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Treatment with Y-40138, a multiple cytokine production modulator, inhibits lipopolysaccharide- or tumour necrosis factor-α-induced production of pro-inflammatory cytokines and augments interleukin-10

Tetsuko Fukuda, Masao Hisadome and Hirotsugu Komatsu

Abstract

N-[1-(4-[4-(pyrimidin-2-yl)piperazin-1-yl]methyl phenyl)cyclopropyl] acetamide · HCl (Y-40138) suppresses liver injury in concanavalin A- and D-galactosamine/lipopolysaccharide (LPS)-induced mouse hepatitis models. However, the mechanism of action of Y-40138 has not been fully investigated. In this study, we examined the effect of Y-40138 on cytokine production in mice. Cytokine production was induced by intraperitoneal injection of LPS (0.5 mg kg^{-1}) or intravenous injection of recombinant mouse tumour necrosis factor (TNF)-α (10 μg mouse^{-1}) in BALB/c mice. TNF-α and interleukin (IL)-10 reached maximum levels 1.5 h after the LPS injection. IL-12 and interferon-γ (IFN-γ) reached maximum levels 3 to 9 h after the injection. When Y-40138 was orally administered 30 min prior to the injection, it inhibited TNF-α, IL-12 and IFN-γ production and augmented IL-10 production. Y-40138 also inhibited IL-12 production and augmented IL-10 production in TNF-α-stimulated mice. In IL-10 knockout mice, Y-40138 inhibited TNF-α and IL-12 production 1.5 h after the LPS injection but not after 3 h or later, unlike in wild mice. In addition, TNF-α production was inhibited by Y-40138 at concentrations that could not augment IL-10 production. These data suggest that Y-40138 modulates pro-inflammatory cytokine production by both IL-10-dependent and -independent mechanisms.

Introduction

Lipopolysaccharide (LPS) is one component of the outer wall of Gram-negative bacteria that has been implicated in sepsis, organ failure and lethal shock. Elevated levels of circulating LPS delivered to the liver via portal blood are known to cause hepatocellular injury (Wheeler et al 2001). LPS activates mononuclear phagocytes to produce inflammatory mediators such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6. TNF-α induces apoptosis in hepatocytes and neutrophil transmigration, a critical step in the necrosis of hepatocytes, occurs at a later stage in liver injury (Chosay et al 1997; Sass et al 2002). The plasma TNF-α level is known to be elevated in patients with acute alcoholic hepatitis (Zhou et al 2003; McClain et al 2004) and chronic hepatitis caused by hepatitis B or hepatitis C virus infection (Kasprzak et al 2004; Koulentaki et al 2004; Perrella et al 2005). TNF-α therefore plays a role in the pathogenesis of not only endotoxin-induced experimental liver injury but also human liver diseases.

On the other hand, IL-10 has been shown to be an anti-inflammatory cytokine, blocking the induced synthesis of TNF-α, IL-1 and IL-8 by human monocytes (Hart et al 1995). IL-10 indirectly suppresses the synthesis of interferon-γ (IFN-γ) by helper T-cells and NK-cells (Cai et al 1999). Subtoxic doses of D-galactosamine (GalN) and LPS are often used for an animal model of fulminant hepatic failure (Josephs et al 2000). TNF-α and IFN-γ release plays a pivotal role in the pathogenesis of liver injury induced by LPS injection in GalN-sensitized mice. Treatment with IL-10 protected mice against GalN/LPS-induced liver injury, and the protective effect was associated with a significant decrease in plasma TNF-α concentrations (Santucci et al 1996). In IL-10 deficient mice, high levels of pro-inflammatory cytokines such as TNF-α were observed (Rennick et al 1997).

Pharmaceuticals Research Unit, Research & Development Division, Mitsubishi Pharma Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama 227-0033, Japan

Tetsuko Fukuda*, Masao Hisadome, Hirotsugu Komatsu

Current address: *Marketing and Planning Department, Sales and Marketing Division, Mitsubishi Pharma Corporation, 2-5-6, Awaji-machi, Chuo-ku, Osaka 541-0047, Japan

Correspondence: Tetsuko Fukuda, Pharmaceuticals Research Unit, Research & Development Division, Mitsubishi Pharma Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama 227–0033, Japan. E-mail: Fukuda.Tetsuko@mf.m-pharma.co.jp

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In order to examine if the suppressive effect of Y-40138, *N*-[1-(4-[4-(pyrimidin-2-yl)piperazin-1-yl]methyl phenyl) cyclopropyl] acetamide·HCl, on TNF-α production appears through augmentation of IL-10 production, we investigated the effect of Y-40138 on the production of multiple cytokines in BALB/c mice and on TNF-α production in IL-10 deficient mice. These results suggest that Y-40138 inhibits inflammatory cytokine production and enhances anti-inflammatory cytokine production.

Materials and Methods

Animals

Female-specific pathogen-free BALB/cAnNCrj (4–6 weeks of age) mice were purchased from Charles River Japan (Kanagawa, Japan). C57BL/6-IL10 + m1Cgn (IL-10 knockout) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed under conditions of controlled temperature ($23 \pm 3^{\circ}C$) and a 12 h light cycle, and fed with standard rodent feed and water ad libitum for at least 5 days before experiments. All experiments were approved by the Animal Ethical Committee of Mitsubishi Pharma Co. and performed in accordance with guidelines of the Japanese Pharmacological Society.

Compounds

Y-40138 was synthesized at Mitsubishi Pharma Co. and dissolved in 0.5% hydroxypropylmethylcellulose (HPMC) solution. LPS (serotype: 0111 B4, 055 B5) was purchased from Difco Laboratories (Detroit, MI, USA) or Sigma Chemical Co. (St Louis, MO, USA) and dissolved in 0.9% pyrogen-free saline. Recombinant mouse TNF-α (mTNF-α) was purchased from Hycut Biotechnology B.V. (Uden, The Netherlands) and dissolved in phosphate buffered saline (PBS).

Cytokine production

Y-40138 was administered orally 30 min prior to LPS injection (0.5 mg kg^{-1}, i.p.) or mTNF-α injection (10 μg mouse^{-1}, i.v.). Blood samples were obtained from the abdominal vein of the anesthetized mice at the indicated times after LPS or mTNF-α injection. Serum was obtained by centrifugation at $2000 \times g$ and stored at $-30^{\circ}C$ until use. The liver and spleen were homogenized in 5 and 2 mL ice-cold PBS, respectively. The samples were centrifuged at $3000 \times g$ for 10 min and supernatants were collected and stored at $-30^{\circ}C$ until use for cytokine determination.

Measurement of cytokine levels

Serum, liver and spleen cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA) with a commercially available kit according to the manufacturers' protocols. The ELISA kits were purchased from the following manufacturing companies: TNF-α ELISA kit, BioSource International, Inc., Camarillo, CA, USA; IL-10 ELISA kit, IFN-γ ELISA kit, total IL-12 ELISA kit, TNF-receptor I (TNF-RI) ELISA kit, Genzyme Techne, Cambridge, MA, USA.

Statistical analysis

The cytokine levels were represented as the mean ± s.e.m. The Mann–Whitney *U*-test, the Kruskal–Wallis test and Friedman's test were used to determine the statistical significance of differences between values for various experimental and control groups. Differences were assessed using an alpha level of 0.05.

Results

Effect of Y-40138 on production of TNF-α and IL-10

In mice with no LPS injection, serum TNF-α and IL-10 levels were below the detection limit (TNF-α, 5.1 pg mL^{-1}; IL-10, 13 pg mL^{-1}). Y-40138 (30 mg kg^{-1}, p.o.) did not influence serum TNF-α and IL-10 levels (data not shown). Serum TNF-α and IL-10 levels increased in response to LPS injection and reached 6.69 ng mL^{-1} and 582 pg mL^{-1} 1.5 h after LPS injection (Figure 1). Y-40138 (3–30 mg kg^{-1}, p.o.) significantly inhibited the elevation of TNF-α level 1.5 h after LPS injection. IL-10 production was significantly augmented by Y-40138 (3–30 mg kg^{-1}, p.o.) at 1.5 h.

Effect of Y-40138 on cytokine production

Y-40138 (30 mg kg^{-1}) was administered orally 30 min prior to LPS injection (0.5 mg kg^{-1}, i.p.). Serum TNF-α, IL-10, IL-12 and TNF-RI levels were measured 0.75, 1.5, 3 and 5 h after LPS injection. Serum IFN-γ levels were measured 3, 5 and 7 h after LPS injection. Serum TNF-α and IL-10 levels reached maximum at 1.5 h (Table 1). Serum TNF RI, IL-12 and IFN-γ levels reached maximum at 0.75, 3 and 7 h, respectively. Y-40138 (30 mg kg^{-1}, p.o.) significantly reduced TNF-α, IL-12 and IFN-γ levels and significantly increased the IL-10 level. Y-40138 had no effect on the TNF-RI level.

Influence of Y-40138 on production of TNF-α, IL-10 and IFN-γ in liver and spleen

TNF-α in liver and spleen reached maximum levels at 1.5 h (Table 2). Y-40138 significantly suppressed these TNF-α increases. IFN-γ levels increased for 7 h in liver and spleen, and these increases were significantly inhibited by Y-40138. IL-10 levels were not elevated in liver and spleen by Y-40138.

Effect of Y-40138 on TNF-α-induced increases in cytokine levels

The serum TNF-α level was 1500 ng mL^{-1} 15 min after mTNF-α injection (10 μg mouse^{-1}, i.v.) and drastically

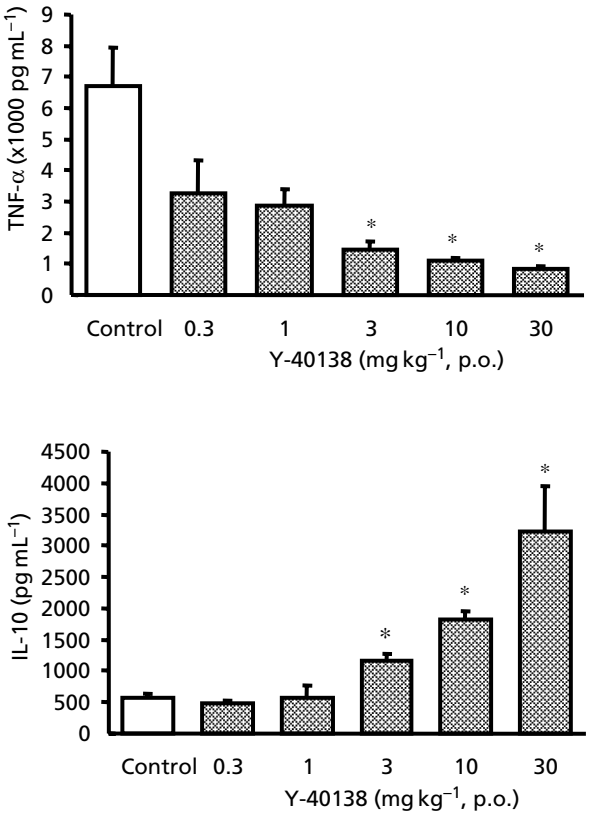

Figure 1 Effect of Y-40138 on LPS-induced TNF-α and IL-10 productions in BALB/c mice. Y-40138 was administered orally 30 min prior to LPS injection (0.5 mg kg^{-1}, i.p.). Results are expressed as the mean ± s.e.m. (n = 5). *$P < 0.05$ significantly different from control.

decreased for 5 h. Y-40138 (30 mg kg^{-1}, p.o.) did not influence TNF-α level (data not shown). Serum IL-10 and IL-12 levels were increased by mTNF-α injection (Figure 2), but IFN-γ could not be detected. Y-40138 (30 mg kg^{-1}, p.o.) significantly augmented IL-10 production and suppressed IL-12 production 1.5 to 3 h and 0.5 to 3 h after mTNF-α injection, respectively.

Involvement of IL-10 in inhibition of cytokine production by Y-40138

Y-40138 (30 mg kg^{-1}, p.o.) was administered 30 min prior to LPS injection to IL-10 deficient mice (Table 3). Y-40138 reduced serum TNF-α and IL-12 levels only 1.5 h after LPS injection.

Discussion

TNF-α plays a role in the pathogenesis of not only LPS-induced experimental liver injury but also human liver diseases. Increased serum TNF-α concentrations in alcoholic liver disease were reported by several groups, and values correlated with disease severity and mortality (McClain et al 2004). Kupffer cells are one of the major sources of TNF-α, and their expression is upregulated in liver injury (Yoshioka et al 1998).

In the present study, oral administration of Y-40138 decreased serum TNF-α levels in LPS-stimulated mice. Y-40138 also decreased TNF-α levels in the liver and spleen of LPS-stimulated mice. Two possibilities are considered, namely that Y-40138 neutralized TNF-α or alternatively suppressed TNF-α production. There are soluble TNF-α receptors which can neutralize TNF-α as TNF-α blocker. Long-term ethanol feeding caused liver injury in TNF-RII knockout mice, but not in TNF-RI knockout mice, providing the hypothesis that TNF-α plays an important role in the development of liver injury via the TNF-RI pathway (Yin et al 1999). Y-40138 did not increase TNF-RI levels in serum, suggesting that Y-40138 is not able to neutralize TNF-α activity. The decrease in TNF-α levels by treatment with Y-40138 might be mediated through the

Table 1 Effect of Y-40138 on LPS-induced increase in TNF-α, IFN-γ, IL-12, IL-10 and TNF-RI levels in serum

Cytokine (pg mL^{-1})	Treatment	Dose (mg kg^{-1}) (p.o.)	Time after LPS injection (h)				
			0.75	1.5	3	5	7
TNF-α	Control	vehicle	907.15 ± 88.85	10827.0 ± 2319.7	761.40 ± 4.71	268.05 ± 6.24	NT
	Y-40138	30	336.90 ± 52.47*	1196.5 ± 79.3*	454.43 ± 32.34*	204.85 ± 22.98*	NT
IFN-γ	Control	vehicle	NT	NT	10.20 ± 1.55	172.35 ± 28.39	347.82 ± 94.19
	Y-40138	30	NT	NT	3.49 ± 0.44*	12.42 ± 3.63*	25.34 ± 5.73*
IL-12 (×1000)	Control	vehicle	2.81 ± 0.08	10.85 ± 0.73	13.05 ± 0.09	6.73 ± 0.48	NT
	Y-40138	30	2.30 ± 0.06*	6.00 ± 0.26*	7.28 ± 0.39*	6.58 ± 0.25	NT
IL-10	Control	vehicle	< 15	159.75 ± 18.99	3.88 ± 3.88	< 15	NT
	Y-40138	30	25.78 ± 18.05	838.00 ± 137.70*	49.23 ± 21.07*	62.06 ± 35.61	NT
TNF-RI	Control	vehicle	276.76 ± 5.88	195.92 ± 2.81	158.12 ± 6.37	151.34 ± 5.10	NT
	Y-40138	30	286.26 ± 12.01	185.62 ± 2.41	165.92 ± 5.37	153.40 ± 5.26	NT

Y-40138 (30 mg kg^{-1}, p.o.) was administered 30 min prior to LPS injection (0.5 mg kg^{-1}, i.p.). Results are expressed as the mean ± s.e.m. (n = 3–4). *$P < 0.05$ significantly different from control. NT: not tested.

Table 2 Effect of Y-40138 on LPS-induced increase in TNF-α, IFN-γ and IL-10 levels in spleen and liver

Cytokine ($pg\,mL^{-1}$)	Organ	Treatment	Dose ($mg\,kg^{-1}$) (p.o.)	Time after LPS injection (h)				
				0.75	1.5	3	5	7
TNF-α	Spleen	Control	vehicle	348.70 ± 42.76	1415.25 ± 98.46	308.07 ± 17.88	198.25 ± 16.14	NT
		Y-40138	30	144.13 ± 9.88*	518.15 ± 40.39*	269.38 ± 16.27*	108.91 ± 14.30*	NT
	Liver	Control	vehicle	111.81 ± 14.59	5355.00 ± 548.95	569.50 ± 53.91	328.77 ± 31.74	NT
		Y-40138	30	34.04 ± 4.87*	1041.28 ± 84.16*	236.45 ± 18.06*	111.62 ± 42.37*	NT
IFN-γ	Spleen	Control	vehicle	NT	NT	20.18 ± 2.08	118.47 ± 8.94	181.86 ± 46.07
		Y-40138	30	NT	NT	9.69 ± 0.60*	24.71 ± 5.28*	22.03 ± 4.55*
	Liver	Control	vehicle	NT	NT	62.73 ± 2.78	92.98 ± 10.67	136.85 ± 22.31
		Y-40138	30	NT	NT	48.45 ± 6.81	55.49 ± 2.49*	66.75 ± 2.08*
IL-10	Spleen	Control	vehicle	364.43 ± 25.37	226.58 ± 24.01	259.20 ± 36.20	232.40 ± 18.61	NT
		Y-40138	30	469.15 ± 84.25	318.68 ± 45.97	219.90 ± 29.54	246.05 ± 19.73	NT
	Liver	Control	vehicle	4207.5 ± 25.7	4586.8 ± 98.3	4417.7 ± 294.7	3805.3 ± 379.0	NT
		Y-40138	30	3689.0 ± 552.2	4794.0 ± 12.9	4019.5 ± 393.3	4473.8 ± 276.9	NT

Y-40138 ($30\,mg\,kg^{-1}$, p.o.) was administered orally 30 min prior to LPS injection ($0.5\,mg\,kg^{-1}$, i.p.). Results are expressed as the mean ± s.e.m. (n = 3–4). *$P < 0.05$ significantly different from control. NT: not tested.

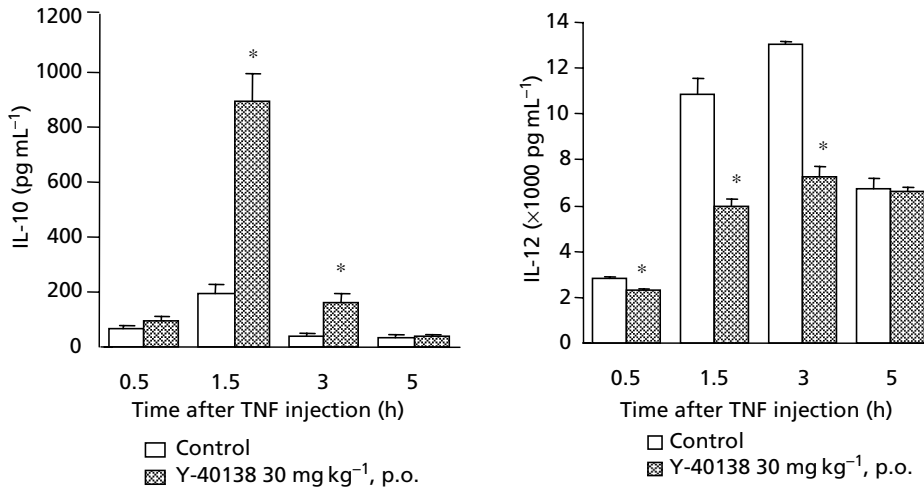

Figure 2 Influence of Y-40138 on TNF-α, IL-10 and IL-12 levels in mTNF-α injected BALB/c mice. Y-40138 ($30\,mg\,kg^{-1}$, p.o.) was administered 30 min prior to TNF-α injection ($10\,\mu g\,kg^{-1}$, i.v.). Results are expressed as the mean ± s.e.m. (n = 4). *$P < 0.05$, significantly different from control.

Table 3 Effect of Y-40138 on LPS-induced productions of TNF-α and IL-12 in IL-10 knockout mice

Cytokine ($pg\,mL^{-1}$)	Treatment	Dose ($mg\,kg^{-1}$, p.o.)	Time after LPS injection (h)			
			1.5	3	5	7
TNF-α	Control	vehicle	26068 ± 1349	21313 ± 4491	7073 ± 1254	3478 ± 565
	Y-40138	30	11361 ± 1520*	19871 ± 1789	4202 ± 856	2454 ± 401
IL-12 (×1000)	Control	vehicle	28.42 ± 2.03	613.8 ± 64.95	1048 ± 62.0	1336 ± 85.8
	Y-40138	30	20.97 ± 2.08*	456.2 ± 33.20	1158 ± 35.8	1202 ± 93.9

Y-40138 ($30\,mg\,kg^{-1}$, p.o.) was administered 30 min prior to LPS injection ($0.5\,mg\,kg^{-1}$, i.p.). Results are expressed as the mean ± s.e.m. (n = 4). *$P < 0.05$ significantly different from control.

suppression of Y-40138 on TNF-α production, probably by inhibition of macrophage/Kupffer cell activation.

In alcoholic liver disease there are not only increased levels of pro-inflammatory cytokines, but also diminished levels of anti-inflammatory cytokines such as IL-10 (McClain et al 2004; Naveau et al 2005). IL-10 produced by monocytes is an anti-inflammatory cytokine that inhibits pro-inflammatory cytokine production and has strong auto-regulatory feedback activity on T-cell activation (DeWaal Malefyt et al 1991; Asadullah et al 2003). Y-40138 augmented IL-10 production in serum when Y-40138 was administered to LPS- or mTNF-α-stimulated mice, but not in non-stimulated mice. It is suggested that Y-40138 is not an IL-10 inducer, but is at least an IL-10 production potentiator. Y-40138 did not increase IL-10 levels in the liver and spleen of LPS-stimulated mice. The reason for the lack of increase is unknown. It is possible that splenic or hepatic IL-10 quickly spills out into the circulation or that the target organ of Y-40138 on IL-10 production is not liver and spleen. From the results using LPS- or mTNF-α-stimulated mice, TNF-α suppression and IL-10 augmentation of Y-40138 are each mediated through independent action mechanisms.

In order to confirm that suppression of Y-40138 on TNF production is not mediated through augmentation of IL-10 production, we evaluated the modulating effects of Y-40138 on pro-inflammatory cytokine production in IL-10 deficient models. IL-10 knockout mice are more susceptible to ethanol hepatotoxicity and exhibit increased levels of pro-inflammatory cytokines such as TNF-α (Rennick et al 1997; McClain et al 2004). In LPS-stimulated IL-10 knockout mice, Y-40138 decreased TNF-α and IL-12 levels at 1.5 h. These data suggested that suppression of Y-40138 on pro-inflammatory cytokine production does not depend on the augmentation of IL-10 production at a later stage. This finding is also supported from results that each effective dose of TNF-α suppression and IL-10 augmentation of Y-40138 differs greatly in LPS-stimulated wild-type mice.

In liver injury IFN-γ and IL-12 are important pro-inflammatory cytokine factors as well as TNF-α. The liver contains a significant number of NKT-cells. In concanavalin A-induced hepatitis, NKT-cells play a critical role in the induction of hepatic injury by cooperating with T-cells and macrophages (Küsters et al 1996). IFN-γ-producing cells are NK-cells, NKT-cells and T-cells. It has been reported that antibodies or knockout mice that block IFN-γ improve hepatitis (Tagawa et al 1997). Y-40138 protects against concanavalin A-induced liver injury under experimental conditions of not only TNF-α participation but also IFN-γ participation (in preparation). In addition to NK-cells, IFN-γ can be produced by macrophages stimulated with LPS in the presence of IL-12 (Munder et al 1998). Interactions between these factors are considered to accelerate the progression of inflammatory reactions in the liver (Gantner et al 1995). IL-12 is a potent IFN-γ inducer and is produced from NK-cells and NKT-cells after LPS stimulation (Emoto et al 1995; Varma et al 2002; Habu et al 2004). Y-40138 significantly suppresses IL-12 and IFN-γ production in LPS-stimulated mice. The hepatoprotective effect of Y-40138 may be mediated through suppressed TNF-α, IL-12 and IFN-γ production and increased IL-10 production.

In conclusion, Y-40138 suppresses pro-inflammatory cytokine production and enhances IL-10 production in LPS- or TNF-α-stimulated mice. Y-40138 inhibits TNF-α, IL-12 and IFN-γ production by both IL-10-dependent and -independent mechanisms.

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