Tocotrienol Suppresses Adipocyte Differentiation and Akt Phosphorylation in 3T3-L1 Preadipocytes

Harumi Uto-Kondo, Reiko Ohmori, Chikako Kiyose, Yoshimi Kishimoto, Hisako Saito, Osamu Igarashi, and Kazuo Kondo

Abstract

In vivo studies show that α-tocotrienol and γ-tocotrienol accumulate in adipose tissue. Furthermore, a recent study reports that the oral administration of γ-tocotrienol from a tocotrienol-rich fraction from palm oil (TRF) decreases body fat levels in rats. The objective of this study was to evaluate the effect of TRF and its components on adipocyte differentiation in 3T3-L1 preadipocytes, which differentiated into adipocytes in the presence of 1.8 μmol/L insulin. TRF suppressed the insulin-induced mRNA expression of adipocyte-specific genes such as PPARγ, adipocyte fatty acid-binding protein (aP2), and CCAAT/enhancer-binding protein-α (C/EBPα) compared with the differentiation of 3T3-L1 preadipocytes into adipocytes only in the presence of insulin. To confirm the suppressive effect of TRF, the major components of TRF, such as γ-tocotrienol, γ-tocotrienol, and α-tocopherol, were investigated. γ-Tocotrienol and γ-tocotrienol decreased the insulin-induced PPARγ mRNA expression by 55 and 90%, respectively, compared with insulin, whereas α-tocopherol increased the mRNA expression. In addition, γ-tocotrienol suppressed the insulin-induced aP2 and C/EBPα mRNA expression, triglyceride accumulation, and PPARγ protein levels compared with insulin. The current results also revealed that γ-tocotrienol inhibited the insulin-stimulated phosphorylation of Akt but not extracellular signal-regulated kinase (ERK)1/2 in the insulin signaling pathway of 3T3-L1 preadipocytes. Thus, the antiadipogenic effect of TRF depends on α-tocotrienol and γ-tocotrienol, and γ-tocotrienol may be a more potent inhibitor of adipogenesis than α-tocotrienol. Therefore, the results of this study suggest that tocotrienol suppresses insulin-induced differentiation and Akt phosphorylation in 3T3-L1 preadipocytes. Furthermore, tocotrienol could act as an antiadipogenic vitamin in the nutrient-mediated regulation of body fat through its effects on differentiation.

Introduction

The collection of obesity-related complications that is often referred to as metabolic syndrome or syndrome X markedly increases the risk of cardiovascular disease and death (1–3). Obesity is characterized at the cellular level by an increase in the number and size of adipocytes that have differentiated from preadipocytes in adipose tissues. During adipocyte differentiation, transcription factors such as PPARγ and CCAAT/enhancer-binding protein-α (C/EBPα) are involved in the sequential mRNA expression of adipocyte-specific proteins such as adipocyte fatty acid-binding protein (aP2) and fatty acid synthase. Thereafter, adipocytes incorporate glucose and FFA to synthesize and accumulate lipids, which results in increased cell size. On the other hand, various adipocyte-secreted proteins (so-called adipo-cytokines), including tumor necrosis factor-α, interleukin-6, and resistin, are upregulated during obesity and induce insulin resistance (4).

Several studies report that certain food components and nutrients inhibit adipogenesis in mouse adipocytes (5,6). Tea catechin suppresses adipocyte differentiation accompanied by
downregulation of PPARγ and C/EBPα in 3T3-L1 cells (5) and the soybean isolavone genistein suppress adipogenesis in 3T3-L1 cells (6). Provitamin A carotenoids and all-trans retinoic acid derived from dietary β-carotene potently inhibits the differentiation of 3T3-L1 cells (7–9) and all-trans retinoic acid effectively inhibits the differentiation of porcine preadipocytes in primary culture, suggesting that retinoids may regulate fat cell differentiation in growing animals (10).

Vitamin E is abundant in cereal grains, soybeans, barley, oats, rice bran, and palm oil. In nature, compounds with vitamin E activity include α-, β-, γ-, and δ- tocopherols and α-, β-, γ-, and δ- tocotrienols, which differ in the number and position of methyl groups on the chroman ring (11). Tocopherols have saturated tails, whereas tocotrienols have 3 double bonds in their phytyl tails. The biological activity of these vitamin E isoforms depends on their structures, and their chemical properties include antioxidative activities. α-Tocopherol has the highest biological activity of the tocopherols. The findings of various studies suggest that tocotrienols exert a hypcholesterolemic or antiatherotic effect on humans, rats, and mice (12–17). In vitro studies show that tocotrienols act as 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors and consequently reduce cholesterol synthesis (12). The tocotrienol-rich fraction from palm oil (TRF), which includes α-tocopherol and α- and γ-tocotrienol, decreases serum total cholesterol, LDL-cholesterol, apolipoprotein B, and triglyceride levels compared with the baseline values (13). From our previous study, we know that α-tocopherol possesses a hormone-like natriuretic function (14) and a recent study by others found that the oral administration of α-tocopherol increases serum lipid levels (15).

Materials and Methods

Materials. TRF consisting of 227 mg/g α-tocopherol, 353 mg/g α-tocotrienol, and 497 mg/g γ-tocotrienol was kindly provided by Lion. It was dissolved in ethanol. α-Tocopherol, γ-tocopherol, and α-tocotrienol were donated by Eisai Food and Chemical and they were also dissolved in ethanol. Polyclonal antibodies against PPARγ (H-100) were purchased from Santa Cruz Biotechnology and polyclonal antibodies against Akt, phospho-Akt (Ser473), extracellular signal-regulated kinase (ERK)1/2, and phospho-ERK1/2 (Thr202/Tyr204) were purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were obtained from GE Healthcare UK for use as secondary antibodies. Recombinant mouse insulin was purchased from US Biological. All reagents used in this study were of reagent grade.

Adipocyte differentiation. 3T3-L1 preadipocytes, which had been grown and maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (200,000 U/L), and streptomycin (200 mg/L), were seeded in 12-well plates at a density of 2 × 10^3 cells per well. 3T3-L1 preadipocytes were cultured for 3 d postconfluency and maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (200,000 U/L), and streptomycin (200 mg/L) in the presence of 1.8 μmol/L insulin.

Real-time RT-PCR. Total cellular RNA was prepared using TRIzol reagent (Invitrogen). One microgram of total RNA was reverse transcribed into cDNA using an Omniscript Reverse Transcriptase kit (Qiagen). The concentration and quality of the purified total RNA were determined spectrophotometrically at 260 nm and by the OD260/280 ratio, and 28s/18s using agarose gel electrophoresis, respectively. mRNA expression was quantified using an ABI 7300 instrument and the SYBR green reagent (Applied Biosystems). Results are expressed as copy number ratio of the target mRNAs/β-actin mRNA. The primers for PPARγ, C/EBPα, p2, and β-actin were as follows: PPARγ, 5'-GGCCATCTTGACAGGAAAGC-3', 5'-CCCTGTAAATTGCTGATGG-3'; C/EBPα, 5'-GCTTTTGCTCTGATTCTTGCC-3', 5'-CGGAATACAAAAACATGCC-3'; p2, 5'-AGCATCAACCTCCGATGCTG-3', 5'-CATACGATCTACCACCAGG-3'; and β-actin, 5'-TCACCCACACTGTGCCCATCTA-3', 5'-TTGCTGATCCCCATCTGCTGG-3'.

Adipocyte triglyceride. The content of cellular protein was determined using a bicinchoninic acid protein assay (Pierce Laboratories) (18). Adipocyte triglyceride was extracted with hexane/isopropyl alcohol (2:3, v:v) for 10 min at 4°C, emulsified with 2% Triton X-100 in isopropyl alcohol for 20 min at 70°C, and measured using a triglyceride test kit (Kyowa Medex).

Oil Red O staining. 3T3-L1 adipocytes were washed twice with PBS and fixed with 4% buffered formalin for at least 30 min at 4°C. The cells were then stained for 30 min at room temperature with a filtered oil red O solution (0.3% oil red O in 60% isopropyl alcohol), washed twice with distilled water, and visualized.

Western blot analysis. 3T3-L1 adipocytes were washed with ice-cold PBS containing 1 mmol/L Na3VO4, lysed with M-PER Mammalian Protein Extraction reagent (Pierce Biotechnology) including proteinase inhibitor cocktail (Pierce Biotechnology). After centrifugation at 20,400 g for 20 min at 4°C, the protein content in the supernatant was determined using a bicinchoninic acid protein assay (Pierce Laboratories) and aliquots of the proteins were separated by 7.5% or 12.5% SDS-PAGE gels (e-PAGE; ATTO). The proteins were electroblotted onto a polyvinylidene difluoride membrane (ATTO) and detected using an ECL Plus detection kit (GE Healthcare UK) and a LAS 3000 image analyzer (Fuji Photo Film).

Statistical analysis. Statistical analyses were performed using the Stat View version 5.0 software (SAS Institute). All results were expressed as the means ± SD. Significance among individual treatment groups was evaluated using 2-way ANOVA with the Scheffe post hoc tests (2 × 2 designs (Fig. 1, 3–6) and the 2 × 4 designs (Fig. 2) with insulin and TRF, insulin and tocotrienol, or insulin and tocopherol as the independent variables. Differences were considered significant at P < 0.05.

Results

TRF suppressed adipocyte differentiation of 3T3-L1 preadipocytes into adipocytes. PPARγ functions as an important adipocyte determination factor and a differentiation marker in fat cells (19). The PPARγ mRNA expression in 3T3-L1 preadipocytes treated with 1.8 μmol/L insulin for 14 d (after 0 d in the presence of 1.8 μmol/L insulin) was higher than the control at each time point (P < 0.001). The mRNA expression of PPARγ was lower in 3T3-L1 preadipocytes treated with 1 μmol/L TRF and 1.8 μmol/L insulin than those treated with 1.8 μmol/L insulin at 0 d (77 ± 6%; P < 0.001) (Fig. 1). In addition, the PPARγ mRNA expression in 3T3-L1 preadipocytes treated with just 1 μmol/L TRF increased by 85 ± 15% (P < 0.001) compared with the control. Furthermore, the insulin-induced mRNA expression for other adipocyte-specific genes, including aP2 and C/EBPα, in 3T3-L1 preadipocytes treated
with 1.8 μmol/L insulin for 14 d increased compared with the control (P < 0.001). The insulin-induced aP2 (55 ± 11%; P < 0.05) and C/EBPa (63 ± 5%; P < 0.001) mRNA expression was decreased by 1 μmol/L TRF (Fig. 3A,D). To determine the duration of the inhibitory effect of TRF during adipocyte differentiation, 3T3-L1 preadipocytes were differentiated in media supplemented with 1.8 μmol/L insulin for 7 or 14 d and then cultured for 14 d in the presence of 1 mg/L TRF and 1.8 μmol/L insulin. The PPARγ mRNA expression in cells treated with 1.8 μmol/L insulin for 14 d after 7 or 14 d in the presence of 1.8 μmol/L insulin increased compared with the control for each number of days (P < 0.001) (Fig. 1). However, for 3T3-L1 preadipocytes differentiated in media in the presence of 1.8 μmol/L insulin for 7 or 14 d and then treated with TRF and insulin, PPARγ mRNA expression did not change compared with insulin only (7 d, 30 ± 18%; and 14 d, 1.3 ± 0.9%, respectively) (Fig. 1). In addition, PPARγ mRNA expression did not change for 3T3-L1 preadipocytes treated with just 1 μmol/L TRF compared with the control after treatment with 1.8 μmol/L insulin for 7 or 14 d (Fig. 1).

**FIGURE 2**  Tocotrienol suppressed PPARγ mRNA expression in the differentiation of 3T3-L1 preadipocytes into adipocytes. Cells were cultured for 14 d with various concentrations of α-tocotrienol (A), γ-tocotrienol (B), or α-tocopherol (C) with (+ Ins) or without 1.8 μmol/L insulin (−Ins). Results are means ± SD, n = 3 separate experiments. Within each treatment group, means without a common letter differ, P < 0.05. *Different from – Ins or + Ins at various concentrations of each vitamin E homologs, P < 0.05.

**FIGURE 3**  Tocotrienol suppressed adipocyte differentiation of 3T3-L1 preadipocytes into adipocytes. Cells were precultured for 0, 7, or 14 d with 1.8 μmol/L insulin and then cultured with 1 mg/L TRF (TRF) with or without 1.8 μmol/L insulin (Ins) for 14 d. Results are means ± SD, n = 3 separate experiments. Within each treatment group, means without a common letter differ, P < 0.05. Asterisks indicate different from 0 d at that group. *P < 0.05.

**Tocotrienol suppressed adipocyte differentiation of 3T3-L1 preadipocytes into adipocytes.** Because the major components of TRF are α-tocotrienol, γ-tocotrienol, and α-tocopherol, we studied the effects of these vitamin E homologs on adipocyte-specific gene expression to further clarify the inhibitory effect of TRF on the differentiation of 3T3-L1 preadipocytes into adipocytes. There was no change in the mRNA expression of PPARγ, aP2, and C/EBPa in 3T3-L1 preadipocytes treated with 2.4 μmol/L α-tocotrienol or 2.4 μmol/L γ-tocotrienol compared with the control differentiation of 3T3-L1 preadipocytes into adipocytes in all experiments (Figs. 2 and 3). The mRNA expression of PPARγ was lower in cells treated with 1.8 μmol/L insulin and 2.4 μmol/L α-tocotrienol (55 ± 4%; P < 0.001) or 0.24 μmol/L α-tocotrienol (50 ± 8%; P < 0.001) than in cells treated only with insulin (Fig. 2A). However, treatment with 2.4 μmol/L α-tocopherol and 1.8 μmol/L insulin produced no changes in the mRNA expression of aP2 (41 ± 19%; P = 0.082) and C/EBPa (28 ± 14%; P = 0.098) compared with insulin (Fig. 3E,B). In addition, the PPARγ mRNA expression in cells treated with 1.8 μmol/L insulin and 2.4 μmol/L γ-tocotrienol (90 ± 2%; P < 0.001), 0.24 μmol/L γ-tocotrienol (73 ± 3%; P < 0.001), or 0.024 μmol/L γ-tocotrienol (70 ± 4%; P < 0.001) was less than in cells treated only with insulin (Fig. 2B). The mRNA expression of other adipocyte-specific genes, including aP2 (42 ± 10%; P < 0.01) and C/EBPa (64 ± 10%; P < 0.01), was also decreased by γ-tocotrienol (Fig. 3C,F). In addition, treatment of 3T3-L1 preadipocytes with 2.4 μmol/L α-tocotrienol or 2.4 μmol/L γ-tocotrienol produced no changes in the mRNA expression of PPARγ, aP2, and C/EBPa compared with the control differentiation of 3T3-L1 preadipocytes into adipocytes. Furthermore, compared with insulin, the level of adipocyte triglycerides was lower in 3T3-L1 cells treated with 2.4 μmol/L γ-tocotrienol and 1.8 μmol/L insulin (40 ± 12%; P < 0.01), although there was no change with 2.4 μmol/L α-tocotrienol and 1.8 μmol/L insulin (30 ± 19%) (Fig. 4). The suppression of insulin-induced adipocyte differentiation by α-tocotrienol or γ-tocotrienol compared with insulin alone was also confirmed by oil red O staining (Supplemental Fig. 1). However, 2.3 μmol/L α-tocopherol and insulin enhanced the mRNA expression of PPARγ (162 ± 35%; P < 0.001) compared with the mRNA expression for insulin (Fig. 2C). Interestingly, the PPARγ mRNA expression in cells treated with

**FIGURE 1**  TRF suppressed the differentiation of 3T3-L1 preadipocytes into adipocytes. Cells were precultured for 0, 7, or 14 d with 1.8 μmol/L insulin and then cultured with 1 mg/L TRF (TRF) with or without 1.8 μmol/L insulin (Ins) for 14 d. Results are means ± SD, n = 3 separate experiments. Within each treatment group, means without a common letter differ, P < 0.05. Asterisks indicate different from 0 d at that group. *P < 0.05.
just 2.3 μmol/L α-tocopherol increased by 205 ± 32% (P < 0.01) compared with the control. In addition, oil red O staining revealed that α-tocopherol also slightly increased the insulin-induced accumulation of intracellular triglycerides compared with insulin (Supplemental Fig. 1).

**Tocotrienol suppressed PPARγ protein level in differentiation of 3T3-L1 preadipocytes into adipocytes.** The PPARγ protein levels in 3T3-L1 preadipocytes were analyzed in the presence of α-tocotrienol and γ-tocotrienol. The PPARγ protein levels in cells treated with 1.8 μmol/L insulin increased compared with the control differentiation of 3T3-L1 preadipocytes into adipocytes (P < 0.001). PPARγ protein levels were lower in cells treated with 2.4 μmol/L γ-tocotrienol and 1.8 μmol/L insulin (54 ± 14%; P < 0.05), although there was no change with 2.4 μmol/L α-tocotrienol and insulin (40 ± 15%; P = 0.066) compared with cells treated only with insulin (Fig. 5). These results were consistent with the results from the analysis of mRNA expression.

**Examination of the inhibitory mechanism using a protein phosphorylation assay of α-tocotrienol and γ-tocotrienol of 3T3-L1 preadipocytes.** Because Akt and ERK1/2 in the insulin signaling pathway are upstream of PPARγ and adipocyte differentiation, we examined the effects of tocotrienols on the levels of phosphorylated Akt and phosphorylated ERK1/2. The tyrosine phosphorylation of Akt (P < 0.001) and ERK 1/2 (P < 0.001) in 3T3-L1 preadipocytes treated with 100 nmol/L insulin increased compared with the control. The phosphorylation of Akt stimulated by 100 nmol/L insulin was inhibited by the presence of 2.4 μmol/L γ-tocotrienol (43 ± 16%; P < 0.05), although α-tocotrienol did not change (27 ± 10%) compared with stimulation by insulin only (Fig. 6A). On the other hand, the insulin-stimulated phosphorylation of ERK1/2 was not affected by the presence of α-tocotrienol (113 ± 20%) or γ-tocotrienol (100 ± 14%) compared with insulin alone (100%) (Fig. 6B).

**Discussion**

The findings of this study showed that through its effects on differentiation, tocotrienol could be an antiadipogenic vitamin similar to vitamin A in the nutrient-mediated regulation of body fat. PPARγ and C/EBPα play roles in the early stage of adipocyte differentiation, because they are transcription factors for numerous genes (20). Further, PPARγ forms a heterodimer with retinoic acid X-receptor (21) and regulates the transcription of adipocyte-specific genes (22–24). Retinoids and carotenoids, which are ligands of retinoic acid X-receptor, inhibit the early stage of differentiation in 3T3-L1 preadipocytes (9). These observations are consistent with the findings of the current study that tocotrienols suppressed PPARγ mRNA expression when 3T3-L1 preadipocytes were postcultured in media supplemented with 1.8 μmol/L insulin for 0 d but not for 7 or 14 d. Therefore, tocotrienols might suppress adipogenesis in preadipocytes but not adipocytes.

In the current study, treatment with 2.4 μmol/L α-tocotrienol and 1.8 μmol/L insulin produced no significant change in the mRNA expression of aP2 and C/EBPα compared with insulin alone, and γ-tocotrienol seemed to be a more potent inhibitor of adipogenesis than α-tocotrienol. However, both aP2 and C/EBPα mRNA expression were suppressed in vitro with α-tocotrienol treatment at much higher doses.

Previous in vivo studies showed that α-tocotrienol and γ-tocotrienol accumulate in adipose tissue and the concentration of these tocotrienols in rats fed a diet containing TRF is ~0.024 μmol/g adipose tissue (16,17). Our study revealed that having 0.024 μmol/L γ-tocotrienol and 1.8 μmol/L insulin in the
culture media suppressed PPARγ mRNA expression compared with insulin alone in the differentiation of 3T3-L1 preadipocytes into adipocytes. Considering that the oral administration of γ-tocotrienol decreases the body fat of rats in another study (15), γ-tocotrienol could decrease adipose tissue mass, although the effects of TRF on body fat mass was not determined in these studies (15–17).

Insulin is known to induce the differentiation of adipocytes from preadipocytes in adipose tissues (25) and these changes are associated with the sequential activation of pro-adipogenic transcription factors, including the mRNA expression of PPARγ and C/EBPα (20,26). To test the antiadipogenic mechanism of tocotrienol, we investigated Akt and ERK1/2 phosphorylation in the insulin signaling pathway. The adipogenic actions of insulin are mediated by the insulin signaling pathway and the binding of insulin to insulin receptor (IR) at the cell surface. This event activates the intrinsic tyrosine kinase activity residing in the β-subunit of the IR and leads to autophosphorylation of the cytoplasmic portion of the β-subunit and further activation of its tyrosine kinase toward several intermediate IR substrate-1 (IRS-1) with several downstream signaling molecules. Two major pathways are located downstream of IRS-1: the Akt and ERK1/2 pathways (27,28). Other studies, however, imply that this is still controversial (29–32). ERK1/2 induces cell growth and potentially affects cell differentiation. Interestingly, retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment to the adipocyte lineage despite its inhibition of the differentiation of clonal preadipocyte cell lines (33,34). In addition, tea polyphenol, which reduces body fat in vivo, and (-)-epigallocatechin gallate inhibit insulin-stimulated phosphorylation of IR and IRS-1 in rat hepatoma cells (35–37). (-)-Epigallocatechin gallate also downregulates resistin mRNA expression via a pathway that is dependent on the ERK pathway (38).

FIGURE 5 Tocotrienol suppressed PPARγ protein levels in the differentiation of 3T3-L1 preadipocytes into adipocytes. Cells were cultured for 14 d in the presence of control medium (Cont), 2.4 µmol/L α-tocotrienol (α-T3), or γ-tocotrienol (γ-T3) with or without 1.8 µmol/L insulin (Ins). Results are expressed as fold of control and are means ± SD, n = 3 separate experiments. Within each treatment group, means without a common letter differ, P < 0.05. A representative Western blot is shown in the insert.

Few studies have considered the inhibitory effects of the phosphorylation of Akt in adipose cells, although the tyrosine phosphorylation of Akt (but not ERK1/2) is inhibited by γ-tocotrienol after insulin stimulation in 3T3-L1 preadipocytes. It should also be noted that tocotrienol inhibited Akt signaling in neoplastic mammary epithelial cells and human umbilical vein endothelial cells (39,40). These previous studies and the current one suggest that γ-tocotrienol might be a potential Akt inhibitor. Because we did not examine insulin signaling in 3T3-L1 adipocytes, further study is needed to evaluate this.

In contrast to the inhibitory effect of the tocotrienols, α-tocopherol enhanced insulin-induced differentiation of 3T3-L1 preadipocytes into adipocytes. These findings are consistent with those of a previous study that found that γ-tocotrienol reduces body fat mass and α-tocopherol is ineffective in this respect in rats fed γ-tocotrienol and α-tocopherol for 8 wk (18). It seems that the opposing effects of α-tocopherol and α-tocotrienol on adipocyte differentiation may depend on differences between them, which are a saturated tail in α-tocopherol and 3 double bonds in the phytyl tail of α-tocotrienol. Also, α-tocopherol (600 mg/kg) increased glucose uptake and ameliorated the inhibitory effect of diabetes on skeletal muscle in vivo (41). Skeletal muscles are a major site of blood glucose utilization and, together with
adipose tissue, are a target tissue for insulin action. Based on these findings, α-tocopherol may effectively prevent diabetes but not obesity.

γ-Tocotrienol is metabolized into 2,7,8-trimethyl-2-(2′-carboxyethyl)-6-hydroxychroman (γ-CEHC), which possesses a hormone-like natriuretic function (14,42). Although it is unclear whether γ-CEHC is incorporated into adipose tissues, the addition of 264.3 μmol/L γ-CEHC and 1.8 μmol/L insulin to the culture media suppressed the PPARγ mRNA expression compared with the insulin of 3T3-L1 preadipocytes into adipocytes. However, the inhibitory effect of γ-CEHC was less than that of γ-tocotrienol (data not shown).

In conclusion, tocotrienols may prevent obesity through suppression of the differentiation of preadipocytes into adipocytes, and the inhibitory effect of TRF depends on both α-tocopherol and γ-tocotrienol but not α-tocopherol. These data show that the possibility that tocotrienol could be an antidiagnosticogenic vitamin similar to vitamin A in regard to nutrient-mediated regulation of body fat through its effects on differentiation. Further study is required on determine whether tocotrienol promotes the loss of body fat in humans.

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Literature Cited


