The Dysregulation of the Monocyte/Macrophage Effector Function Induced by Isopropanol Is Mediated by the Defective Activation of Distinct Members of the AP-1 Family of Transcription Factors

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Received July 18, 2011; accepted October 11, 2011

Isopropanol is the second most common cause of short-chain alcohol acute intoxication. Nonethanolic short-chain alcohols mediate their immunomodulatory effect by interfering with nuclear factor of activated T cells (NFAT) activation with or without additional activator protein-1 (AP-1) involvement. In the present study, we examined the immunomodulation induced by isopropanol in conditions that are not reliant on NFAT: the inflammatory cytokine response of lipopolysaccharide (LPS)-stimulated monocytes. Our hypothesis was that isopropanol acute exposure would have an attenuated effect or no consequence in this setting. To our surprise, the impairment of AP-1 activation was sufficient to mediate a severe and dose-dependent phenotype in human monocytes in vitro at alcohol concentrations as low as 0.16% (or 26mM). There were three outcomes: interleukin (IL)-1β/IL-8 were unaltered; IL-6 was upregulated; and tumor necrosis factor alpha (TNF-α)/CCL2 were downregulated. The effector function of human monocyte-derived macrophages was also compromised. Our results showed that Toll-like receptor 4 early signaling was preserved, as isopropanol did not change the kinase activity of the IL-1 receptor-associated kinase 1 in LPS-stimulated cells. The nuclear factor-κB signaling cascade and the p38/c-Jun N-terminal kinase modules of the mitogen-activated protein kinase pathway were alcohol insensitive. Conversely, the activation of extracellular signal-regulated protein kinase and, ultimately, of c-Fos and JunB were impaired. The alcohol-induced cytokine dysregulation was confirmed in a mouse model of LPS-induced toxic shock. Our data contribute to the dismal body of this alcohol effect was sufficiently high to rescue animals from the inflammatory cytokine response of lipopolysaccharide (LPS)-stimulated monocytes. Our hypothesis was that isopropanol acute exposure would have an attenuated effect or no consequence in this setting. To our surprise, the impairment of AP-1 activation was sufficient to mediate a severe and dose-dependent phenotype in human monocytes in vitro at alcohol concentrations as low as 0.16% (or 26mM). There were three outcomes: interleukin (IL)-1β/IL-8 were unaltered; IL-6 was upregulated; and tumor necrosis factor alpha (TNF-α)/CCL2 were downregulated. The effector function of human monocyte-derived macrophages was also compromised. Our results showed that Toll-like receptor 4 early signaling was preserved, as isopropanol did not change the kinase activity of the IL-1 receptor-associated kinase 1 in LPS-stimulated cells. The nuclear factor-κB signaling cascade and the p38/c-Jun N-terminal kinase modules of the mitogen-activated protein kinase pathway were alcohol insensitive. Conversely, the activation of extracellular signal-regulated protein kinase and, ultimately, of c-Fos and JunB were impaired. The alcohol-induced cytokine dysregulation was confirmed in a mouse model of LPS-induced toxic shock. Our data contribute to the dismal body of information on the general biological effects of isopropanol in animal models of acute, subchronic, and chronic exposure (Burleigh-Flayer et al., 1994, 1997; Kapp et al., 1996; Kasuga et al., 1992), surprisingly little is known about its immunotoxicology. Conversely, it is well documented that ethanol modulates the immune system directly or indirectly by interfering with the function of a variety of cells such as T lymphocytes, monocytes, macrophages, dendritic cells, neutrophils, and endothelial cells (Goral and Kovacs, 2005; Oak et al., 2006; Saeed et al., 2004; Szabo et al., 2007; Taieb et al., 2002; Zhao et al., 2003). In our previous work, we have demonstrated that the molecular events that underlie the immunomodulation induced by short-chain alcohols have different flavors that are specific to each alcohol despite their considerable structural similarities (Désy et al., 2008, 2010). Although many of the biological effects of ethanol on the immune system have been attributed to a dysregulation of the nuclear factor-κB (NF-κB) signaling pathway, other short-chain alcohols seem to keep this signaling cascade unaltered (Désy et al., 2008, 2010; Oak et al., 2006; Saeed et al., 2004; Szabo et al., 2007). Instead, they mediate their immunomodulation by interfering with the activation of the nuclear factor of activated T cells (NFAT) family of transcription factors with or without additional involvement of the activator protein-1 (AP-1). Thus, whereas methanol upregulates NFATc2 nuclear...
translocation in lymphocytes (Désy et al., 2010), isopropanol downregulates the activation of NFATc1 and AP-1 in T lymphocytes and natural killer (NK) cells (Désy et al., 2008). In the present study, we sought to examine the immunomodulation induced by isopropanol in a stimulation model that is less reliant on the NFAT family of transcription factors. We chose to study the monocyte inflammatory cytokine response to lipopolysaccharide (LPS) because it involves a well-defined signal transduction pathway that leads to NF-κB and AP-1 activation and does not require NFAT (Kawai and Akira, 2010). Our hypothesis was that isopropanol acute exposure would have an attenuated effect or no consequence in this setting. Our results revealed that the impairment of AP-1 activation was sufficient to cause a severe and dose-dependent phenotype in human monocytes in vitro at alcohol concentrations as low as 0.16% (or 2.6 mM). Similar to what was reported for T lymphocytes and NK cells (Désy et al., 2008), isopropanol did not change the NF-κB signaling cascade in activated monocytes; nevertheless, it produced an immune dysregulation that was mediated by extracellular signal-regulated protein kinase (ERK) and, ultimately, by the c-Fos and JunB members of the AP-1 family of transcription factors. The immunosuppressive potential of this alcohol was validated in vivo and had sufficiently high magnitude to rescue mice from LPS-induced toxic shock.

**MATERIALS AND METHODS**

**Cell isolation, culture, and stimulation.** This study was approved by the Institutional Clinical Research Ethics Committee (L’Hôpital-Dieu de Québec/Centre Hospitalier Universitaire de Québec—L’Hôpital CHUQ). Mononuclear cells were prepared from the peripheral blood from healthy volunteers by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare, Piscataway, NJ). Written informed consent was obtained from all donors. Monocytes were isolated from mononuclear cells by plastic adherence (Fuss et al., 2009; Szabo and Mandrekar, 2008) and maintained in RPMI 1640 (Invitrogen Canada, Burlington, Canada) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (BioCell Inc., Drummondville, Canada). Monocyte-derived macrophages were generated by culturing monocytes in RPMI 1640 supplemented with 18% heat-inactivated autologous serum for 8 days as described (Szabo and Mandrekar, 2008). Human primary cells were used in most in vitro experiments to strengthen the quality of the data. Established cell lines were used only in a few instances to limit the volume of blood drawn from the donors.

The human monocytic line Mono Mac 6 (Ziegler-Heitbrock et al., 1988) and the murine macrophage cell line P388D1 (Koren et al., 1975) were cultivated in RPMI 1640 containing 10% FBS. Murine monocytes were purified from bone marrow or from spleens (Swirski et al., 2009) by using an antibody-based negative selection kit with magnetic nanoparticles (StemCell Technologies, Vancouver, Canada) according to the manufacturer’s instructions.

Monocytes and macrophages were activated for cytokine production for 24 h at 37°C with 1 μg/ml ultrapure LPS from Escherichia coli O111:B4 (List Biological Laboratories, Campbell, CA). When indicated, alternative cell activation protocols were used: protocol a, 15 min stimulation with 1 μg/ml LPS at 37°C (Western blot and in vitro kinase assay); protocol b, 1 h stimulation with 1 μg/ml LPS at 37°C (transcription factor activation assays); protocol c, 1 h stimulation with labeled E. coli at 37°C (flow cytometry–based phagocytosis analysis); and protocol d, 2 h stimulation with labeled E. coli at 37°C (microscopy–based phagocytosis analysis).

The data on primary cells in the various assays utilized in this work were obtained from multiple donors. The means ± SEMs from independent experiments are indicated in each figure. In addition, two technical replicates per sample were used within each independent ELISA experiment. Isopropanol was purchased from BDH (Toronto, Canada) and was 99.5% pure.

**Cytokine analysis.** Measurements of human tumor necrosis factor alpha (TNF-α), IL-1β, IL-6, and IL-8 or murine IL-6 in cell culture supernatants and murine TNF-α, IL-6, and CCL2 in serum samples were performed with specific cytokine ELISA kits according to the manufacturer’s instructions (BioLegend, San Diego, CA). Human CCL2 levels were measured with the human CCL2 (MCP-1) ELISA Ready-SET-Glo® kit (Ebioscience, San Diego, CA) as recommended by the manufacturer. Supernatants from human cells were used undiluted for IL-1β analysis or diluted 1:20, 1:10, and 1:4 for TNF-α, IL-6, IL-8, and CCL2 measurements, respectively. Supernatants from murine cells were diluted 1:4 for IL-6 analysis. Murine sera were diluted 1:25, 1:50, and 1:5 for TNF-α, IL-6, and CCL2 measurements, respectively. Briefly, 96-well plates were coated with the relevant capture antibody and incubated with serially diluted standards or unknown samples; then, they were washed and incubated with the biotinylated detection antibody followed by streptavidin-horseradish peroxidase. The plates were read at 450 nm after sequential treatment with 3,3’,5,5’-tetramethylbenzidine (TMB) substrate solution and phosphoric acid.

It is worth mentioning that short-chain alcohols may interfere with the sensitivity of certain TNF-α ELISA kits at high concentrations (von Maltzan and Pruett, 2011). In our experiments, TNF-α ELISA assays were conducted with supernatants diluted 20 times and harvested after a 24-h-long incubation period with LPS at 37°C. Isopropanol has an evaporation rate of 2.83 as compared with 1.4 for ethanol and 0.3 for water (n-BuAc = 1.0); thus, only negligible isopropanol amounts were still present at the time of processing of diluted supernatants. As remarked for the ELISA assays for TNF-α measurement in cell supernatants, the serum samples were also diluted (25 times) before analysis. Only trace amounts of isopropanol were left in the diluted serum, < 0.01% or < 1.5 mM, without accounting for metabolic clearance, which is likely to reduce these levels even further.

Inhibitor compounds (Sigma, St Louis, MO) were used as follows: BAY 11-7082: 5 μM; SB202190: 10 μM; SP600125: 25 μM; and PD98059: 50 μM.

**Western blot.** Purified cells were activated for 15 min at 37°C with LPS as described above with or without 0.6% (wt/vol) isopropanol. The cells were washed in alcohol-free buffer and lysed in sodium dodecyl sulfate (SDS) sample buffer (2% wt/vol SDS, 0.25M β-mercaptoethanol, 10% vol/vol glycerol, 0.05M Tris-HCl, pH 6.8, 0.004% wt/vol bromophenol blue); lysates were separated in 12% polyacrylamide gels and blotted onto nitrocellulose filters (Hybond-C, GE Healthcare). All antibodies were purchased from Cell Signaling Technology (Danvers, MA) unless indicated otherwise. The membranes were first probed with the following antibodies: IκB kinase (IKK) α/β detection, anti-phospho-IKKβ/IKKα rabbit monoclonal antibody (16A6, 1:1000) and anti-β-tubulin mouse monoclonal antibody for protein loading control (TUB 2.1, 1:4000, Sigma); p38 detection, anti-phospho-p38 mitogen-activated protein kinase (MAPK) mouse monoclonal antibody (28B10, 1:2000) and anti-total p38 rabbit polyclonal antibody (In-house, 1:10,000); c-Jun terminal kinase (JNK) detection, anti-phospho-SAPK/JNK rabbit polyclonal antibody (Thr¹⁸³/Tyr¹⁸⁵, 1:4000) and anti-β-tubulin mouse monoclonal antibody for protein loading control (TUB 2.1, 1:4000); and ERK detection, anti-phospho-p44/42 MAPK mouse monoclonal antibody (E10, 1:2000) and anti-total p42 MAPK rabbit polyclonal antibody (In-house, 1:5000; Huot et al., 1995). The membranes were subsequently washed and incubated with 1:15,000 dilutions of the antibodies IRDye 800CW goat anti-rabbit immunoglobulin G (IgG) and/or IRDye 680 goat anti-mouse IgG (Li-Cor Biosciences, Lincoln, NE). Detection and quantification were performed with the Odyssey Infrared Imaging System (Li-Cor Biosystems).

**IL-1 receptor–associated kinase 1 immunoprecipitation and in vitro kinase assay.** Mono Mac 6 cells were stimulated with LPS for 15 min at 37°C in the presence or absence of 0.6% (wt/vol) isopropanol. Cells were washed
in alcohol-free PBS and submitted to lysis and IL-1 receptor-associated kinase 1 (IRAK1) immunoprecipitation with reagents from the Roche protein G immunoprecipitation kit (Roche Diagnostics, Laval, Canada) according to the manufacturer’s instructions. The anti-IRAK1 rabbit polyclonal antibody (Millipore, Billerica, MA) was used to generate immunocomplexes. Sample input was equalized for protein content after quantification with the DC Protein Assay (Bio-Rad, Hercules, CA). Washed immunocomplexes were resuspended in kinase buffer (25mM Tris-HCl, pH 7.2, 10mM MgCl₂, 5mM β-glycerophosphate, 0.1mM Na₂VO₄, and 2mM dithiothreitol) and added to the myelin basic protein substrate in the presence of 32P-γ-ATP and cold ATP for 30 min at 30°C. The kinase reaction was stopped by adding SDS sample buffer, and an aliquot was separated in 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were dried and subsequently analyzed and quantified in a phosphoimager.

**Phagocytosis assays.** P388D1 cells were incubated at 37°C with E. coli bioparticles labeled with either pHrodo or Alexa Fluor 488 (Invitrogen Canada) in the presence or absence of isopropanol as indicated. Phagocytosis was analyzed with a TE300 microscope (Nikon, Melville, NY) and quantified with the MetaVue software (Molecular Devices, Sunnyvale, CA). Confocal imaging was performed with a FluoView FV1000 microscope and the FluoView application software (Olympus Canada, Markham, Canada). Alternatively, samples were analyzed in an XL flow cytometer (Beckman Coulter Inc., Miami, FL). Quenching of surface-bound Alexa Fluor 488 bacteria was achieved by incubating the cells with 0.2% (wt/vol) trypan blue in PBS.

**ELISA-based transcription factor activation assay.** Cells were washed in alcohol-free buffer before the generation of nuclear lysates. Nuclear proteins were extracted using the Active Motif Nuclear Extract kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions; the total protein concentration of the lysates was determined by the Bradford assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions; the total protein concentration of the lysates was determined by the Bradford assay (Bio-Rad). c-Fos, Fra1, FosB, c-Jun, JunD, and JunB activation was measured with the TransAm AP-1 kit; p65 activation was measured with the TransAm NF-kB kit. ELISA-based TransAm kits were used according to the manufacturer’s instructions (Active Motif). Briefly, nuclear extracts were incubated with plate-bound transcription factor–specific oligonucleotides; the plates were washed and further incubated with transcription factor–specific antibodies. Addition of a horseradish-conjugated secondary antibody and the TMB substrate produced a colorimetric reaction measurable in a spectrophotometer.

**In vivo studies.** Female BALB/c mice, 7–13 weeks old, were bought from The Jackson Laboratory (Bar Harbor, ME). All tests respected the ethical guidelines set by the Institutional Animal Protection Committee (CPA-CHUQ). Food and water were provided ad libitum. Animals received 5 μg LPS sc for cytokine induction and were sacrificed by CO₂ asphyxiation 90 min or 180 min after administration for TNF-α or IL-6/CCL2 serum analysis, respectively. Toxic shock was induced with an sc injection of 0.2 μg LPS after presensitization with 20 mg n-galactosamine (Sigma) as reported elsewhere (Tsytsykova and Goldfeld, 2000). Animals were checked hourly in the beginning of the protocol and were followed for 5 days. Isopropanol was injected ip (2 g/kg) in the experiments for cytokine induction analysis and for toxic shock protection.

**Statistical analysis.** One-way ANOVA followed by Dunnett’s multiple comparison post test was performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA) on data presented in all figures, except when indicated otherwise. The Student’s t-test was used in Figures 3A, 3B, and 4A. Survival curves were determined by the Kaplan-Meier method (Fig. 5E). p values < 0.05 were considered significant.

**RESULTS**

**Isopropanol Changes the Cytokine/Chemokine Release Profile of Activated Human Monocytes**

Monocytes are capable of releasing several cytokines in response to Gram-negative bacteria (da Silva Correia et al., 2001; Miyake, 2006). The acquisition of effector function is a highly choreographed process that may begin with the recognition of pathogen-derived lipoglycans, as illustrated by the engagement of LPS by the MD2/Toll-like receptor 4 (TLR4) complex, and culminates with the transcriptional activation of several genes encoding immunologically relevant proteins (Miyake, 2006). In the present study, we have investigated whether isopropanol exposure in vitro would have any impact on the ability of human monocytes to produce cytokines once activated by LPS. Three distinct outcomes were observed as illustrated in Figure 1. The releases of the proinflammatory cytokine IL-1β and of the chemokine IL-8 were unaltered even at the highest isopropanol concentrations tested. The production of IL-6 was upregulated by 53% at the lowest alcohol dilution and more than doubled at the highest isopropanol concentration as measured in the culture...
The Immunomodulatory Effect of Isopropanol Extends to Differentiated Macrophages

Monocytes are attracted to injured sites where they differentiate into macrophages (Swirski et al., 2009), which play a pivotal role in the regulation of the inflammatory response. The finding that cytokine release by human monocytes is affected by isopropanol led us to predict a similar impact on the function of derived macrophages. Panel A in Figure 2 shows that exposure to this alcohol in vitro creates an identical profile of cytokine dysregulation in monocytes and macrophages with downregulation of TNF-α/CCL2 and upregulation of IL-6 production. Besides their secretory function, tissue macrophages are also active phagocytes involved in pathogen clearance and removal of necrotic material (Park et al., 2011). Thus, it was conceivable that the alcohol-induced dysfunction could extend to their ability to ingest large particles. We have tested this assumption by examining the internalization of fluorescent bacteria in a murine

**FIG. 2.** Biological effect of isopropanol treatment in vitro on macrophages. (A) Isopropanol treatment affects the cytokine production of human primary macrophages: Primary cultures of human macrophages (Mφ) and monocytes (Mo) were stimulated with LPS in the presence or absence of 0.6% (wt/vol) isopropanol and the supernatants were harvested after 24 h for measurement of TNF-α, IL-6, and CCL2 by ELISA. Concentrations are shown as means ± SEMs. The positive controls (LPS stimulation in the absence of isopropanol) are shown as white columns; the negative controls (unstimulated cells in the absence of isopropanol) are shown as black columns; the experimental samples (LPS stimulation in the presence of isopropanol) are shown as gray columns (**p < 0.01 relative to the positive control [black] group in each histogram; n = 4 for Mo/Mφ TNF-α, n = 3 for Mo/Mφ IL-6, n = 5 for Mo CCL2, and n = 3 for Mφ CCL2). The arrows indicate the mean cytokine production in alcohol-treated unstimulated macrophages. (B) Isopropanol interferes with macrophage phagocytosis: P388D1 cells were incubated with pHrodo* Escherichia coli for 2 h in the presence or absence of 0.6% (wt/vol) isopropanol. These bioparticles fluoresce in acidic environment upon internalization. The panels show representative micrographs of cells without bioparticles (B-I), of cells incubated with bioparticles on ice in the absence (B-II) or presence (B-III) of isopropanol, and of cells incubated with bioparticles at 37°C in the absence (B-IV) or presence (B-V) of isopropanol. (C) Phagocytosis quantification by microscopy: P388D1 cells were incubated with pHrodo* E. coli at 37°C for 2 h in the presence of 0.16, 0.3, and 0.6% (wt/vol) isopropanol. The fluorescence intensity values above background measured with the MetaVue software are shown as means ± SEMs (mean fluorescence intensity [MFI]; **p < 0.01 relative to the [+] control group; n = 4). Background levels were established by incubating P388D1 cells with bioparticles in absence of isopropanol on ice for 2 h. (+) indicates incubation of P388D1 cells with bioparticles and without isopropanol at 37°C for 2 h. IPA indicates isopropanol. (D) Phagocytosis quantification by flow cytometry: P388D1 cells were incubated with Alexa Fluor 488 E. coli bioparticles at 37°C for 1 h in the presence of 0.16, 0.3, and 0.6% (wt/vol) isopropanol. Quenching of surface-bound bacteria was performed as described in the “Materials and Methods” section. The mean fluorescence intensity ± SEM is presented (MFI; **p < 0.01 relative to the [+] control group; n = 6). (−) Indicates incubation of P388D1 cells in absence of bioparticles and without isopropanol at 37°C for 1 h; ICE indicates incubation of P388D1 cells with bioparticles and without isopropanol on ice for 1 h. IPA indicates isopropanol. [Cautionary note: Panel B cannot be properly analyzed without color. The reader is advised to consult the color version of this article available online at http://toxsci.oxfordjournals.org/]
Isopropanol could exert its modulatory effect via direct interaction with molecules that initiate or facilitate signaling in response to lipoglycans. The LPS-binding protein is a lipid transferase that initially promotes the incorporation of LPS aggregates into the plasma membrane and their subsequent transfer to CD14 (Miyake, 2006). The latter ultimately presents LPS to the MD2/TLR4 complex, inducing receptor clustering, recruitment of key adaptor molecules to the TLR4 cytoplasmic domain, and downstream signaling (da Silva Correia et al., 2001; Miyake, 2006). In order to address the possibility that isopropanol would interfere with the membrane-based early events that characterize lipoglycan recognition, we have examined the kinase activity of IRAK1. This enzyme is recruited by the MyD88 adaptad protein early on following LPS stimulation and is central to the activation of NF-κB and AP-1 transcription factors and the resulting production of proinflammatory cytokines (Kawai and Akira, 2010). Panel A in Figure 3 shows that IRAK1 is not affected by alcohol exposure as measured by its ability to phosphorylate a model substrate.

**FIG. 3.** Isopropanol acts downstream of the cell membrane and does not compromise the NF-κB signaling pathway. (A) Isopropanol does not interfere with the activation of IRAK1. Mono Mac 6 cells were stimulated with LPS for 15 min in the presence ([+]IPA) or absence (+) of 0.6% (wt/vol) isopropanol; cell lysates were equalized for protein content and were used for IRAK1 immunoprecipitation as described in the “Materials and Methods” section. The enzymatic activity in the immunocomplexes was assessed by measuring [32P]-γ-ATP incorporation into the myelin basic protein (pMBP) substrate after SDS-PAGE. One representative autoradiograph of four is depicted (mean densitometric units ± SEM: 16.25 ± 2.0, [+]; 15.31 ± 1.8, [+IPA]; p > 0.05). (−) Represents the unstimulated control in absence of isopropanol. (−IPA) represents the unstimulated control in the presence of 0.6% (wt/vol) isopropanol. (B) Isopropanol treatment does not affect IKKα/β phosphorylation following LPS stimulation: Human primary monocytes were stimulated for 15 min with LPS in the presence ([+]IPA) or absence (+) of 0.6% (wt/vol) isopropanol and processed for SDS-PAGE. One representative Western blot is shown. The relative quantification of phosphorylated IKKα/β (pIKK) in relation to β-tubulin expression is depicted in mean densitometric units (DU) ± SEM on the right hand side histogram ([p] p > 0.05, n = 3). (−) Represents the unstimulated control in the absence of isopropanol. (−IPA) represents the unstimulated control in the presence of 0.6% (wt/vol) isopropanol. LC indicates loading control (β-tubulin). (C) Nuclear translocation of the NF-κB p65 subunit is not affected by isopropanol: Primary human monocytes were stimulated with LPS for 1 h in the presence or absence of isopropanol. Nuclear extracts were incubated with immobilized NF-κB-binding oligonucleotides in 96-well plates; the amount of bound transcription factor was assessed with a p65-specific antibody by ELISA. The nuclear transcription factor ratio was calculated by dividing the sample value by the value of the unstimulated control in absence of isopropanol. Data are presented as means ± SEMs (⁎p > 0.05, *p < 0.05, **p < 0.01 relative to the [+] control group, n = 6). The dashed line represents the baseline of p65 nuclear content in unstimulated cells. (−IPA) represents the unstimulated control in the presence of 0.6% (wt/vol) isopropanol. (+) indicates LPS stimulation in absence of isopropanol. (+IPA) indicates LPS stimulation in the presence of 0.6% (wt/vol) isopropanol. BAY indicates LPS stimulation in the presence of the BAY 11-7082 inhibitor compound without isopropanol.
Isopropanol Selectively Modulates the Activation of the ERK Module of the MAPK Superfamily and Reduces the Nuclear Translocation of Discrete Components of the AP-1 Transcription Factor in LPS-Treated Human Monocytes

As our results have revealed that the NF-κB pathway is insensitive to isopropanol exposure, we have decided next to examine the three modules of the MAPK signaling cascade, p38, JNK, and ERK, in LPS-stimulated monocytes. Panel A in Figure 4 shows that there is no clear alcohol effect on the phosphorylation of p38 and JNK. Conversely, the phosphorylation of the activation loop residues Thr185 and Tyr187 in ERK2 was 37% less efficient in the presence of isopropanol. One of the best studied effects of ERK is the initiation of transcription of the immediate early gene c-fos through the activation of the ETS-like transcription factor 1 (Elk-1) (Yoon and Seger, 2006). The c-Fos transcription factor is detectable as early as 20 min after stimulation and is readily phosphorylated at Ser174 by ERK and on additional sites by ERK-activated kinases or by an extended ERK activity (Lallemand et al., 1997; Murphy et al., 2002; Yoon and Seger, 2006). In order to dissect the impact of isopropanol on ERK substrates, we have measured the nuclear translocation of phosphorylated c-Fos in TLR4-stimulated monocytes exposed to different concentrations of the alcohol. Figure 4B, left panel, shows that monocyte activation by LPS led to a threefold increase in the amount of c-Fos in the nucleus. The same stimulation in the presence of 0.16% isopropanol led to a 2.4-fold increase in nuclear c-Fos (or 68% of the maximal c-Fos nuclear content above the unstimulated cell baseline). The alcohol effect was dose dependent, and the increase of this transcription factor in the nucleus was down to 1.7-fold (or 33% of the maximal content) at the highest isopropanol concentration. The same stimulatory conditions in the presence of a combination of inhibitor drugs of the MAPK signaling cascade, PD98059 (MEK1)/SB202190 (p38)/SP600125 (JNK), produced levels of nuclear c-Fos lower than those of the unstimulated cells.

We have examined next the fate of other members of the AP-1 family of transcription factors in the same experimental conditions as above. Our results have revealed that isopropanol exposure does not have any impact on the nuclear translocation of c-Jun, JunD, Fra1, and FosB (data not shown). We have found, however, a measurable alcohol effect on JunB activation. Similar to c-Fos, the JunB protein appears rapidly after cell activation and is encoded by an immediate early gene whose transcription is induced by two important types of ERK substrates, the kinases RSK2 and MSK1/2 (Cargnello and Roux,
The right panel in Figure 4B shows that LPS activation produced a 2.2-fold augmentation in nuclear JunB. Monocytes stimulated in the presence of 0.16% isopropanol experienced a 1.8-fold increase in nuclear JunB (or 68% of the maximal JunB nuclear content above the unstimulated cell baseline). The alcohol effect was also dose dependent, and the LPS-induced increase in nuclear JunB was 1.4-fold (or 36% of the maximal content) at the highest alcohol concentration. The JunB nuclear content in TLR4-activated monocytes was similar to that of control unstimulated cells when the PD98059/SB202190/SP600125 kinase inhibitors were used.

**Isopropanol Inhibits the Production of TNF-α and CCL2 In Vivo and Confers Protection from LPS-Induced Toxic Shock**

The biological effect of isopropanol on the immune response to LPS was subsequently tested in a mouse model of acute alcohol intoxication. Mice were administered isopropanol ip, 2 g/kg, to generate a mean blood alcohol concentration of 200 mg/dl after 30 min. The production of TNF-α, IL-6, and CCL2 was induced in vivo by sc injection of LPS. As anticipated, LPS triggered TNF-α levels of >1700 pg/ml after 90 min (Fig. 5A) and >13,000 pg/ml CCL2 after 180 min (Fig. 5D).

Isopropanol exposure led to a statistically significant down-regulation of the serum release of these cytokines: 86% or 240 pg/ml TNF-α detected at 90 min and 76% or 3195 pg/ml CCL2 detected at 180 min. The differences in cytokine serum levels between animals treated and untreated with the alcohol were statistically significant as indicated in Figures 5A and 5D. Similarly, we expected that isopropanol would increase IL-6 production in this mouse model in a way that would resemble its impact on LPS-stimulated human monocytes in vitro. To our surprise, LPS challenge in vivo was not associated with higher serum levels of IL-6 in the context of isopropanol injected as above, and the animals were sacrificed after 180 min. Serum IL-6 was measured by ELISA. Means ± SEMs are shown (***p < 0.05, **p < 0.01 relative to the LPS/PBS group, n = 6/group). (C) Isopropanol treatment in vitro does not interfere with the ability of mouse monocytes to produce IL-6 in response to LPS. Purified primary murine monocytes were stimulated with 1 μg/ml LPS for 24 h in the presence (+ IPA) or absence (-) of 0.6% (wt/vol) isopropanol. The levels of IL-6 in the supernatants were measured by ELISA and are shown as means ± SEMs (***p < 0.05, **p < 0.01 relative to the [-] control group; n = 3). (-) Represents the unstimulated control in absence of isopropanol. (+)IPA represents the unstimulated control in the presence of 0.6% (wt/vol) isopropanol. IPA indicates isopropanol. (D) CCL2 production is compromised in mice acutely exposed to isopropanol: Experimental groups were treated and labeled as in panel A. Animals were sacrificed 180 min after injections, and serum CCL2 levels were quantified by ELISA. Results are presented as means ± SEMs (***p < 0.01 relative to the LPS/PBS group, n = 6/group). (E) Isopropanol confers full protection from LPS-induced toxic shock syndrome: BALB/c mice were presensitized with 20 mg D-galactosamine; then, they were injected with 0.2 μg LPS sc plus 2 g/kg isopropanol ip (LPS + IPA group). Alternatively, the presensitized animals were injected with 0.2 μg LPS sc plus saline ip (LPS + PBS group). The Kaplan-Meier survival curve is presented (p < 0.0001, n = 10/group).
intoxication (Fig. 5B). This finding made us consider the possibility that murine and human primary monocytes would respond differently to LPS as regards the alcohol modulation of IL-6 release. Indeed, upon LPS stimulation, purified murine monocytes were not responsive to isopropanol in vitro as illustrated in Figure 5C.

Our results revealed that isopropanol is a powerful negative regulator of the inflammatory cytokine TNF-α both in vitro and in vivo. TNF-α is copiously produced and plays a central role in the pathophysiology of LPS-induced lethal shock (Galanos and Freudenberg, 1993; Tsytyskova and Goldfeld, 2000). Therefore, it was our assumption that isopropanol treatment could protect mice from toxic shock. In order to address this issue, we have sensitized BALB/c mice with 20 mg d-galactosamine by ip injection. All sensitized animals developed toxic shock within 16 h following sc LPS challenge (10/10) (Fig. 5E).

A very different picture was produced by alcohol administration, a single injection of 2 g/kg had a dramatic effect because it completely prevented LPS-induced toxic shock, and all animals survived. These mice were followed for 5 days after LPS challenge and were indistinguishable from sensitized control groups receiving PBS or isopropanol. No death occurred in groups injected with isopropanol (10/10) or PBS (10/10) in absence of LPS.

DISCUSSION

Short-chain alcohols mediate a variety of biological effects through nonspecific mechanisms at high concentrations, usually in the 500mM range (Dwyer and Bradley, 2000). Nevertheless, they may interact with specific targets and induce loss of function of ion channels, neurotransmitter receptors, enzymes, and adhesion molecules at more physiologically relevant levels (Jung et al., 2005; Ren et al., 2003; Shahidullah et al., 2003). The inhibitory effect of ethanol on LPS-induced TLR4 triggering in macrophages has been suggested to result from its partition into cell membranes (Dai et al., 2005; Szabo et al., 2007); in this setting, the alcohol would reduce the capacity of microdomains to recruit and/or retain relevant molecules and compromise the cascade that relays the signal generated by receptor ligation on the cell membrane to the nucleus. In a similar fashion, it was conceivable that isopropanol could change LPS binding to CD14 and the stability and/or conformation of the MD2/TLR4 complex, thereby dampening or virtually aborting downstream signaling. This scenario would be compatible with our findings that mostly revealed loss of function of LPS-stimulated monocytes after isopropanol treatment, IL-6 upregulation being the notable exception. Our results, however, showed that TLR4 early signaling is preserved during isopropanol acute exposure (Fig. 3A). We found that isopropanol acts downstream of the cell membrane as it does not change the kinase activity of IRAK1 in LPS-stimulated cells. This is in sharp contrast with ethanol, which affects IRAK1 activity in line with the model described above.

Further down in the activation cascade, the kinase TAK1 is capable of triggering the two major pathways leading to proinflammatory cytokine production during LPS stimulation, MAPKs and NF-κB signaling (Cargnello and Roux, 2011; Israël, 2010; Kawai and Akira, 2010). Our data suggest that isopropanol initiates its effect downstream of TAK1, as the function of this enzyme remains unchanged because the activation of the p38, JNK, and NF-κB signaling cascades proceeds normally (Figs. 3B, 3C, and 4A). We believe that isopropanol may interact directly with ERK2 because ERK1 phosphorylation is not affected, indicating that the upstream MEK kinases are functional (Fig. 4A). Although MEK1/2 are not completely interchangeable, MEK1 can phosphorylate both ERK1/2 in vitro (Xu et al., 2001) and in vivo in MEK2-deficient mice (Bélanger et al., 2003).

Thus, it is unlikely that MEK2 loss of function could account for the lower ERK2 phosphorylation observed in isopropanol-treated samples because the MEK2 kinase activity could be operationally replaced by that of MEK1.

Unphosphorylated ERK2 is virtually idle but undergoes a dramatic conformational change upon cell activation resulting from the posttranslational modification of the Thr183 and Tyr185 residues (Yoon and Seger, 2006). This change is accompanied by the acquisition of a catalytic activity five orders of magnitude higher than basal levels and the ability to translocate into the nucleus (Chuderland and Seger, 2005). There are multiple ERK substrates, and, among these, c-Fos has been studied extensively (Yoon and Seger, 2006). The nuclear content of c-Fos is determined by the level of direct ERK-mediated phosphorylation, which stabilizes the c-Fos protein, and by the very fast transcriptional activation of the c-fos gene via Elk-1, which is also an ERK-dependent process (Cargnello and Roux, 2011; Murphy et al., 2002; Yoon and Seger, 2006).

In agreement with the results presented in Figure 4, lower ERK activity is expected to be associated with a diminished c-Fos phosphorylation and nuclear presence (Murphy et al., 2002). c-Fos dimerizes with Jun proteins to form the AP-1 transcription factor (Lallemand et al., 1997). It is noteworthy that the promoter region of the gene that encodes the chemokine CCL2 displays two AP-1 binding sites (Kok et al., 2009; Martin et al., 1997; Shy et al., 1995; Sutcliffe et al., 2009).

Thus, the lower activation and nuclear translocation of c-Fos that are associated with isopropanol exposure could account for the downregulation of CCL2 production by stimulated monocytes as illustrated in Figure 1. Nevertheless, although there are AP-1 sites also in the proximal TNF-α promoter, monocytes stimulated with whole bacteria or LPS assemble rather unique TNF-α enhanceosomes that include AP-1 heterodimers lacking c-Fos (Barthel et al., 2003; Tsai et al., 2000). It is conceivable that the other transcription factor that we found to be altered in our experiments, JunB, mediates the TNF-α downregulation induced by acute alcohol exposure. The transcription of the
immediate early gene junB is coordinated by the ERK-activated substrate kinases RSK2 and MSK1/2, which activate CREB by phosphorylation at Ser112, thereby allowing the recruitment of CBP and p300 to the junB promoter (Cargnello and Roux, 2011). JunB appears as a protein 20–40 min after the initiation of MAPK signaling and is expected to be downregulated when the catalytic activity of ERK is diminished (Lallemand et al., 1997). Figure 4B, right panel, shows that the nuclear content of transcriptionally active JunB is indeed compromised by isopropanol. In support to this scenario, a recent report described the participation of JunB in the induction of TNF-α by LPS-stimulated myeloid cells and showed JunB binding to the TNF-α promoter by chromatin immunoprecipitation (ChIP) (Gomard et al., 2010). At this point in time, we cannot exclude the possibility that additional factors encoded by immediate early genes downstream of ERK may contribute to the alcohol effect.

The Elk-1–dependent Egr-1 transcription factor, for instance, participates in the transcriptional activation of this gene in LPS-stimulated monocytes (Barthel et al., 2003; Shi et al., 2002; Tsai et al., 2000). This is in line with the observation that the MEK1 (ERK) inhibitor PD98059 blocks Elk-1, Egr-1, and TNF-α expression in LPS-stimulated cells (Guha et al., 2001; Shi et al., 2002).

The finding that isopropanol acute exposure was associated to JunB downregulation can also help us to understand the paradoxical upregulation of IL-6 presented in Figure 1. It has recently been shown by ChIP and luciferase reporter assays that JunB-containing AP-1 complexes act as powerful repressors of transcriptional activity in the context of the IL-6 promoter (Pflegerl et al., 2009). Most importantly, JunB-deficient cells produce larger amounts of IL-6 than their wild-type counterparts (Meixner et al., 2008; Pflegerl et al., 2009). Therefore, the selective and simultaneous reduction of c-Fos and JunB in the nucleus could account for the alcohol-induced downregulation of TNF-α/CCL2 and upregulation of IL-6 in LPS-stimulated cells in vitro.

It is not surprising that isopropanol induces a similar pattern of cytokine modulation in monocytes and macrophages (Figs. 1 and 2A) as these cells represent partially overlapping differentiation stages of the same lineage (Valledor et al., 1998). This modulation is likely to be mediated by the dysregulation of the c-Fos and JunB transcription factors as discussed above. Moreover, we have found that macrophages were less efficient in internalizing bacteria in the presence of isopropanol (Figs. 2B–D), a finding that is reminiscent of the impact of ethanol on phagocytosis (Boe et al., 2010; Goral et al., 2008; Karavitis et al., 2008). ERK has been reported to participate in the Fcγ receptor- and complement-mediated phagocytosis in neutrophils and macrophages (García-García and Rosales, 2002; García-García et al., 2002; Jehle et al., 2006; Mansfield et al., 2000). MAPK signaling also contributes to the uptake of nonopsonized particles by macrophages, although the p38 pathway is believed to play the predominant role (Blander and Medzhitov, 2004). The reduced phagocytosis observed in our experiments with isopropanol may result from impaired ERK signaling. With over 150 direct phosphorylation targets (Yoon and Seger, 2006), ERK kinases are truly pleiotropic and have several possible paths to impact phagocytosis. Thus, the alcohol effect could be indirectly mediated by one of the first identified targets of ERK, cytosolic phospholipase A2, which has been suggested to be involved in phagocytosis (García-García and Rosales, 2002; García-García et al., 2002; Lin et al., 1993); alternatively, such an effect could be a consequence of reduced phosphorylation of cytoskeletal elements by ERK (Mansfield et al., 2000; Yoon and Seger, 2006). This matter will be addressed in future investigations.

The immunomodulation by isopropanol of the monocyte/macrophage effector function was demonstrated in vitro at concentrations as low as 0.16% (26 mM), which are comparable to the concentrations of ethanol associated to a biological effect on immune cells in other studies (Goral and Kovacs, 2005; Oak et al., 2006; Saed et al., 2004; Szabo et al., 2007; Taieb et al., 2002; Zhao et al., 2003). The potential of isopropanol to modulate the TLR4-mediated inflammatory cytokine response to LPS was confirmed in vivo in a mouse model of acute alcohol intoxication. To put in perspective the relevance of the alcohol doses used in our in vivo experiments, we should consider the blood alcohol concentrations in acutely poisoned patients. Although there are thousands of cases of isopropanol intoxication recorded each year (Bronstein et al., 2010), clinical reports with detailed information on time and volume of ingestion are relatively scarce in the medical literature. Patients may survive blood isopropanol concentrations as high as 560 mg/dl (0.56% or 93 mM), whereas others may succumb to much lower concentrations (Lacouture et al., 1983). This disparity comes from the fact that clinical measurements have often been made hours after ingestion and underestimate the serum alcohol levels present in the early phase of the intoxication (Daniel et al., 1981; Gaudet and Fraser, 1989; King et al., 1970; Mueller-Kronast et al., 2003; Rich et al., 1990; Rosansky, 1982). Concentrations above 400 mg/dl are considered life threatening and generally require dialysis (Emadi and Coberly, 2007; Lacouture et al., 1983). We have injected mice with 2 g/kg isopropanol to generate a blood alcohol concentration of 200 mg/dl (0.2% or 33 mM) after 30 min; this level is lower than the reported average sublethal isopropanol blood concentration in severely intoxicated humans (310 mg/dl after 7 h) (Ekwall and Clemedson, 1997) and is comparable to the concentration range that is active in vitro (starting at 0.16% or 26 mM). As suggested by the results of our in vitro experiments, intoxicated mice had a severe impairment in their response to LPS as measured by a substantial drop in serum TNF-α and CCL2 (7.4- and fourfold, respectively). There was, however, no significant effect of isopropanol on IL-6 production in animals challenged with LPS. The latter result suggested that mouse and human cells are differently susceptible to alcohol modulation of IL-6.
production. This assumption was confirmed by exposing purified murine monocytes in vitro to LPS in the presence or absence of isopropanol. We found that murine cells responded to LPS but were indeed insensitive to alcohol modulation of the IL-6 release. The regulation of the IL-6 promoter is rather intricate and involves the concerted action of several transcription factors (Dendorfer et al., 1994; Vanden Berghe et al., 1999). Although the proximal region of the mouse and human IL-6 promoters share consensus sequences for AP-1, NF-κB, NFAT, C/EBP, and CREB, there is divergence further upstream from the transcription start site (Allen et al., 2010; Samuel et al., 2008). It is conceivable that the differential alcohol modulation of the IL-6 response to LPS reflects a distinct set of interactions of JunB-containing AP-1 dimers with other factors that are recruited in accordance with the contextual profile of each promoter; this issue, however, goes beyond the scope of this paper. Overall, the immunological impact of isopropanol in vivo is suppressive and corroborates our previous data obtained with lymphocytes. The magnitude of the TNF-α downregulation led us to speculate that isopropanol could rescue mice from LPS-induced toxic shock syndrome. We found that all animals injected with LPS after presensitization with D-galactosamine developed a fulminant toxic shock with a median survival of 10.5 h. In stark contrast, all mice treated with isopropanol survived without any signs of the syndrome.

Our results have clinical implications, given the possibility that patients acutely intoxicated with isopropanol may also be acutely immunosuppressed. This scenario should be considered in cases of severe poisoning, especially if underlying infection and/or trauma are present. Moreover, the general consumer has easy access to a wide range of products that contain high concentrations of isopropanol, such as hand sanitizers and rubbing alcohol. Isopropanol is readily absorbed by the gastrointestinal tract, but its absorption through intact adult skin is very poor (Brown et al., 2007; Kirschner et al., 2009; Kraut and Kurtz, 2008). With that in mind, limited transdermal absorption may occur with an estimated skin permeation coefficient \( k_p \) of 4–15 × 10^{-4} cm/h (Boatman et al., 1998; Clewell et al., 2001; Cronin et al., 1999; Frasch, 2002; Turner et al., 2004). Although topical application was documented in a few cases of isopropanol poisoning (Arditi and Killner, 1987; Dyer et al., 2002; Leeper et al., 2000), most conventional topical uses are likely to produce only negligible systemic alcohol levels (Brown et al., 2007; Kirschner et al., 2009). In addition, the direct immunomodulatory impact of isopropanol on healthy skin, if any, is likely to be transitory and confined to resident/infiltrating immune cells. From another standpoint, however, one should perhaps be cautious when applying isopropanol-containing gels or solutions to diseased skin. It should be noticed that a typical 70% alcohol solution is over 400 times more concentrated than the minimal biologically active amount in vitro (0.16% or 26mM). In this regard, a recent report has identified the downregulation of JunB and the associated upregulation of IL-6 production in keratinocytes in systemic lupus erythematosus (Pflegerl et al., 2009). As the JunB/IL-6 dysregulation could potentially be exacerbated by isopropanol, one should be prudent in using topical isopropanol in these patients until further investigation is conducted to clarify the risks, if any.

The current work extends our previous findings in that it reveals yet another mechanism of isopropanol-induced immunosuppression, which is based on the disablement of downstream events in the TLR4 signaling cascade. Acute exposure to this alcohol dysregulates the effector function of monocyte/macrophages in vitro and compromises the cytokine response to LPS challenge in vivo to an extent that protects animals from otherwise lethal toxic shock. Our data contribute to the existing dismal body of information on the immunotoxicology of isopropanol, one of the most ubiquitous chemicals in the world.

FUNDING

National Sciences and Engineering Research Council of Canada (327062-07); Canada Foundation for Innovation (4087) to P.O.deC.-L.

ACKNOWLEDGMENTS

The authors wish to thank M. Caruso for helpful discussions and critical reading of the manuscript; D. Richard for access to the infrared imaging system; J. P. McNamee for providing the Mono Mac 6 cell line; Y. Fradet and A. Bergeron for providing the P388D1 cell line; D. L’Hérauld, S. Comeau, and H. Dombrowski for blood collection; C. St-Pierre for microscopy technical support; J. Charron, L. Caron, H. Lambert, and E. Chouinard for helpful discussions. The authors declare no financial or commercial conflict of interest.

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