The effect of substances extracted from *Toona sinensis* leaves with 50% alcohol solution on lipolysis was investigated in cultured 3T3-L1 differentiated adipocytes. The amount of glycerol released from cells into culture medium was used to measure lipolysis activity. Glycerol release was increased by *Toona sinensis* leaf extract in a dose-dependent and time-dependent manner. Following treatment of 3T3-L1 adipocyte cells with various concentrations of *Toona sinensis* leaf extract (0.001, 0.01, and 0.1 mg/mL) for 6 hours, the amounts of glycerol released from 3T3-L1 cells increased from a control value of 99 nmol/mg protein to 127, 144, and 154 nmol/mg protein, respectively. The lipolytic effect of *Toona sinensis* leaf extract was not inhibited by pretreatment of cells with cycloheximide, econazole, baicalein, or indomethacin. However, the lipolytic activity induced by *Toona sinensis* leaf extract was diminished by dibutyryl cyclic adenosine-5′-monophosphate (dibutyryl cAMP) and the protein kinase C inhibitor calphostin C. These results indicate that the lipolytic effect induced by *Toona sinensis* leaf substances may be involved in the protein kinase C pathway and may be down-regulated by cAMP.

**Key Words:** lipolysis, glycerol release, 3T3-L1 adipocytes, *Toona sinensis* leaf

5'-triphosphate (ATP), phosphoenolpyruvate (PEP), reduced nicotinamide adenine dinucleotide (NADH), pyruvate kinase, lactate dehydrogenase, isoproterenol, dibutyryl cyclic adenosine-5'-monophosphate (dibutyryl cAMP), calphostin C, cycloheximide, econazole, baicalein, and indomethacin were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Other drugs were obtained from Merck (E. Merck, Darmstadt, Germany).

**Cell culture**

3T3-L1 cells, obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), were grown in culture plates containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 3.8 mM L-glutamine, and 50 μg/mL gentamicin. The cultures were kept at 37°C in a humidified chamber with 10% CO₂. The medium was changed every 2 or 3 days.

**Differentiation of 3T3-L1 cells to adipocytes**

3T3-L1 pre-adipocytes were differentiated to adipocytes as described previously by Hong et al [5]. Briefly, 2 days after confluence (Day 0), the medium was removed and fresh medium containing 0.5 mM IBMX and 0.25 mM dexamethasone was added. After another 3 days, the medium was replaced with fresh culture medium and the cultures were maintained as described above. By Day 8, more than 90% of the cells had differentiated into rounded cells with lipid droplets.

**Preparation of leaf extract**

Substances were extracted from *Toona sinensis* leaves by boiling in 50% v/v alcohol/water for 3 hours. Following centrifugation at 2,000g for 10 minutes, the extracted substances were lyophilized.

**Measurement of lipolysis**

Lipolytic activity was measured by assaying glycerol released from cells into incubation buffer. Briefly, on days 8 to 12, the medium was removed and the differentiated adipocytes were incubated for 10 minutes in serum-free medium with 1% BSA and Krebs-Ringer phosphate (KRP) buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 1.35 mM MgSO₄, and 10 mM disodium hydrogen phosphate [Na₂HPO₄] pH 7.4) containing 1 U/mL adenosine deaminase. Various concentrations of *Toona sinensis* leaf extract in KRP buffer at concentrations of 0.001, 0.01, and 0.1 mg/mL were then added to cells for 1, 3, and 6 hours. The incubation mixture was aspirated and used to assay glycerol. In agonist and antagonist experiments, $10^{-5}$ M isoproterenol, 0.1 mM dibutyryl cAMP, $5.6 \times 10^{-8}$ M calphostin, $10^{-5}$ M cycloheximide, $5 \times 10^{-5}$ M econazole, $5 \times 10^{-5}$ M baicalein, or $5 \times 10^{-5}$ M indomethacin was added to cells 30 minutes before the addition of 0.01 mg/mL *Toona sinensis* leaf extract.

**Glycerol assay**

Glycerol was assayed enzymatically as described previously [5]. The glycerol assay reagent contained 5 mM MgSO₄, 0.9 mM ATP, 0.9 mM PEP, 6 U pyruvate kinase, 2 U lactate dehydrogenase, and NADH to a final absorbance at 340 nm of 0.9 to 1.0 in potassium phosphate buffer, pH 7.0. The decrease in absorbance at 340 nm was proportional to the concentration of glycerol.

**Statistical analysis**

Overall significant differences between groups were determined using one-way ANOVA. The least significant difference was used to determine significant differences between individual samples. Values were considered to be significantly different from the control if $p$ was less than 0.05.

**RESULTS**

The control value for basal release of glycerol was $99 \pm 7$ nmol/mg protein. Following incubation with *Toona sinensis* leaf extract 0.001, 0.01, and 0.1 mg/mL for 6 hours, cultured 3T3-L1 adipocytes released significantly more glycerol: $127 \pm 8$, $144 \pm 6$, and $154 \pm 9$ nmol/mg protein, respectively (Figure 1). The effect was dose-dependent; glycerol release was $128\%$, $145\%$, and $156\%$ of the control value. The lipolytic effect induced by *Toona sinensis* leaf extract was also time-dependent. Following incubation with 0.01 mg/mL of leaf extract for 1, 3, and 6 hours, cultured 3T3-L1 adipocytes released significantly more glycerol: $102 \pm 9$ nmol/mg protein at baseline was increased to $124 \pm 10$, $136 \pm 11$, and $145 \pm 9$ nmol/mg protein (Figure 2). In the presence of $10^{-5}$ M isoproterenol for 30 minutes, glycerol release increased to $155 \pm 9$ nmol/mg protein, $151\%$ of the control value.
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A combination of 0.01 mg/mL *Toona sinensis* leaf extract and isoproterenol changed glycerol release to 140 ± 6 nmol/mg protein (Figure 3). Pretreatment of cells with 0.1 mM dibutyryl cAMP for 30 minutes followed by incubation with 0.01 mg/mL *Toona sinensis* leaf extract for 6 hours significantly inhibited glycerol release to 107 ± 9 nmol/mg protein (Figure 3). Similarly, pretreatment of cells with a protein kinase C inhibitor (5.6 × 10⁻⁸ M calphostin C) also inhibited *Toona sinensis* leaf extract-induced cellular lipolytic effects (Figure 3).

Various other compounds such as the protein synthesis inhibitor cycloheximide (10⁻⁵ M), the cytochrome P-450 inhibitor econazole (5 × 10⁻⁵ M), the lipoxidase inhibitor baicalein (5 × 10⁻⁵ M), and the cyclooxygenase inhibitor indomethacin (5 × 10⁻⁵ M) failed to diminish the *Toona sinensis* leaf extract-induced lipolytic effect (Figure 4).

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**Figure 1.** Dose-dependent effects of substances extracted from *Toona sinensis* (TS) leaves on lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated with various concentrations of extract for 6 hours and lipolysis was measured by glycerol release from cells into culture medium. Values are mean ± standard error from three experiments with triplicate determination. *p < 0.05 compared with control (n = 9).

**Figure 2.** Time-dependent effects of substances extracted from *Toona sinensis* (TS) leaves on lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated in the presence of 0.01 mg/mL *Toona sinensis* leaf extract for 1, 3, and 6 hours. Lipolytic activity was measured from the amount of glycerol in the culture medium. Values are mean ± standard error from three experiments with triplicate determination. *p < 0.05 compared with control (n = 9).

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**Figure 3.** Effects of isoproterenol, calphostin C, and dibutyryl cAMP on *Toona sinensis* (TS) leaf extract-stimulated lipolysis in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were pretreated with 10⁻⁵ M isoproterenol, 5.6 × 10⁻⁸ M calphostin C, or 0.1 mM dibutyryl cAMP for 30 minutes and then incubated with *Toona sinensis* leaf substances for 6 hours. Lipolytic activity was measured from the amount of glycerol in the culture medium. Values are mean ± standard error from three experiments with triplicate determination. *p < 0.05 compared with control (n = 9). †p < 0.05 when compared to TS (n = 9).
DISCUSSION

The present study demonstrated that exposure to various concentrations of *Toona sinensis* leaf extract significantly enhanced lipolytic activity in 3T3-L1 adipocytes. Up to now, there are few data on the lipolytic effects of *Toona sinensis* leaves on cultured adipocytes in vitro. Our results show that the regulatory mechanism of *Toona sinensis* leaves on cellular lipolysis may be mediated by the protein kinase C pathway, because glycerol release is inhibited by the addition of calphostin C, a protein kinase C inhibitor. cAMP may down-regulate *Toona sinensis* leaf extract-induced lipolysis. Although extracts from oolong tea also possess potent lipolytic activity, the active substance was identified as caffeine [3]. The present study showed that a combination of isoproterenol and *Toona sinensis* leaf extract did not have an additional enhancing effect when compared to *Toona sinensis* alone. Thus, the regulatory mechanism of the lipolytic effect of *Toona sinensis* leaf extract may be similar to that of isoproterenol.

Isoproterenol is a non-selective adrenergic agonist that stimulates lipolysis activity. It is popularly used as a lipolysis-stimulating agent in the study of adipocyte function [6]. Compounds acting selectively on the β3-adrenoceptor are promising tools to achieve sustained stimulation of lipolysis and energy expenditure [7]. However, isoproterenol has more effective lipolytic activity than specific β3-adrenergic agonists such as BRL37344 and CGP12177 in large mammals [8,9]. Thus, isoproterenol was used as the stimulating agent in this study.

Pretreatment of cells with cycloheximide failed to inhibit the enhanced lipolysis seen with *Toona sinensis* leaf extract, indicating that protein synthesis is not required. Similarly, arachidonic acid metabolic pathways such as the cytochrome P-450, lipoxidase, and cyclooxygenase pathways may also not regulate *Toona sinensis* leaf extract-induced lipolysis.

In summary, this study demonstrated that *Toona sinensis* leaf extract induced lipolysis in 3T3-L1 adipocytes. Our results suggest that components of *Toona sinensis* leaves have potent lipolytic activity in vitro. Their roles in anti-obesity effects in vivo need to be further explored. *Toona sinensis* leaf extract may potentially be used as an anti-obesity agent in the future.

ACKNOWLEDGMENTS

This work was supported by research grants from the National Science Council of Taiwan (NSC 90-2316-B-037-004), and the Department of Health of Taiwan (DOH92-TD-1014).

REFERENCES

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