Effects of Ethanol and Lipopolysaccharide on the Sphingomyelin Cycle in Rat Hepatocytes

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Lipopolysaccharide toxin added to primary hepatocyte culture slightly modified the basal concentrations of ^{3}H-serine-labeled sphingomyelin, sphingosine, and ceramide. Ethanol reduced the levels of sphingomyelin and sphingosine by 20-25 and 15-20%, respectively, but increased ceramide content by 7-17%. Tumor necrosis factor reduced the concentrations of sphingomyelin and sphingosine, but did not modify the content of ceramide. Combined treatment with lipopolysaccharide toxin and ethanol potentiated the effect of alcohol.

Key Words: *sphingomyelin; ceramide; hepatocytes; lipopolysaccharide; ethanol*

Ethanol and lipopolysaccharide toxin (LPS) produce a deleterious effects on parenchymal cells of the liver (hepatocytes) *in vivo* and *in vitro*, sometimes even leading to their apoptosis [3,4,6]. The effect of ethanol and LPS is often mediated through TNF, which is released in the liver mainly by Kupffer's cells, but not hepatocytes. Many toxic events in cells are associated with activation of the sphingomyelin cycle [2], which, similarly to the glycerophospholipid cycle, plays the key role in signal transmission cascade during cell proliferation, differentiation, and apoptosis. Sphingomyelin (SM), one of the main lipids of the cell membrane, plays the structural role and participates in the generation of important second messengers. Ceramide (CA) formed under the action of sphingomyelinase stimulates differentiation, inhibits proliferation, and causes apoptosis. Ceramide, in turn, is the substrate for ceramidase, catalyzing the formation of sphingosine (SP) converted then into sphingosine-1-phosphate (SP-P). Sphingosine inhibits proteinkinase C and cell growth, causes apoptosis, while SP-P stimulates cell growth and blocks apoptosis. The effects of endogenous sphingomyelinases can be simulated by bacterial sphingomyelinases, which hydrolyze SM on the cell surface with the formation of CA.

We studied the time relationship between basal concentrations of SM, CA, and SP in primary culture of rat hepatocytes exposed to combined treatment with ethanol and LPS and evaluated the role of TNF.

MATERIALS AND METHODS

Primary hepatocyte culture derived from Sprague-Dawley rats (5×10^6 cells/dish/5 ml medium) was prepared as described previously [9]. No less than 97% isolated cells were not stained by trypan blue (*i.e.* were viable). Wild type purified lyophilized LPS (*Salmonella typhimurium*), neutral sphingomyelinase (*Staphylococcus aureus*), TNF-α, lipid reference samples, and reagents from Sigma were used. Hepatocyte culture was labeled with ^{3}H-serine (Amersham, 0.1 mCi/dish, 37°C, 4 h). After experiments, the cells were scraped and lipids were extracted [1]. The extract was hydrolyzed with 0.1 M methanol KOH (37°C, 1.5 h) in order to remove all acyl lipids (SM and its metabolites were retained). Then ^{3}H-labeled SM, SP, SP-P, and CA were ana-

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lyzed by a specially developed two-stage thin-layer chromatography (10×20 cm Silica Gel plates, Merck). At stage I, the plates with cell lipid spots were chromatographied in a hexane:ethanol:acetic acid (20:30:2) system. Neutral lipids migrated close to the front-line (R_f<0.65), while for CA R_f=2.5, and other components remained at the start. At stage II and directly after stage I the TLC plates were chromatographied in the same direction in a methylacetate:n-propanol:chloroform:methanol:0.5 KCl (50:50:50:20:18) system until the solvent front-line approached the neutral lipid zone (Fig. 1). In this case, R_f values were 0.90-0.93 for CA, 0.41-0.42 for SP, and 0.10-0.12 for SM, while SP-P remained at the start. The spots corresponding to CA, SP, and SM were scraped off from the plate; radioactivity was measured in a scintillation counter.

RESULTS

Treatment of hepatocyte culture with exogenous sphingomyelinase led to a rapid significant reduction of SM level and increase in CA concentrations, which persisted throughout the entire experiment (Table 1). Incubation of hepatocytes with ethanol caused similar changes: degradation of SM, reduction of SP level, and accumulation of CA, higher concentrations of ethanol induced more pronounced effects. It can be hypothesized that ethanol activates endogenous neutral sphingomyelinase in hepatocytes [4], presumably without stimulating ceramidase. In addition, the function of phospholipase D is impaired in the presence of ethanol (phosphatidylethanol is formed instead of phosphatide acid), which, in turn, can promote the increase in CA level [5].

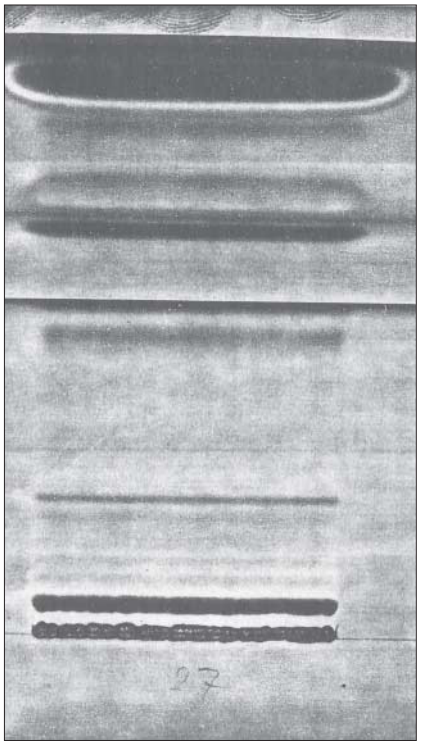

Fig. 1. Unidimensional two-stage TLC method, developed for analysis of sphingomyelin cycle components: chromatogram of control hepatocyte lipid extract after acyl lipid hydrolysis.

Treatment of hepatocytes with LPS caused no appreciable activation of the sphingomyelin cycle (Table 1): basal concentrations of SM, SP, and CA virtually did not change. However, not LPS, but TNF, a second messenger released by other hepatic

TABLE 1. Concentrations of ^{3}H-Labeled Components of Sphingomyelin Cycle (% of control) in Rat Hepatocytes Depending on Activator and Time of Incubation

Sample	Duration of incubation, min														
	5			15			30			60			120		
	SM	SP	CA	SM	SP	CA	SM	SP	CA	SM	SP	CA	SM	SP	CA
Sphingomyelinase, 0.258 U/ml	83.6*	94.9	102	78.2*	101	107.6*	60.3*	90.1	121.5*	55.1*	92.3*	131*	48.5*	93.5	133*
Ethanol, 100 mM	97.7	93.2	97.9	95.2	92.1	93.1	86.2*	80.2*	94.8	78.4*	82.1*	105	79.1*	80.3*	106.7*
Ethanol, 300 mM	89.4*	101	97.5	92.6*	82.7*	93.3	81.3*	81.5*	94.4	73.5*	85.2*	112*	74.8*	83*	116.9*
TNF, 100 ng/ml	94.5	107	98.3	95.1	93.9	94.1	83.8*	85.5*	99.2	84.8*	88.1*	102	82.3*	85*	99.8
LPS, 1 μg/ml	98.7	96.7	98.9	95.8	99.7	98.9	93.7	99.7	103.5	94.2	96.3	99.6	95.4	97.2	98.4
LPS (1 μg/ml)+ ethanol (100 mM)	96.5	99.2	98.8	90.9*	94.4	101.1	80.1*	87.3*	104.4	76.2*	88.8*	108.2*	74.6*	89.9*	114.7*

Note. Each time point had a special control (intact cell culture without additives). Mean radioactivity for the control (per dish; cpm): SM: 8230; SP: 740: CA: 11,040; start (SP-P): 1800; neutral lipids: 20,100. Mean values of three experiments are presented. *p<0.05 compared to the control.

cells (Kupffer and endothelial cells) in response to LPS, can destroy hepatocytes during intoxication *in vivo*. Direct effect of TNF on SM metabolism in hepatocytes was tested. Though TNF activated sphingomyelinase leading to a reduction of SM level (Table 1), no appreciable accumulation of CA was noted. Presumably, the forming CA was subjected to further hydrolysis by ceramidase (or degraded by a different path [7]) or its *de novo* synthesis was inhibited. Hence, direct effect of LPS on SM metabolism in hepatocytes differed from the effect of TNF alone.

The effect of LPS in the presence of ethanol attracted special interest, because a synergic effect was previously detected in hepatic cells under these conditions [3,6,8]. We also showed that LPS potentiated the effect of ethanol on the sphingomyelin cycle in hepatocytes: basal concentration of SM decreased and CA level increased, while SP concentration was reduced. In order to clear out the molecular mechanisms of this phenomenon, further studies are needed, because both agents (LPS and ethanol) can cause activation of a variety of metabolic pathways in cells.

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