). The pleural exudate volume reached a basal level 6 days after CAR injection. In alcohol-fed rats, the pleural exudate volume remained elevated for up to 6 days (Fig. 1). The exudate cells from basal control rats (no CAR) contained less than 10^5/μl immune cells content. At 1 h after CAR injection, although the exudate volume did not change, the cell count increased to 10^5 to 10^6 cells/μl. Then exudate volume and cell count both increased and peaked at 2 days after CAR injection (600–800 x 10^6 cells/μl). Neutrophils were the first to appear, followed by monocytes. The neutrophil count peaked at 2 days after CAR injection (Fig. 2). Then a gradual decrease was noted. There was a late increase in lymphocytes, occurring at 3 days after the injection. In alcohol-fed rats, the cell count remained elevated for up to 6 days.

Figure 3 shows the flow cytometric analysis of exudate cells. Control animals prior to CAR injection poorly contained the inflammatory cells (Fig. 3, basal). At day 2, the exudate cell count increased significantly. Neutrophil was the predominant cell group. At day 4, the cell count decreased but a new group of cells, possibly lymphocytes, appeared (Fig. 3, day 4). In exudate from alcohol-fed rats, the cell counts at day 4 were greater than the count in the day 2 samples (Fig. 3).

Apoptosis

In control rats, exudates cells collected 6 or 12 h after CAR injection poorly labeled with annexin-V or propodium iodide (Fig. 4 control). The annexin-V labeling increased thereafter. Propodium iodide

![FIG. 1. Pleural exudate volume in water-drinking or alcohol-drinking P rats subjected to CAR-IP. Values are mean ± standard deviation, n = 4. *Significant (P < 0.05) when compared with corresponding control values.](image)

![FIG. 2. Distribution of neutrophils (plot i), monocytes (plot ii) and lymphocytes (plot iii) in pleural exudates from water-drinking or alcohol-drinking P rats subjected to CAR-IP. Values are mean ± standard deviation, n = 4. *Significant (P < 0.05) when compared with corresponding control values.](image)

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staining appeared at 4 days after CAR injection. Propodium iodide staining was abundant at 4 and 6 days after CAR injection. Apoptosis was delayed in alcohol-fed rats (Fig. 4 alcohol). A suppression of apoptosis in alcohol-fed rats may account for the persistence of inflammation cells in the exudate.

Electrophoretic mobility shift assay (Fig. 5)

**Control rats.** At 1 h after CAR injection, the mobility shift assay showed that probes P1–P4 yielded a band at 80 kDa that corresponded to the RelA-p50–probe complex and probe P5 did not bind to any protein. Thus, the probe P5 binding protein was not expressed at 1 h after CAR injection. At 2 days after CAR injection, P1, P2, and P4 yielded the 80 kDa band that corresponded to the RelA-p50–probe complex, while P3 also yielded a 60 kDa band that corresponded to the p50-p50–probe complex. P5 yielded only one band that corresponded to the p50-p50–probe complex. This showed that the probes P1 or P2 selectively bound RelA-p50, P6 selectively bound p50-p50, and P3 bound to both complexes. This was further confirmed by studying the p202a supershift assay. Only P3 and P5 probes interacted with p202a, which has been shown to specifically bind p50-p50. The radioactivity incorporation in RelA-p50 band increased 0.5 h after CAR injection and then declined gradually (Fig. 6). The radioactivity incorporation in the p50-p50 band increased at 12 h after, and peaked at 24 h after, CAR injection (Fig. 6).

**Alcohol-drinking rats.** Binding of probes to RelA-p50 in samples from control and alcohol-fed rats was comparable. However, alcohol-fed rats lacked the p50-p50 band (Fig. 5). Thus alcohol suppresses induction of the anti-inflammatory NFκB, p50-p50. The radioactivity incorporation in the RelA-p50 band increased 0.5 h after CAR injection and then declined gradually (Fig. 6). The values were greater than the control values. The radioactivity incorporation in the p50-p50 band increased poorly at 24 h after injection (Fig. 6). Radioactivity levels in p50-p50 bands from
alcohol-fed rats were several fold lower than that in control rats.

Discussion

This study showed that plasma fluid began to accumulate into the pleural cavity 0.5 h after CAR injection. The fluid level peaked 2 days and returned to the basal level 5–6 days after CAR injection in control rats. Neutrophils were detected first, followed by monocytes and lymphocytes in the pleural fluid. The time-course of accumulation of cells in the pleural exudate corresponded to that of fluid accumulation into the pleural cavity. Apoptotic cells were not detected during the first 12 h, but began to appear thereafter. In alcohol-drinking rats, however, considerable fluid accumulation and neutrophils were noted 6 days after CAR injection. Apoptosis was significantly suppressed in alcohol-drinking rats subjected to CAR-IP. Thus, alcohol augmented the CAR-induced inflammation but suppressed its resolution in rats.

An activation of the pro-inflammatory NFκB, RelA-p50, was observed in exudate neutrophils collected from control rats at 0.5 h after CAR injection. Activation peaked at 1 h after CAR injection. RelA-p50 activation has been shown to induce the transcription

![FIG. 5. Binding of different oligonucleotide probes to exude neutrophil nuclear proteins form water-drinking or alcohol-drinking P rats subjected to CAR-IP. Lanes 1–5 represent probes 1–5, respectively. i: RelA-p50 band; ii: unidentified band; iii: free probe; iv: p50-p50 band; v: p50-p50:p202a supershift.]

![FIG. 6. Radioactivity incorporation in different bands representing RelA-p50 (triangles) or p50-p50 (circles). Open triangles or circles represent control rats and solid triangles or circles represent alcohol-drinking rats. Values are mean ± standard deviation, n = 4. *Significant (P < 0.05) when compared with corresponding control values. Radioactivity incorporation in RelA-p50 band from control (i) and alcohol-drinking (ii) rats, and in p50-p50 band from control (iii) and alcohol-drinking (iv) rats.]

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of pro-inflammatory genes including tumor necrosis factor, interferon, interleukin-1 and interleukin-10 genes. However, 12 h after CAR injection in control rats, activation of an anti-inflammatory NFκB, p50-p50, occurred that peaked 24 h after CAR injection. The NFκB:p50-p50, but not NFκB:RelA-p50, band exhibited a super-shift in the presence of p202A. NFκB:p50-p50 has been shown to resolve inflammation by inducing transcription of anti-inflammatory and pro-apoptotic genes and suppressing transcription of pro-inflammatory genes.73 Similar to this study, earlier studies have also shown that CAR injection activated NFκB:RelA-p50 during initiation of inflammation.16,30 thus, a suppression in pro-inflammatory NFκB:RelA-p5050 activation will suppress inflammation, while a suppression in anti-inflammatory NFκB:p50-p50 activation will delay or prevent resolution of inflammation, causing chronic inflammatory diseases. This study also showed that RelA-p50 activation was augmented, while p50-p50 activation was suppressed in exudate neutrophils from alcohol-drinking rats subjected to CAR injection. Earlier studies have associated p50-p50 activation with resolution of inflammation.16,37 Since activation of p50-p50 also induces apoptosis possibly through bcl-2,18,19 we propose that the suppressive effects of alcohol on p50-p50 activation may be responsible for dysregulation of CAR-IP. As discussed earlier, appropriate activation of RelA-p50 and p50-p50 is essential for optimal inflammatory response following injury or bacterial infection. An impaired p50-p50 activation has been shown to be associated with dysregulated inflammation,22,24,38–40 liver function,41–43 and cancer growth.44,45 Since alcoholism has also been associated with dysregulated inflammation,46 hepatitis,47,48 and an increased cancer incidence,49,50 it is possible that an alcohol-induced impairment in RelA-p50 and p50-p50 activation may be causally related to its peripheral toxicity.

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