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A Low Concentration of Ethanol Reduces the Chemiluminescence of Human Granulocytes and Monocytes but Not the Tumor Necrosis Factor Alpha Production by Monocytes after Endotoxin Stimulation

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The ability of polymorphonuclear neutrophils (PMNs) and monocytes (Mφ) to produce reactive oxygen species (ROS) has been related closely to their potential in the killing of microorganisms. Ethanol has been shown to impair the generation of ROS in these phagocytes after stimulation with some immunogens and to increase the susceptibility of alcohol abusers to infectious diseases. As endotoxemia is common in alcohol abusers, we investigated the effect of ethanol (21.7 mmol/liter) on the luminol-amplified chemiluminescence of PMNs and Mφ after endotoxin stimulation and the release of tumor necrosis factor alpha (TNF-α) from Mφ. Further, the efficiency of ethanol to inactivate chemically generated ROS was tested. Significant stimulation of ROS release occurred at endotoxin concentrations of 1 ng/ml or higher in both PMNs and Mφ. Ethanol significantly suppressed the formation of ROS in both cell types, the decrease being more pronounced in Mφ (−73.8%) than in PMNs (−45.7%). The correlations between endotoxin concentration and the amount of released ROS showed a dose-dependent, sigmoidal course. Concentrations of endotoxin necessary for half-maximum stimulation were nearly identical (6 to 8 ng/ml) in both PMNs and Mφ, independent of the presence of ethanol. In contrast to ROS formation, ethanol had no effect on the amount of TNF-α produced by endotoxin-stimulated Mφ. Ethanol was shown to be unable to decrease the levels of chemically generated ROS under physiological conditions. Therefore, ethanol cannot be assumed to be an “antioxidative” compound but rather seems to modify processes of endotoxin recognition, intracellular signal transduction, or metabolism.

One of the most important functions of polymorphonuclear neutrophils (PMNs) and monocytes (Mφ) is the phagocytosis and destruction of invasive microbes. This task is, at least in part, fulfilled by the release of reactive oxygen species (ROS) (4). The potential of endotoxin (lipopolysaccharide [LPS]), a constituent of the outer membrane of gram-negative bacteria and a strong immunogen, to induce the release of ROS in both cell types has been demonstrated previously (12, 18, 29). The activation of phagocytes results in the formation of numerous oxidizing agents such as superoxide, hydrogen peroxide, and hypochlorite (36). After stimulation with endotoxin, monocytes produce a number of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), which is a central mediator of the inflammatory response to LPS activation (10). The simultaneous presence of hydrogen peroxide and hypochlorite has been shown to be essential for the enhancement of chemiluminescence by luminol (2, 33). A close correlation between the ability of phagocytes to kill microbes and to induce luminol-amplified chemiluminescence has been demonstrated previously (11, 16).

The immune response of alcohol abusers is suppressed, leading to an increased infection rate and a higher mortality rate due to infectious diseases (1, 31). This suppression in host defense may be due to an impaired formation of reactive oxygen compounds, as ethanol concentrations in blood observed in alcoholics has been shown to decrease PMN-associ-
Chemiluminescence measurement. Microtiter plates (Maxisorp; Nunc, Wiesbaden Germany) additionally depyrogenized by ethylene oxide treatment were used for chemiluminescence measurements. Cavities of the plates were filled with RPMI medium and blood serum (final concentration, 10%). In every second cavity, ethanol (ad injectable; Braun, Melsungen, Germany) was added to a final concentration of 0.1% (21.7 mmol/liter). One blank (LPS-free) and five final endotoxin (E. coli B4:O111 [Sigma, Deisenhofen, Germany]) concentrations from 0.1 ng/ml to 1.0 μg/ml each concentration 10-fold higher than the previous one, were used for stimulation. Finally, 2.0 × 10⁶ PMNs or 8.0 × 10⁶ PBMCs in RPMI medium were added to each cavity. After computer-controlled addition of 50 μl of 10 mM luminol (5-amin-2,3-dihydro-1,4-phthalazinedione; Sigma) solution in 0.2 M borate buffer (pH 9.0), the chemiluminescence measurement was started immediately in a luminescence microplate reader (BMG Labtechnologie, Offenburg, Germany). To avoid any effects of the luminol or the associated change in pH on the luminescence, the light intensity measurement started with luminol addition and lasted for 1 s. The luminescence intensity was measured every 8 min (final measurement after 56 min).

Direct interactions of ethanol with ROS. To determine the direct (chemical) potential of ethanol to destroy highly reactive oxygen species under comparable conditions, we mixed RPMI medium, luminol in borate buffer, endotoxin, and serum to provide identical concentrations, as described above. Hydrogen peroxide (0.1%, 25 μl) and potassium ferricyanide (1.0 × 10⁻⁵ M, 25 μl) were added in parallel from two computer-controlled syringes, and chemiluminescence was measured during the same time. This experiment was repeated 10 times with and without 0.1% ethanol (21.7 mmol/liter).

TNFα assay. For measurement of TNFα release, 20.0 × 10⁶ PBMCs were kept for 1.5 h (37°C, 5% CO₂) until Mø became adherent. The nonadherent cells were discarded, and the adherent Møs were incubated for 4 h (37°C, 5% CO₂) with increasing LPS concentrations, with and without ethanol. The concentrations of blood serum, ethanol, and LPS were identical to those used in the chemiluminescence assays. After 4 h of incubation, the supernatant was taken and kept frozen (–20°C) until TNFα quantification by enzyme-linked immunosorbent assay (ELISA). After endotoxin challenge, the cells that were adherent to the plastic bottom of the cavities were counted under the microscope.

TNFα sandwich ELISA was performed according to standard ELISA procedures by incubating the supernatants in microtiter plates coated with a monoclonal mouse anti-human TNFα antibody (IC Chemikalien GmbH, Ismaning, Germany). After a wash, adherent TNFα was double labeled with a polyclonal rabbit anti-human TNFα antibody (Genzyme Corp., Cambridge, Mass.) and a peroxo-conjugated monoclonal goat immunoglobulin G Fab anti-rabbit antibody (Medac GmbH, Hamburg, Germany). TNFα was quantified by incubating the antibody complex with an o-phenylene diamine–hydrogen peroxide solution in citrate buffer (pH 5.0) in parallel with a recombinant human TNFα standard (Genzyme Corp.). TNFα concentrations were related both to the number of applied cells (PBMCs) and to the number of adherent cells, which were assumed to be Møs, as only these cells are capable of adherence (see Table 2). The viability of the cells was tested at the end of the experiment with the trypan blue test.

Data evaluation. Values (relative luminescence units [RLU]) are given as means ± standard deviations (SD), if not otherwise indicated. Significance was tested with the Mann-Whitney U test (CSS software; StatSoft Inc.). Mathematical approximation of the luminescence-time solutions was achieved by fitting the last measurement at 56 min, the chemiluminescence intensity declined against the background level (Fig. 1).

All time courses of chemiluminescence intensity were fitted by equation 1 with regression coefficients higher than 0.95.

The total amount of released ROS (calculated as the AUC below function 1 and approximated by equation 2) was significantly increased at an endotoxin concentration of 1.0 ng/ml or higher without alcohol (P < 0.05). In the presence of 0.1% ethanol (21.7 mmol/liter), the AUC was significantly elevated at LPS concentrations of 10 ng/ml or higher in comparison to the unstimulated cells, for both PMNs (Fig. 2) (P < 0.05) and Mø (Fig. 3) (P < 0.05).

For PMNs stimulated with an endotoxin concentration of 1.0 ng/ml or more, ethanol depressed significantly the amount of ROS produced (P < 0.05) (Fig. 2). ROS formation by Mø after being challenged with endotoxin was also significantly depressed (P < 0.05) in the presence of ethanol (21.7 mmol/liter) at LPS concentrations of 0.1 and 1 ng/ml. This depression was more pronounced (P < 0.01) at endotoxin concentrations of 10 ng/ml or higher (Fig. 3).

Results

Luminol-amplified luminescence values and, therefore, increased production of ROS was already detectable at 8 min after endotoxin (LPS) stimulation in both PMNs and PBMCs. The highest values were reached after 24 min, and, approximating the last measurement at 56 min, the chemiluminescence intensity declined against the background level (Fig. 1). All time courses of chemiluminescence intensity were fitted by equation 1 with regression coefficients higher than 0.95.

The total amount of released ROS (calculated as the AUC below function 1 and approximated by equation 2) was significantly increased at the endotoxin concentration of 1.0 ng/ml or higher without alcohol (P < 0.05). In the presence of 0.1% ethanol (21.7 mmol/liter), the AUC was significantly elevated at LPS concentrations of 10 ng/ml or higher in comparison to the unstimulated cells, for both PMNs (Fig. 2) (P < 0.05) and Mø (Fig. 3) (P < 0.05).

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Points of inflection of the sigmoidal regression (corresponding to half-maximum stimulation of the cells) were 6.0 ng of LPS per ml without ethanol and 8.0 ng of LPS per ml with ethanol in PMNs (Fig. 2). Monocytes reached half-maximum

![Graph showing chemiluminescence intensity over time with and without ethanol.](image-url)

FIG. 1. Time course of chemiluminescence (expressed as mean RLU ± standard error of the mean) of granulocytes with (solid lines) or without (dashed lines) 10 ng of endotoxin (E. coli O111:B4) per ml in the presence (☐, ☐) or absence (●, ○) of ethanol (21.7 mmol/liter). The AUC for comparative statistics was calculated from the function given in equation 1.

![Graph showing correlation between endotoxin concentration and release of ROS.](image-url)

FIG. 2. Correlation between endotoxin concentration and release of ROS from PMNs in the presence (solid line) or absence (dashed line) of 21.7 mmol of ethanol per liter. ROS release was calculated as the AUC (mean ± SD) from the function given in equation 1. The course of AUC dependence on endotoxin concentration was approximated by equation 2 for calculation of the LPS concentration necessary for half-maximum stimulation (point of inflection, as given in the text). * P < 0.05.
release of ROS (point of inflection) at 7.8 ng of LPS per ml without alcohol and 6.9 ng of LPS per ml with alcohol (Fig. 3).

Maximum LPS-induced chemiluminescence was weaker in monocytes within the PBMC fraction (68.7% of the value reached by polymorphonuclear neutrophils, calculated for identical cell numbers [P < 0.01]). At endotoxin concentrations of maximum ROS stimulation (100 and 1,000 ng/ml), Mφ in the presence of other PBMCs were more sensitive to ethanol in ROS production (~73.8%) than were PMNs (~45.7%), if only endotoxin-induced stimulation was taken into account and background signals were neglected.

Ethanol did not decrease the chemiluminescence when a chemical system (hydrogen peroxide-potassium ferricyanide) was used for the generation of ROS (Table 1). Also, different endotoxin concentrations had no influence on the concentration of active, luminescence-inducing ROS.

Endotoxin addition resulted, as expected, in increased expression of TNF-α protein in Mφ. The TNF-α release was dose dependent and significantly increased at LPS concentrations of 10 ng/ml or higher (Table 2). In the present system, ethanol did not have any influence on the production of TNF-α from monocytes, either the applied (PBMCs) or adherent (Mφ) cells (Table 2).

For each experiment, viability was found to be higher than 97% at the end of the experiment.

**DISCUSSION**

The results presented above confirm findings showing a release of ROS in PMNs (19) and activation of Mφ by LPS (18). They are not consistent with investigations postulating the failure of endotoxin in the stimulation of ROS production of phagocytes (38). According to the results of this study, endotoxin is a potent activator of ROS release from Mφ and PMNs if blood serum factors are present.

Furthermore, a clear inhibition of ROS formation in PMNs and Mφ by ethanol at low concentrations after LPS elicitation in vitro is evident. Ethanol also inhibits zymosan-induced chemiluminescence of phagocytes in whole blood (5), indicating a similar effect of ethanol on immunogen-stimulated chemiluminescence in complex cell systems and isolated cell types. The course of chemiluminescence impairment in PMNs and Mφ indicates a classical, noncompetitive inhibition (Fig. 2 and 3). From the present experiments, the exact type of impairment in receptor binding or enzyme activity cannot be deduced, and further experiments are necessary to clarify this result. Simple noncompetitive inhibition of an enzyme, which is responsible for the formation of one of the ROS after endotoxin stimulation by ethanol, could be a possible explanation for the reduced ROS generation.

A direct reaction of ethanol with the formed ROS and a direct chemical inactivation of these compounds is not consistent with the fact that ethanol is not able to reduce the concentration of chemically generated ROS. This is evident from the failure of ethanol to reduce the chemiluminescence within the hydrogen peroxide-potassium ferricyanide system (Table 1). Based on chemical theory, ethanol is unable to provide mesomerically stabilized forms after splitting off of an unpaired electron and therefore does not possess the ability to form stabilized radicals itself, lacking essential structural demands. Therefore, a direct “antioxidative” effect of ethanol (7, 15, 35) is unlikely.

A reduced phagocytosis in PMNs and Mφ has been shown to occur after incubation with ethanol, and the impaired phagocytosis has been discussed as a possible reason for reduced ROS release (6, 21). In the experiments of the present study, no particles for phagocytosis were presented to the phagocytes. Nevertheless, a reduction in ROS formation was evident. Therefore, phagocytosis does not seem to be an indispensable trigger for ROS production in this case.

Ethanol or its first metabolite, acetaldehyde, has been assumed to be cytotoxic (22); such a cytotoxic effect could be responsible for reduced ROS formation. However, the high percentage of viable cells at the end of all experiments is inconsistent with this hypothesis.

An increase of the cytosolic calcium concentration is assumed to serve as an activating signal of the physiological response during the respiratory burst of leukocytes (23), but low ethanol concentrations were not found to change basal levels of calcium after stimulation (25, 27). A short-term increase in the intracellular concentration of cyclic AMP (cAMP) occurs after phagocyte stimulation (30), whereas a long-term elevated cytosolic cAMP concentration in phagocytes is associated with a decline in phagocytic functions (9, 14, 20). Ethanol has been demonstrated to increase intracellular cAMP levels (3, 17, 35). Hence, the ethanol-associated elevation of cytosolic cAMP concentration has to be taken into account.

![Graph](image)

**FIG. 3.** Correlation between endotoxin concentration and release of ROS from PBMCs in the presence (solid line) or absence (dashed line) of 21.7 mmol of ethanol per liter. ROS release (AUC [mean ± SD]) and the point of inflection were calculated as described for PMNs. *, P < 0.05; **, P < 0.01.

**TABLE 1.** Influence of endotoxin and ethanol (21.7 mmol/liter) on the inactivation of chemically generated ROS from a hydrogen peroxide-potassium ferricyanide system

<table>
<thead>
<tr>
<th>Exptl condition</th>
<th>Chemiluminescence (AUC [10^3 RLU · r]/cavity) at endotoxin concn (ng/ml) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Without ethanol</td>
<td>29.4 ± 3.2</td>
</tr>
<tr>
<td>With ethanol</td>
<td>31.0 ± 1.8</td>
</tr>
</tbody>
</table>

* Values are means ± SD. There are no significant differences between any of the values.
account as a major mechanism in the depression of ROS generation.

Endotoxemia is common in alcoholics (8), but the LPS concentration in the plasma of these patients is still nearly 3 orders of magnitude lower (15 to 40 pg/ml [13]) than the measured concentration necessary to reach half-maximum stimulation (6 to 8 ng/ml). This effect may be due to isolation from the complex environment in whole blood and the modified interaction between the investigated and the endothelial cells, resulting in reduced sensitivity of the cells to endotoxin challenge. On the other hand, the response to the endotoxin stimulus is very fast, indicating a short-term intracellular response which is unlikely to be due to complex cell interactions or cytokine network-associated reactions.

Furthermore, as LPS-binding protein, a plasma factor important for LPS recognition, is present at milligram-per-liter concentrations in human blood (34) and a 10% autologous serum concentration was used during stimulation, an important for LPS recognition, is present at milligram-per-milliliter concentrations in human blood (34) and a 10% autologous serum concentration was used during stimulation, an impairment of LPS recognition by PMNs and Mφ is unlikely. It may be possible that ethanol-accompanied impairment starts to play a role in cases of local bacterial infections, in which the LPS concentration determined for half-maximum stimulation can be easily reached.

The influence of physiologically relevant ethanol concentrations on LPS-stimulated ROS formation of phagocytes parallels its effect on these cells when other stimuli are used (5, 25–28, 35). Therefore, ethanol-induced suppression of ROS released from these cells does not seem to depend on the kind of applied stimulus but rather is related to changes in intracellular metabolism or signal transduction.

TNF-α production has been reported by Szabo et al. and Verma et al. to be suppressed after acute ethanol exposure (32, 37). Although we showed a significant increase of TNF-α release at an LPS concentration as low as 10 ng/ml in comparison to unstimulated controls, we were not able to confirm the findings of those authors, as no suppression of TNF-α production by 0.1% ethanol over the applied LPS concentration range was evident. The inconsistency of this result may be due to different experimental conditions, as the suppression of TNF-α release from LPS-activated Mφ by ethanol was reported after preincubation with alcohol, without the presence of ethanol during LPS challenge, and with the application of fetal calf serum instead of autologous human serum.

ACKNOWLEDGMENTS

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REFERENCES


TABLE 2. TNF-α production by Mφ after 4 h of incubation with increasing concentrations of endotoxin with or without 0.1% ethanol

<table>
<thead>
<tr>
<th>Exp1 condition</th>
<th>TNF-α production (pg)/1,000 PBMCs or Mφ (mean ± SD) at endotoxin concn (ng/ml) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Applied PBMCs without ethanol</td>
<td>0.4 ± 0.9</td>
</tr>
<tr>
<td>Applied PBMCs with ethanol</td>
<td>0.4 ± 0.9</td>
</tr>
<tr>
<td>Adherent Mφ without ethanol</td>
<td>17 ± 51</td>
</tr>
<tr>
<td>Adherent Mφ with ethanol</td>
<td>23 ± 41</td>
</tr>
</tbody>
</table>

* After incubation with LPS.

** No significant differences were found between results with and without ethanol at a single endotoxin concentration. Asterisks (*, P < 0.05; **, P < 0.01) indicate significantly higher yields of TNF-α compared to zero stimulation (endotoxin [LPS] concentration of 0 ng/ml). Compared to zero stimulation, Mφ produced significantly higher amounts of TNF-α at LPS concentrations of 10 ng/ml or higher, independently of the presence of ethanol.


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