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Protective effect of vitamin D_3 analogues on endotoxin shock in mice

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Abstract

The effect of vitamin D_3 analogues on endotoxin shock in mice was investigated. Male ICR mice were orally administered vitamin D_3 analogues or vehicle, accompanied by an intraperitoneal injection of endotoxin (*E. Coli* lipopolysaccharide, LPS, 20 mg/kg). Endotoxin caused a decrease in survival rate in a time-dependent manner. Increases in plasma immunoreactive (i) eicosanoid and hepatic malondialdehyde (MDA) levels were also observed. Administration of 1α-hydroxyvitamin D_3 (1α-OH-D_3) improved the survival rate 24 to 48 h after endotoxin treatment. The effects were markedly observed at a dose of 20 ng/kg. In addition, 1α-OH-D_3 restored the plasma iTXB_2 and hepatic MDA levels 8 h after endotoxin injection. However, it did not affect plasma iPGE_2, i6-keto-$PGF_{1\alpha}$ and blood iLTB_4 levels. At a dose of 20 ng/kg, both 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and 1,24(R)-dihydroxyvitamin D_3 (1,24(R)-$(OH)_2D_3$) restored the survival rate, the plasma iTXB_2 and hepatic MDA levels. These results suggest that vitamin D_3 analogues may inhibit endotoxemia through regulation of the formation of TXA_2 and free radicals.

Introduction

Endotoxin (lipopolysaccharide, LPS), a major component of the outer cell wall of gram-negative bacteria, is considered a primary factor of septic shock, disseminated intravascular coagulation and adult respiratory distress syndrome. Further, it is associated with the last stage of the sepsis, causing multiple organ failure [1]. These effects are mediated partly through increases of eicosanoid metabolism. Namely, in the leukocytes, endotoxin stimulates release of arachidonic acid [2], which is metabolized by either cyclooxygenase or lipoxygenase pathways to prostaglandins, thromboxane, leukotrienes, 5-HETE and 12-HETE [3–7].

Endotoxin is metabolized in the reticuloendothelial system in the liver. It is suggested that oxygen radicals are released from liver macrophages (Kupffer cells) by endotoxin, causing lipid peroxidation and injury the liver [8, 9].

It has been reported that 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) possesses immunoregulatory properties. Differentiation of mouse [10] and human [11–14] myeloid leukemia cells into macrophages is augmented by 1,25-$(OH)_2D_3$. It also activates [15, 16] or suppresses [17, 18] the function of maturated monocyte/macrophages. So, It is possible that 1,25-$(OH)_2D_3$ regulates the functions of monocyte/macrophages activated by endotoxin. We investigated the effect of vitamin

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D_3 analogues on endotoxin shock and found that it protected mice from endotoxin shock.

Materials and methods

Laboratory animals

Male ICR mice aged 7–8 weeks old were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Animals were kept in an air-conditioned room and given standard chow and water ad libitum for the duration of the study.

Endotoxin shock

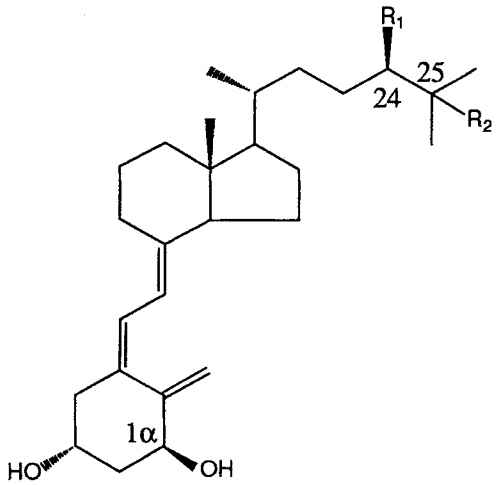
Endotoxin (*E. coli* 055:B5, Difco Laboratories, Detroit, MI) was suspended in saline and was intraperitoneally injected into mice at a dose of 20 mg/kg.
Survival rate of mice was recorded up to 48 h after endotoxin treatment.

Radioimmunoassay of $iTXB_2$, $iPGE_2$, i6-keto-$PGF_{1\alpha}$ and $iLTB_4$

Eight h after endotoxin injection, blood samples were collected by cardiac puncture under ether anaesthesia. For radioimmunoassay of $iTXB_2$, $iPGE_2$ and i6-keto-$PGF_{1\alpha}$, blood was collected into a 1 ml plastic syringe with a 26G needle, filled with 1/10 volume of solution containing 3.8% EDTA-2Na and 10^{-4} *M* indomethacin. For $iLTB_4$ measurement, heparinized blood was collected, treated with 10 times volume of ethanol and mixed gently. Sample preparations were clarified by centrifugation and stored at −20 °C until assayed.
Plasma $iTXB_2$ (a stable metabolite of TXA_2), $iPGE_2$, i6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) and blood $iLTB_4$ levels were measured using RIA kits (Du Pont, Boston, MA).

Hepatic malondialdehyde (MDA) level

Hepatic MDA level was assayed according to the method of Ohkawa et al. [19], with a slight modification. The liver was excised and perfused with cold saline via the portal vein to remove the blood 8 h after endotoxin injection. Then, 10% tissue homogenates were prepared with saline and the hepatic MDA level was measured as thiobarbituric acid (TBA) reactant. Protein content was analyzed with an automatic analyzer (Flexigem).



R_1=H, R_2=H : 1α-OH-D_3
R_1=H, R_2=OH : 1,25-(OH)$_2D_3$
R_1=OH, R_2=H : 1,24(R)-(OH)$_2D_3$

Figure 1
Structures of vitamin D_3 analogues.

Treatment

All vitamin D_3 analogues shown in Fig. 1 were dissolved in ethanol, and subsequent dilution was made in saline containing 0.2% Triton X. The final ethanol concentration used was 1%. The drugs were administered orally, simultaneously with endotoxin treatment. Control mice were received only the dosing vehicle at a constant volume of 10 ml/kg.

Statistical analyses

The data are presented as means ± SEM, and the results have been statistically evaluated by Dunnett's *t*-test.

Results

Effects of 1α-OH-D_3 on endotoxin shock

Survival rate. The deaths occurred from 20 h after endotoxin injection, and a time-dependent decrease in survival rate was observed. Orally administered 1α-OH-D_3 decreased endotoxin lethality.

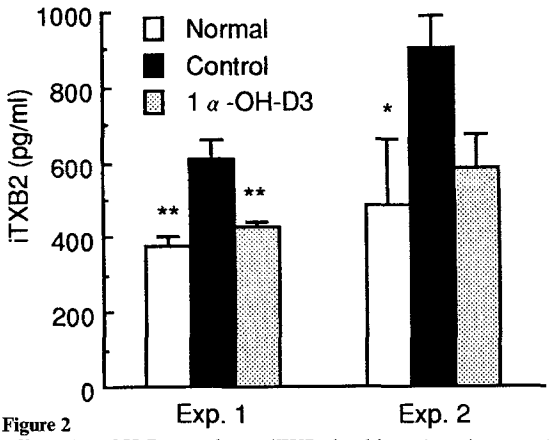


Figure 2
Effect of 1α-OH-D_3 on plasma $iTXB_2$ level in endotoxin-treated mice. Simultaneously with endotoxin injection, 1α-OH-D_3 was administered orally at a dose of 20 ng/kg. Eight h after endotoxin injection, blood was collected and plasma $iTXB_2$ level was measured using a RIA kit. Results are represented as the means ± SEM of 5 mice. * $p<0.05$ and ** $p<0.01$: statistically different from control (Dunnett's t-test).

Table 1
Effect of 1α-OH-D_3 on survival rate of endotoxin-treated mice.

Exp. No.	Treatment	Dose (ng/kg)	No. of survivals (%) 24 h	30 h	48 h
1	Control		7/20 (35.0)	NT	1/20 (5.0)
	1α-OH-D_3	4	13/20 (65.0)	NT	2/20 (10.0)
		20	16/20 (80.0)	NT	4/20 (20.0)
		100	11/20 (55.0)	NT	2/20 (10.0)
2	Control		13/19 (68.4)	6/19 (31.6)	2/19 (10.5)
	1α-OH-D_3	4	14/18 (77.8)	9/18 (50.0)	4/18 (22.2)
		20	14/18 (77.8)	10/18 (55.6)	4/18 (22.2)
		100	12/18 (66.7)	8/18 (44.4)	3/18 (16.7)

Oral administration of 1α-OH-D_3 was simultaneous with endotoxin (*E. coli*; 20 mg/kg, i.p) injection. Survival rate was recorded up to 48 h after endotoxin injection. NT: not tested.

This effect was markedly observed at a dose of 20 ng/kg (Table 1).

Eicosanoid levels. The plasma $iTXB_2$, $iPGE_2$, i6-keto-$PGF_{1\alpha}$ and blood $iLTB_4$ levels were significantly increased 8 h after endotoxin treatment, but returned to normal levels by 16 h except for blood $iLTB_4$ level (data not shown). As shown in Fig. 2, 1α-OH-D_3 significantly attenuated the $iTXB_2$ level 8 h after endotoxin treatment at a dose of 20 ng/kg. However, the elevation in $iPGE_2$, i6-keto-$PGF_{1\alpha}$ and blood $iLTB_4$ levels was not altered by 1α-OH-D_3 (Table 2).

Hepatic MDA levels. Endotoxin induces the release of free radicals directly or indirectly and causes tissue injury via lipid peroxidation. So, we next investigated the effect of vitamin D_3 on the hepatic MDA level of endotoxin-treated mice. As illustrated in Fig. 3, the hepatic MDA level was considerably increased in comparison with that of normal mice 8 h after endotoxin injection. The increase in hepatic MDA level was inhibited 39.9% by 1α-OH-D_3 at a dose of 20 ng/kg (Fig. 3).

Effects of 1,25-$(OH)_2D_3$ and 1,24(R)-$(OH)_2D_3$ on endotoxin shock

To ascertain whether other vitamin D_3 analogues were also capable of preventing endotoxemia, we next examined the effects of other vitamin D_3 analogues, 1,25-$(OH)_2D_3$ and 1,24(R)-$(OH)_2D_3$, observing the parameters which were improved by 1α-OH-D_3 treatment.

At a dose of 20 ng/kg, both 1,25-$(OH)_2D_3$ and 1,24(R)-$(OH)_2D_3$ increased the survival rate of endotoxin-injected mice (Table 3). Furthermore, these vitamin D_3 analogues normalized the in-

Table 2
Effects of 1α-OH-D_3 on immunoreactive eicosanoid levels in the plasma or blood of endotoxin-treated mice.

Exp. No.	Treatment	Dose (ng/kg)	$iPGE_2$ (pg/ml)	i6-keto-$PGF_{1\alpha}$ (pg/ml)	$iLTB_4$ (ng/ml)
1	Normal	–	52.2 ± 5.0**	NT	2.04 ± 0.10*
	Control	–	162.7 ± 30.5	NT	2.98 ± 0.33
	1α-OH-D_3	20	118.9 ± 20.6	NT	2.98 ± 0.39
2	Normal	–	57.6 ± 4.4**	261.7 ± 34.9**	NT
	Control	–	116.6 ± 8.7	760.5 ± 52.8	NT
	1α-OH-D_3	20	150.8 ± 29.8	731.3 ± 93.4	NT

Experimental conditions are as described in the legend of Table 1. Eight h after endotoxin injection, blood was collected and plasma $iPGE_2$, i6-keto-$PGF_{1\alpha}$ and blood $iLTB_4$ levels were measured using RIA kits. Results are represented as the means ± SEM of 5 to 8 mice. NT: not tested. * $p<0.05$ and ** $p<0.01$: statistically different from control (Dunnett's t-test).

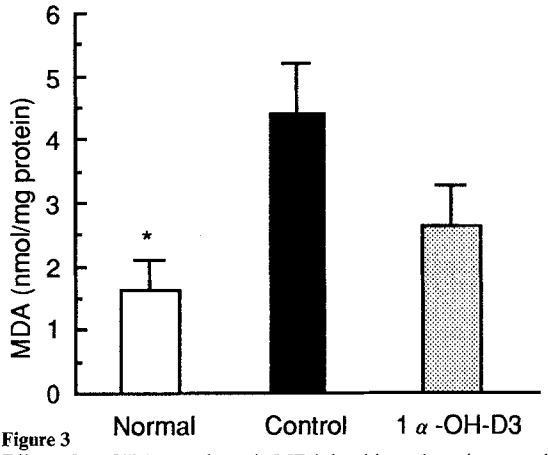


Figure 3
Effect of 1α-OH-D_3 on hepatic MDA level in endotoxin-treated mice. Experimental conditions are described in the legend of Fig. 2. Eight h after endotoxin injection, the liver was excised and 10% tissue homogenate was prepared with saline. The hepatic MDA level was measured as TBA reactant. Results are represented as the means ± SEM of 3 to 6 mice. * $p<0.05$: statistically different from control (Dunnett's *t*-test).

Table 3
Effects of vitamin D_3 analogues on survival rate in endotoxin-treated mice.

Treatment	Dose (ng/kg)	No. of survivals (%)		
		24 h	30 h	48 h
Control	–	9/15 (60.0)	7/15 (46.7)	0/15 (0)
1,25-$(OH)_2D_3$	20	12/12 (80.0)	9/15 (60.0)	2/15 (13.3)
1,24(R)-$(OH)_2D_3$	20	11/13 (84.6)	8/13 (61.5)	5/13 (38.5)

Vitamin D_3 analogues were administered orally at a dose of 20 ng/kg, simultaneously with endotoxin (*E. coli*; 20 mg/kg, i.p.) injection. Survival rate was recorded up to 48 h after endotoxin injection.

creased plasma $iTXB_2$ and hepatic MDA levels (Table 4).

Discussion

Although the effects of vitamin D_3 analogues on lymphocyte functions have been well investigated, those on macrophages remain unclear. It has been reported that vitamin D_3 analogues induced monocyte/macrophage differentiation [10–14] and activated their function to produce interleukin-1 (IL-1) [15] and hydrogen peroxide [16]. Conflicting experimental results have been reported about monokine production. Iho et al. [17] and Tsoukas et al. [18] reported that vitamin D_3 analogues suppressed the IL-1 production by monocytes. To estimate the effect of vitamin D_3 on macrophages, we investigated endotoxemia and observed that vitamin D_3 analogues prevented endotoxin lethality.

Endotoxin itself facilitates the release of oxygen radicals from macrophages [20]. Endotoxin is metabolized by the reticulo-endothelial system of liver, liver macrophages (Kupffer cells). In this process, oxygen radicals are released from Kupffer cells. It is postulated that oxygen radicals cause lipid peroxidation and injure the liver [9]. Moreover, endotoxin activates the leukocytes to release eicosanoids [2], which are thought to participate in the experimental endotoxemia [3–7]. It is well known that experimental endotoxemia is prevented by biosynthesis inhibitors or antagonists of eicosanoids and antioxidants such as thromboxane synthetase inhibitors [5], thomboxane A_2 antagonists [4], 5-lipoxygenase inhibitors [7], leukotriene antagonists [5, 21], superoxide dismutase,

Table 4
Effects of vitamin D_3 analogues on plasma $iTXB_2$ and $iTXB_2$ and hepatic MDA levels in endotoxin-treated mice.

Treatment	Dose (ng/kg)	$iTXB_2$ (pg/ml)	Inhibition %	Hepatic MDA (nmol/mg protein)	Inhibition %
Normal	–	201.4 ± 19.8**	–	1.57 ± 0.19**	–
Control	–	638.6 ± 55.5	–	4.59 ± 0.42	–
1α-OH-D_3	20	549.2 ± 60.8	14.0	3.03 ± 0.72	34.0
1,25-$(OH)_2D_3$	20	501.1 ± 31.3	21.5	3.45 ± 0.65	24.8
1,24(R)-$(OH)_2D_3$	20	455.4 ± 34.0*	28.7	2.97 ± 0.36	35.3

Experimental conditions are as described in the legend of Table 1. Eight h after endotoxin injection, plasma $iTXB_2$ and liver MDA levels were measured as described in the legends of Fig. 2 and 3, respectively. Results are represented as the means ± SEM of 4 to 6 mice. * $p<0.05$ and ** $p<0.01$: statistically different from control (Dunnett's *t*-test).

catalase [22], coenzyme Q_{10} [9], α-tocopherol, glutathione and allopurinol [8]. So, we measured hepatic MDA, plasma $iTXB_2$, $iPGE_2$, i6-keto-$PGF_{1\alpha}$ and blood $iLTB_4$ levels 8 h after endotoxin injection when death had not occurred. Hepatic MDA and plasma $iTXB_2$ levels were reduced by vitamin D_3 analogues studied. However, 1α-OH-D_3 did not affect plasma $iPGE_2$, i6-keto-$PGF_{1\alpha}$ and blood $iLTB_4$ levels. We recently observed that 1,25-$(OH)_2D_3$ significantly decreased $iTXB_2$ release from *Propionibacterium acnes*-elicited liver adherent cells and from oyster glycogen-elicited peritoneal macrophages both stimulated by LPS (unpublished data). Thus our results suggest that vitamin D_3 analogues improve the survival rate of LPS-treated mice through regulation of the function of macrophages including Kupffer cells.

Vitamin D_3 analogues are reported to regulate the gene expression of metallothionein [23] or heat shock protein [24, 25]. These proteins are well known to scavenge the free radicals [25, 26] and protect some types of cells from oxidative stress. But protective effects of vitamin D_3 analogues may not be due to the induction of these proteins because these proteins were induced 24–72 h post treatment of vitamin D_3 analogues [23, 25]. At that time, endotoxic shock were already observed in our experiments.

It has been established that 1α-OH-D_3 is converted into 1,25-$(OH)_2D_3$ in the liver [27, 28]. Therefore, the effects of 1α-OH-D_3 should be considered to result from 1,25-$(OH)_2D_3$ converted. However, the effect of 1α-OH-D_3 itself cannot be denied.

In the present study, both 1,25-$(OH)_2D_3$ and 1,24(R)-$(OH)_2D_3$ showed almost the same effects as 1α-OH-D_3 did. 1,24(R)-$(OH)_2D_3$, one of systemic analogues of vitamin D_3, is known to bind to 1,25-$(OH)_2D_3$ receptors in some tissues with almost the same binding affinity [29, 30]. Furthermore, several pharmacological profiles of 1,24(R)-$(OH)_2D_3$, such as intestinal calcium absorption [31] and immunoregulatory effects [32] are similar to those of 1,25-$(OH)_2D_3$. So, it is considered that 1,24(R)-$(OH)_2D_3$ might affect on endotoxemia in the same mechanism of 1,25-$(OH)_2D_3$.

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