MICROBIOLOGY AND IMMUNOLOGY

Effects of Hydroxysterols and Atorvastatin on Lipopolysaccharide-Induced Secretion of Tumor Necrosis Factor and Interleukin-10 by Mouse Macrophages

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Preincubation of macrophages with atorvastatin, cholesterol, 25-, 27-hydroxycholesterol, and 7-ketocholesterol reduced the level of TNF-α to 10, 61, 13, 64.5, and 82%, respectively. Addition of mevalonate to the preincubation medium canceled the effects atorvastatin, cholesterol, and 7-ketocholesterol, but not the effects of 25- and 27-hydroxycholesterols. Atorvastatin increased the level of IL-10 by 41%, while 7-ketocholesterol and 25-hydroxycholesterol inhibited its secretion by 48 and 55%.

Key Words: *macrophages; hydroxysterols; atorvastatin; tumor necrosis factor-α; interleukin-10*

Hydroxysterols, products of enzymatic or free radical oxidation of cholesterol (CH), are accumulated during macrophage transformation into foamy cells in atherosclerotic plaques [8], and the increase of their blood level is regarded as a factor promoting coronary atherosclerosis [11,13]. Being bioactive molecules, hydroxysterols can modify various functions of the cells, including macrophage functions, under conditions of inflammation. Macrophages expressing more than 100 inflammatory mediators in atheromatous plaques [7] play a key role in the development of atherosclerosis, a variant of chronic inflammation in the vessels. Production of proinflammatory cytokines and mediators (TNF-α, IL-1β, IL-6, metalloproteinases) leads to destabilization of the atherosclerotic plaque, thrombus formation, and development of acute myocardial ischemia. High level of IL-10 is regarded as a factor of plaque stabilization and reduction of atherosclerosis complications [10]. Statins, with their numerous antiatherogenic properties, can reduce the levels of proinflammatory cytokines and stimulate the secretion of antiinflammatory IL-10 by inhibiting mevalonate transformation into nonsteroid products isoprenoids, essential for prenylation of signal proteins [12]. We previously showed that 7-keto-CH and 25-hydroxy-CH inhibit LPS-induced macrophage activation [3,5]. We found that LPS-tolerant macrophages are characterized by increased intracellular content of 25- and 27-hydroxy-CH [1]. These hydroxysterols are natural ligands for hepatic X-receptors on macrophages, whose stimulation leads to reduction of cell activation [4,9]. However, the effects of hydroxysterols on the balance of pro- and

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antiinflammatory cytokines deserve more detailed studies.

We studied the effects of products of enzymatic (25- and 27-hydroxy-CH) and nonenzymatic (7-keto-CH) oxidation of CH and hydroxymethyl glutaryl coenzyme A-reductase (HMG-CoA reductase) inhibitor atorvastatin on the production of TNF-α and IL-10 in peritoneal macrophages (PM), induced by LPS.

MATERIALS AND METHODS

The study was carried out on primary culture of PM from male C57BL/6 mice, isolated on day 4 after injection of 4% starch using DMSI without calcium and magnesium and cultured in 35-mm Petri dishes (2×10^6 cells/dish) in RPMI-1640 with 10% FCS and 50 μg/ml gentamicin at 5% CO_2 and 95% air [2]. Macrophages were incubated for 24 h with or without 1 mM mevalonic acid lactone (ISN Pharm. Inc.) with CH (Sigma), 25-hydroxy-CH (Steroloid Inc.), 27-hydroxy-CH (Research Plus Inc.), 7-keto-CH (Sigma), or 5 μmol/ml atorvastatin (Pfizer). Hydroxysterols and CH were dissolved in absolute ethanol and added to incubation medium (1 μl/ml) to the final concentration of 5 μg/ml medium. Equivalent volumes of ethanol were added into control dishes. After 24-h incubation cell monolayer was washed 3 times in Hanks' medium and *E. coli* LPS (055:B5) (Sigma) was added into fresh incubation medium to a final concentration of 1 μg/ml. Control dishes were incubated in amedium without LPS. The medium for evaluation of TNF-α and IL-10 was collected after 3 and 6 h, respectively, of incubation with LPS and frozen at -70°C. The content of TNF-α and IL-10 in the incubation medium was measured using ProCon reagent kits for solid phase enzyme immunoassay (R&D Systems Inc.). The results were registered using an automated photometer (Spectra Max Plus) for microplates at λ=450 nm. All samples were measured twice. Cytokine content in the medium was standartized to mg cell protein measured by Bredford's method. Experiments were repeated 3 times. The results were expressed in percent of LPS-stimulated production of TNF-α and IL-10, which was taken for 100%. LPS-stimulated secretion of cytokines varied within 2-5 ng/ml culture medium for TNF-α and 70-200 pg/ml culture medium for IL-10. Differences between the cells incubated with LPS with hydroxysterols and atorvastatin were analyzed using paired Student's *t* test.

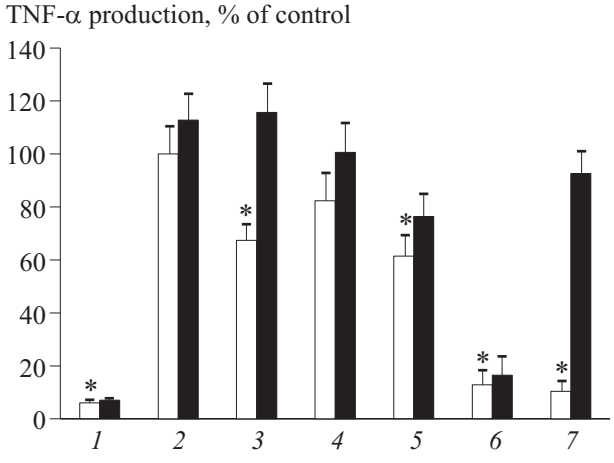

Fig. 1. Effects of hydroxysterols and atorvastatin on LPS-induced secretion of TNF-α by mouse peritoneal macrophages incubated in the absence (light bars) and presence of 1 mM mevalonic acid (dark bars). Here and in Fig. 2: *1*) no LPS; *2*) LPS; *3*) cholesterol; *4*) 7-ketocholesterol; *5*) 27-hydroxycholesterol; *6*) 25-hydroxycholesterol; *7*) atorvastatin. *$p<0.05$ compared to LPS without mevalonic acid.

RESULTS

Preincubation of macrophages with CH, hydroxysterols, or atorvastatin for 24 h reduced LPS-induced secretion of TNF-α in the absence of mevalonic acid (Fig. 1). 25-Hydroxy-CH exhibited the highest capacity to inhibition of LPS-induced production of TNF-α, reducing its level to 13%. 27-Hydroxy-CH and CH under these conditions reduced the production of TNF-α to 61 and 64.5%, respectively. The effect of 7-keto-CH on LPS-induced secretion of TNF-α was minimum (the parameter decreased to 82%, $p=0.05$). Our results are in line with the data of other authors, obtained under similar conditions in a culture of human mononuclears [6]. Selective HMG-CoA reductase inhibitor atorvastatin in a concentration of 5 μM 5-fold inhibited the production of TNF-α in the presence of LPS. Mevalonic acid added to the preincubation medium in a concentration of 1 mM canceled the inhibitory effect of atorvastatin and restored LPS-induced secretion of TNF-α by macrophages to the control level. Hydroxysterols in a concentration of 5 μg/ml can inhibit CH production by reducing the expression of HMG-CoA reductase gene [13], and we therefore studied the effect of mevalonic acid on hydroxysterol capacity to suppress TNF-α production (Fig. 1). Mevalonic acid abolished the inhibitory effect of CH and 7-keto-CH. However, 25- and 27-hydroxy-CH retained their capacity to decrease LPS-induced secretion of TNF-α after addition of mevalonic acid to macrophage preincubation medium, reducing significantly the level of TNF-α (to 13.5 and 62.2%, respectively).

Atorvastatin in the absence of mevalonic acid significantly increased the production of IL-10 (by

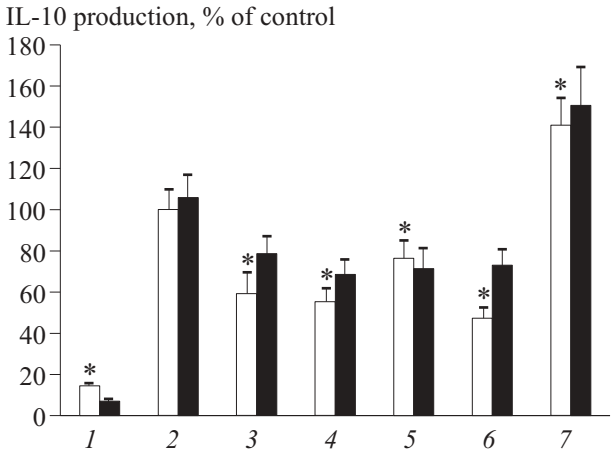

Fig. 2. Effects of hydroxysterols and atorvastatin on LPS-induced secretion of IL-10 by mouse peritoneal macrophages incubated in the absence (light bars) and presence of 1 mM mevalonic acid (dark bars).

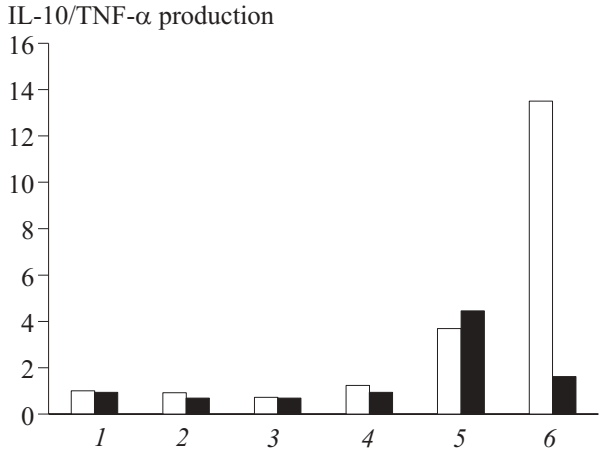

Fig. 3. Hydroxysterol effects on the ratio of IL-10 and TNF-α produced by LPS-activated macrophages. Light bars: no mevalonic acid; dark bars: with mevalonic acid. *1*) LPS; *2*) cholesterol; *3*) 7-ketocholesterol; *4*) 27-hydroxycholesterol; *5*) 25-hydroxycholesterol; *6*) atorvastatin.

41%); the increase in IL-10 level was not prevented by mevalonic acid added to the preincubation medium (Fig. 2). Hydroxysterols and CH inhibited IL-10 secretion in the incubation medium without mevalonic acid. 25-Hydroxy-CH and 7-keto-CH reduced IL-10 level in the medium to 48 and 55%, respectively. IL-10 secretion was significantly inhibited in the presence of CH (by 40%); 27-hydroxy-CH produced a less pronounced effect (by 35%). Addition of mevalonic acid increased IL-10 level in the incubation medium with 25-hydroxy-CH and CH by 22 and 15%, respectively, but did not modify the inhibitory effect of 7-keto-CH and 27-hydroxy-CH.

The increase of IL-10 is an antiatherogenic factor, promoting stabilization of atherosclerotic plaques [10]. Simvastatin increases the level of anti-inflammatory cytokine IL-10 in the blood of patients with unstable angina [12]. On the other hand, for evaluation of macrophage activity during atherosclerosis development it is important to assess changes in the balance of pro- and antiinflammatory cytokines. Atorvastatin increases the IL-10/TNF-α ratio (13.5 times) under conditions of macrophage activation by LPS, and this increase was abolished by mevalonic acid (Fig. 3). These data indicate that 25-hydroxy-CH increased the IL-10/TNF-α ratio 3.7 and 4.45 times in the presence of mevalonic acid and without it, respectively. 7-Keto-CH, an atherogenic product of CH autooxidation in oxidized low density lipoproteins [13], reduced this ratio by 30%, and mevalonic acid did not modify this value.

Hence, 25-hydroxy-CH shifts the balance between pro- and antiinflammatory cytokines towards antiinflammatory ones, while 7-keto-CH produced an opposite effect after LPS activation of macrophages. We conclude that the mechanisms underlying the effect of 7-keto-CH and 25-hydroxy-CH on cytokine production by macrophages are not linked with HMG-CoA reductase inhibition. Since 25-hydroxy-CH is a natural ligand for nuclear hepatic X-receptors, whose activation leads to inhibition of inflammatory cytokine gene expression [11], the antiinflammatory effect of 25-hydroxy-CH can be realized through activation of hepatic X-receptor macrophage ligands.

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