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An *in vitro, ex vivo*, and *in vivo* demonstration of the lipolytic effect of slimming liposomes: An unexpected α_2-adrenergic antagonism

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Synopsis

Most of the slimming products already developed for cosmetic applications did not result from strategies that integrate whole lipolysis-regulating mechanisms. We thus focused our attention on a more complete integration of these mechanisms and we developed slimming liposomes (SLC) containing two microcirculation activators, i.e., esculoside and *Centella asiatica* extracts, one phosphodiesterase inhibitor, i.e., caffeine, and one fatty acid-β oxidation activator, i.e., L-carnitine.

The validity of our approach was assessed through (a) *in vitro* tests demonstrating that SLC induced a dramatic increase in the cyclic adenosine monophosphate (cAMP) content in human adipocytes, with a subsequent rise in the nonesterified fatty acids (NEFA) content of human adipocyte incubation medium, and (b) *in vivo* studies showing that SLC could provide an actual potent slimming effect on human volunteers.

Moreover, we give here, through binding experiments, the unambiguous demonstration that SLC is able to antagonize the α_2-adrenergic receptor that is known to reduce intracellular AMPc content and, subsequently, to down-regulate lipolysis. This α_2-adrenergic antagonism has never been reported for any component of SLC, and this work is the first demonstration of the α_2-adrenergic antagonism of such a combination of active liposome compounds.

INTRODUCTION

In order to develop potent slimming products we have to (a) enhance the external skin grain (smoothness) and (b) produce a lipolytic effect on human adipocytes acting through main mechanisms regulating lipolysis.

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To reach the first goal, i.e., enhance skin smoothness, we can resort to microcirculation activators, which present some draining properties. Esculin, for example, which presents anti-inflammatory properties (1), is able to stimulate venous resistance (2,3) and to enhance capillary resistance (1). Esculin is also active in quenching free radicals (4). It can therefore protect compounds especially prone to oxidation from this phenomenon, notably epinephrine, a β_3-adrenergic receptor agonist that is known to play an important role in lipolysis regulation. *Centella asiatica* extracts are potent veinotonics as well. Their activities are mediated via effects on metabolic pathways implicated in vascular wall connective tissue homeostasis (5). Such extracts have also been found to stimulate the synthesis of collagen, fibronectin, and proteoglycans by normal dermal human fibroblasts in tissue culture (6). This double activity makes these extracts ideal for (a) stimulating the rate of capillary blood flow and (b) improving the texture of the skin. This regeneration of the cutaneous extracellular matrix implies enhanced elasticity and subsequent reduction of the "orange peel" aspect.

For the second part of our approach one needs to well understand lipolysis regulation. Two enzymes are involved in this process: a hormone-sensible lipase (HSL) and a monoglyceride lipase (MGL); these two enzymes catalyze successive hydrolysis reactions leading to the breakdown of lipids stored in adipose tissue (7,8). The final step of this process, which results in the release of free monoglycerides and glycerol, is rapid and occurs spontaneously, but the initial step is dependent on the status of HSL, which exists in two different forms, where only one is active. An understanding of intracellular mechanisms controlling the interconversion of this enzyme between active and inactive forms is essential to developing active fat-reducing products. In other words, HSL represents the key enzyme in the control of lipolysis.

The HSL-modulating mechanisms are now well characterized. This enzyme is activated by a phosphorylation catalyzed by protein kinase A (PKA) (9). Phosphatase enzymes can subsequently inactivate the HSL by removing the phosphate group. The regulation of HSL activity can be viewed as the control of the balance between the amounts of active phosphorylated and inactive dephosphorylated enzymes present in the cell.

Certain compounds have the capacity to modulate the activity of PKA and thereby modify this balance. Such compounds act by altering the intracellular concentration of the molecule that controls PKA activity, i.e., the cyclic adenosine monophosphate (cAMP) (10). There are two ways of increasing intracellular concentration of cAMP: (a) by increasing the activity of the enzyme responsible for its production, i.e., adenylate cyclase (AC), or (b) by inhibiting the enzyme responsible for its degradation, i.e., phosphodiestarase (PDE) (11). Any factor that tends to increase the activity of AC will lead to the activation of PKA and, thereby, of HSL. Similarly, any factor that inhibits PDE will have the same ultimate effect. Caffeine, for instance, is a PDE inhibitor that can diffuse across cell membranes and directly interact with its target (12).

A third efficient way coexists with the two preceding ones: in order to increase lipolysis it is possible to act on the intracellular transport of free fatty acids derived from the breakdown of triglyceride. As enzyme-catalyzed reactions inducing triglyceride breakdown are reversible, the balance between triglyceride degradation and/or triglyceride synthesis depends on the intracellular free fatty acid concentration. In the presence of a high level of free fatty acids, the balance will be shifted to favor triglyceride synthesis. In these conditions, L-carnitine, which stimulates free fatty acid transfer to mitochondria

(13), is able to reduce the intracytosolic free fatty acid concentrations favoring the breakdown rather than the synthesis of the triglycerides. As a consequence, L-carnitine acts as a major stimulator of the lipolysis.

The rationale to evaluate the lipolytic effect of SLC through *in vitro* and *ex vivo* models to test its slimming activity in human subjects is provided by (a) the demonstration by Deguercy *et al.* in 1995 (14) that human adipocytes in suspension represent a good predictive model to evaluate the *in vivo* activity of slimming products and (b) the relationship existing between the adrenergic control of lipolysis and the weight loss observed in humans in *in vivo* studies (15).

We have therefore demonstrated in a first series of experiments that SLC can act through the main lipolysis regulating mechanisms to show its actual potent slimming effect in human subjects. Moreover, and in an interesting way, we demonstrate for the first time, the α_2-adrenergic antagonism of such a combination of encapsulated active compounds.

MATERIALS AND METHODS

DRUGS, CHEMICALS, AND REAGENTS

All the culture media came from GIBCO-BRL (France). The [3H]RX821002 (specific activity 67.0 Ci/mmol) was purchased from Amersham Pharmacia Biotech Europe (Saclay, France). The (−)epinephrine bitartrate, clonidine hydrochloride, prazosin hydrochloride, propanolol hydrochloride, and indomethacin were obtained from Sigma (St. Quentin Fallavier, France). Phospholipon 90 G came from Nattermann (Cologne, Germany). *Centella asiatica* came from Indena. L-carnitine was purchased from Quimdis S.A. (Levallois-Perret, France). Esculoside was purchased from Zileco (Evry, France). Phenonip came from Clariant (Puteaux, France). Butylene glycol came from Lambert-Riviere (Pierre-Benite, France). Carbopol was purchased from Gattefosse (Genevilliers, France). All other reagents came from Aldrich (St. Quentin Fallavier, France), Carlo Erba (Val de Reuil, France), Merck (Nogent sur Marne, France), or Sigma.

SLC PREPARATION

To prepare one kilogram of SLC, 2 g of *Centella asiatica* extract was dissolved in 150 g of butylene glycol at 80°C. Twenty grams of caffeine and 113 ml of deionized water were then added to the mixture under agitation until complete dissolution. One hundred grams of L-carnitine and 2 g of esculoside were then solubilized in the blend before addition of 30 g of phospholipon 90 G. When phospholipon 90 G was completely dissolved, the mixture was left to cool overnight under agitation.

Added to the mixture at room temperature were 125.8 ml of deionized water, 7.5 g of a phenonip/butylene glycol mixture (10–2.5), 2 g of preservatives, and 250 g of a collagen/chondroïtine sulfate solution. After homogenization, liposomes were formed by strong agitation (10 min). Finally, 166.7 g of a 3% carbopol solution was added, and the pH of the mixture was adjusted to 5.7 with citric acid.

ISOLATION OF HUMAN ADIPOCYTES

Adipose tissue samples from abdominal fat deposits were obtained after plastic surgery. Tissue samples were rinsed in physiological medium to remove adhering blood. Visible fibrous material and blood vessels were carefully dissected. The remaining adipose tissue was cut into small fragments with a scalpel and then incubated in the dissociation medium [MEM medium without phenol red added, with 50 IU/ml of penicillin, 50 µg/ml of streptomycin, and 0.1% (w/v) collagenase], at 37°C under slow shaking for 60 min. The ratio of adipose tissue mass to digestion solution was approximately 4 g for 10 ml. The dispersed tissue was then filtered onto a 250-µm nylon filter to discard non-digested pieces. The suspension was allowed to stand for 10 min at room temperature to allow adipocytes to float. The medium under the cells was removed and replaced twice by the incubation medium [MEM medium without phenol red added, with 50 IU/ml of penicillin, 50 µg/ml of streptomycin, and 0.5% (w/v) lipid-free bovine serum albumin]. The integrity of the adipocytes was checked by microscopic observation.

cAMP AND NEFA MEASUREMENTS

Adipocyte suspensions were incubated 2 hr at 37°C in a water bath in the absence (control) or in the presence of increasing concentrations of SLC. At the end of the incubation period, cell lysates were obtained by sonication and the intra-adipocyte cAMP contents were quantified using a sensitive and specific enzyme immunoassay (EIA) (Amersham, Saclay, France). The NEFA were measured in the incubation medium using a colorimetric enzymatic test (Wako Chemicals, Japan).

BINDING EXPERIMENTS

Human α_2-adrenoreceptor binding assay. Competition binding experiments were performed as described by Bylund *et al.* (16), with slight modifications. Briefly, membranes from HT-29 human adrenocarcinoma cells (40 µg protein) were incubated for 20 min at 22°C with 0.7 nM of [^{3}H]RX821002 in the presence of increasing concentrations of SLC in 250 µl of assay buffer containing 1 mM of EDTA, 2 mM of $MgCl_2$, and 50 mM of Tris-HCl (pH 7.4). The reaction was terminated by rapid filtration under vacuum through glass fiber filters (Filtermat A, Wallac), using a 48-sample cell harvester (Mach IV, Tomtec). The filters were cooled with 50 mM of ice-cold Tris-HCl (pH 7.4), dried, and counted for radioactivity in a scintillation counter (1204 Betaplate, Wallac), using a solid scintillant (MeltiLex B/HS, Wallac). Non-specific binding was determined in the presence of 100 µM of (−)epinephrine. The specific binding was defined as the difference between total and non-specific binding. Assays were performed in duplicate and the results expressed as a percent of the control specific binding (mean values). IC50 values and Hill slopes (nH) were calculated by computer-assisted nonlinear regression analysis of the competition curves.

ISOLATED ORGAN EXPERIMENTS—α_2-ADRENORECEPTOR ANTAGONISM

In vitro α_2-adrenoreceptor bioassay. The bioassay was performed using the rabbit isolated ear vein as described by Daly *et al.* (17). Male New Zealand white rabbits weighing

about 3 kg (Charles River, St. Aubin les Elbeuf, France) were sacrificed, and the main ear veins were removed quickly, cleaned of connective tissue, and cut into rings 3 mm in length. These preparations were suspended in a 20-ml organ bath containing Krebs solution of the following composition (in mM): NaCl, 118.0; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $NaHCO_3$, 25.0, and glucose 11.0 (pH 7.4). It also contained 1 μM of prazosin, 1 μM of propranolol, and 1 μM of indomethacin to block α_1- and β-adrenergic receptor activation and prostanoid release, respectively. This solution was continuously aerated with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C.

The tissues were connected to isometric force transducers (UF1, Phymep, Paris, France) for tension recordings, stretched to a resting tension of 1 g, and allowed to equilibrate for 60 min. During this time, they were washed repeatedly and the resting tension was readjusted at 15-min intervals.

For the evaluation of α_2-receptor antagonistic activity, the tissues were exposed to a submaximal concentration of clonidine (0.1 μM) to obtain a sustained contraction. Thereafter, they were exposed to cumulative increasing concentrations of SLC. Each concentration was added after the response to the previous one had stabilized, and the resulting change in the clonidine-induced contraction was measured. SLC was thereby investigated on two tissues and the results expressed as a percent of the clonidine-induced contraction (mean values).

IN VIVO EXPERIMENTS

Eighteen healthy human volunteers were included in this study. All the volunteers applied daily a 3% SLC formulation on one of their thighs; their other thigh served as a control. Before and at the end of the study, twenty-eight days later, the volunteers' thigh circumferences were measured.

STATISTICAL ANALYSIS

Results were presented as means ± standard error (SE). The level of significance was assessed using a one-way analysis of variance (ANOVA 1), followed by a Dunnett's test.

RESULTS AND DISCUSSION

The product we have developed consists of a liposome containing esculoside, *Centella asiatica* triterpenes, caffeine, and L-carnitine. The liposome form allows the enhancement of the penetration of active compounds through the skin (18).

In order to assess the magnitude or the SLC lipolytic effect, we first conducted some *in vitro* experiments on primary normal human adipocytes in suspension. The capability of SLC to induce lipolysis in this model was evaluated by measuring NEFA liberation from adipocytes. As shown in Figure 1, SLC induced a dose-related increase in the NEFA content of the adipocyte incubation media. The SLC effect was detectable for a concentration as low as 0.1% (v/v) and reached a maximum at 1% (v/v).

To decipher the intracellular signaling pathway underlying this lipolytic effect and because the main intracellular messenger regulating lipolysis is cAMP, we measured

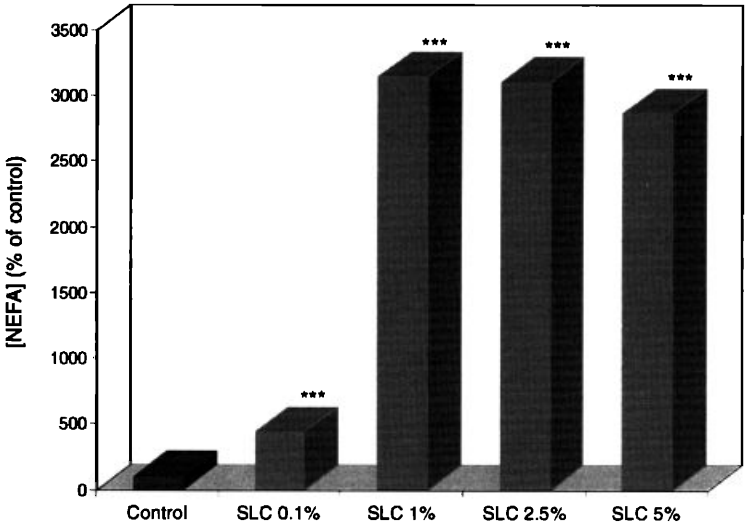

Figure 1. Effect of SLC on the concentration of NEFA present in the human adipocyte incubation medium. ***p < 0.001 vs control value.

under the same experimental conditions the intracellular cAMP content of adipocytes. As shown in Figure 2, SLC increased the cAMP content of adipocytes in a dose-dependent manner. The SLC effect regarding this endpoint was detectable for a concentration as low as 0.1% (v/v) and reached a maximum at 5% (v/v).

It should be noted here that the rate of NEFA appearance increased in parallel with the increase in intracellular cAMP content. This result strongly suggests that these two phenomena are causally interrelated. Therefore, at concentrations over 1%, although SLC continued to induce higher intracellular cAMP levels, the rate of lipolysis did not further

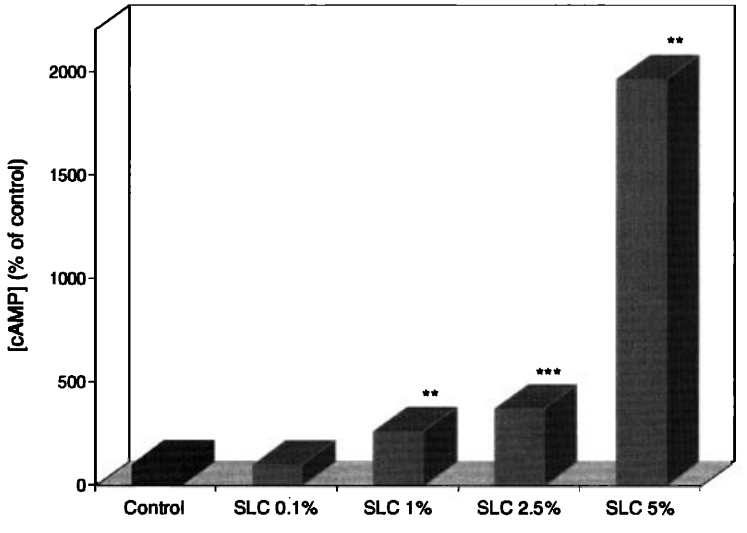

Figure 2. Effect of SLC on the intracellular cAMP concentration of human adipocytes. **p < 0.01 and ***p < 0.001 vs control value.

increase. This is a common pattern in pharmacology and shows that SLC can increase the rate of lipolysis only to a certain extent. This cAMP-modulating activity of SLC could prove to be very useful in pathological conditions in which intra-adipocyte cAMP baseline levels are abnormally low.

Moreover, we can note that 5% of SLC is able to induce a 20-fold increase in intracellular cAMP content. As the only component of SLC that is able to directly increase the intracellular cAMP levels is the caffeine, and since the caffeine effect in this regard cannot reach this magnitude, we conducted further experiments aimed to clarify this point.

Lipolysis is mainly regulated by three types of receptors that are positively (β_3-adrenergic receptor) (19) or negatively (α_2-adrenergic and PYY receptors) (20) coupled to the enzyme responsible for the cAMP synthesis, the adenylate cyclase (AC). In other words, the binding of certain effector molecules to their specific receptors results in an increase in intracellular cAMP concentration and the level of lipolysis, whereas activation of some other receptors has the opposite effect. The key molecules in this case are the G-proteins, which are closely associated with the relevant transmembrane receptors. Briefly, the G-proteins, which are a family of heterotrimeric proteins (consisting of α, β, and γ subunits), can be broken down into two main subfamilies on the basis of their α subunit: one subfamily tends to have a stimulatory effect (G-proteins are then called PGs), and the other tends to have an inhibitory effect (G-proteins are then called PGi). This is true for the AC system in which certain G-proteins tend to stimulate the enzyme and some others tend to inhibit it (21,22), which reflects the fact that different receptors have the capability of either stimulating or inhibiting AC and, in turn, lipolysis.

We have thus examined through radioactive binding experiments whether SLC can bind to the β_3- or to the α_2- or to the PYY receptors, and we have also checked if it could present some agonistic action on the first one or some antagonistic action on the latter two.

As shown in Figure 3, SLC was able to bind the α_2-adrenergic receptor; 1% of SLC inhibited 21% of the radioactive ligand binding. In order to demonstrate that this binding activity was partially responsible for the dramatic increase in the intra-adipocyte cAMP levels observed in the *in vitro* study, we performed some *ex vivo* experiments aimed to confirm the antagonistic effect of SLC on α_2-adrenergic receptors.

As shown in Figure 4, SLC dose-dependently antagonizes α_2-adrenergic receptors. The SLC effect regarding this point was detectable for a concentration as low as 0.1% (v/v) and reached a maximum at 5% (v/v). At concentrations of 0.1%, 1% and 5% (v/v), SLC inhibited the activation of α_2-adrenergic receptors by factors of 35, 74, and 99%, respectively.

The dramatic increase in the intra-adipocyte cAMP levels observed in the presence of SLC was thus the result of the caffeine effect and this surprising α_2-adrenergic antagonism. As far as we can know, none of the SLC components was shown to antagonize this receptor.

The capacity of SLC to dramatically increase intracellular cAMP levels, combined with the well known effects of L-carnitine, esculoside, and *Centella asiatica* triterpenes, should

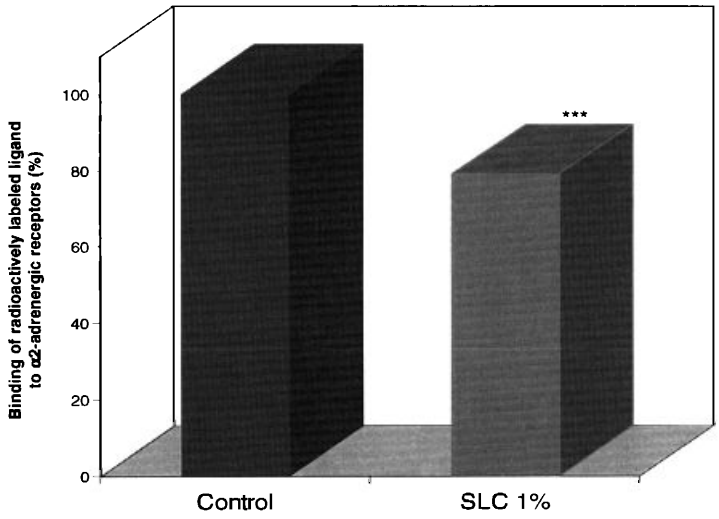

Figure 3. Binding activity of SLC on α_2-adrenergic receptors. ***p < 0.001 vs control value.

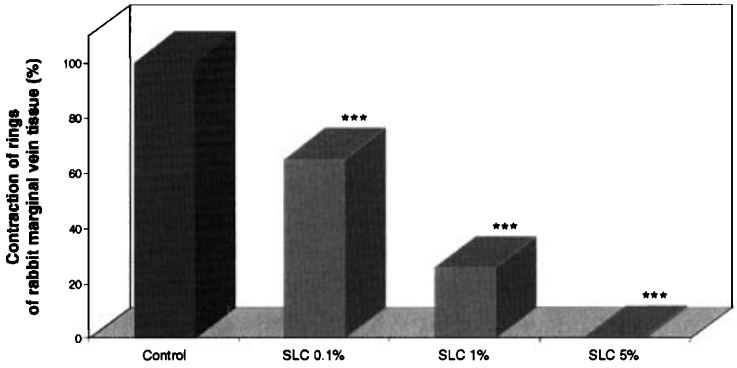

Figure 4. Antagonistic activity of SLC on α_2-adrenergic receptors. ***p < 0.001 vs control value.

confer to SLC a potent slimming effect. In order to check this point, we thus conducted some *in vivo* experiments on eighteen normal, healthy, human volunteers.

As shown in Figure 5, 3% SLC-containing formulation is able to reduce the thigh circumferences of human volunteers by more than 10 mm. More than 20% of the human volunteers participating in the test had their thigh circumferences reduced by more than 10 mm, whereas, in the same time, their other, non-treated, thigh did not show any circumference reduction. This last study shows the potent slimming effect of SLC and furthermore emphasizes the relevance of our "slimming approach."

CONCLUSION

In conclusion, we provide here the experimental demonstration of the validity of a global approach concerning lipolysis. This study is not only based on *in vitro* and *ex vivo* demonstrations of effective product efficacy, but it also shows the real relevance of SLC

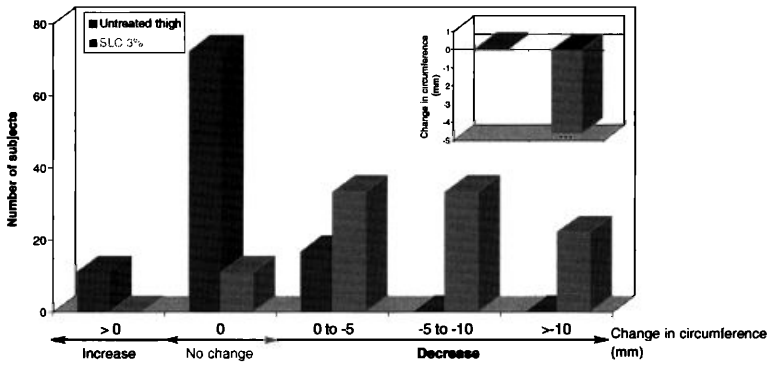

Figure 5. Effect of SLC in reducing the circumference of the thighs of eighteen normal, healthy volunteers. Insert: Mean of decrease in thigh circumference in control conditions versus thighs treated by SLC 3% (mm). ***p < 0.001 vs control value.

in physiopathological fields. Moreover, this work provides the first unambiguous demonstration for the α_2-adrenergic antagonism of such a combination of encapsulated active compounds. Further experiments are running in our laboratories to check which SLC components are responsible for this unexpected effect.

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REFERENCES

(1) P. Tetenyi, Les principes actifs du marron d'inde, *Plant Med. Phytither.*, 11, 158–164 (1977).
(2) J. Lavollay and J. Sevestre, The nature of vitamin P activity of esculoside on capillary resistance, *Compt. Rend. Soc. Biol.*, **218**, 979 (1944).
(3) J. Lavollay, Vitamin P action of esculoside and esculetol on capillary resistance, *Compt. Rend. Soc. Biol.*, **139**, 270 (1945).
(4) N. Yanishlieva and E. Marinova, Antioxidative effectiveness of some natural antioxidants in sunflower oil, *Z. Lebensm. Unters Forsch.*, **203**, 220–223 (1996).
(5) M. Arpaia, R. Ferrone, M. Amitrano, C. Nappo, G. Leonardo, and R. del Guercio, Effects of *Centella asiatica* extract on mucopolysaccharide metabolism in subjects with varicose veins, *Int. J. Clin. Pharmacol. Res.*, **10**, 229–233 (1990).
(6) R. Tenni, G. Zanaboni, M. De Agostini, A. Rossi, C. Bendotti, and G. Cetta, Effect of the triterpenoid fraction of *Centella asiatica* on macromolecules of the connective matrix in human skin fibroblast cultures. *Ital. J. Biochem.*, **37**, 69–77 (1988).
(7) P. Belfrage, G. Fredrikson, P. Stralfors, and H. Tornqvist, Adipose tissue lipases, in *Lipases*, B. Borgström and H. Brockman, Eds. (Elsevier, Amsterdam, 1984).
(8) D. Langin, C. Holm, and M. Lafontan, Adipocyte hormone-sensitive lipase: A major regulator of lipid metabolism, *Proc. Nutr. Soc.*, **55**, 93–109 (1996).
(9) M. Anthonsen, E. Degerman, and C. Holm, Partial purification and identification of hormone-sensitive lipase from chicken adipose tissue, *Biochem. Biophys. Res. Commun.*, **236**, 94–99 (1997).
(10) I. Pastan, Cyclic AMP, *Sci. Amer.*, **227**, 97–105 (1972).
(11) E. Degerman, P. Belfrage, and V. Manganiello, Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3), *J. Biol. Chem.*, **272**, 6823–6826 (1997).
(12) J. Smith and D. Mills, Inhibition of adenosine 3′,5′-cyclic monophosphate phosphodiesterase, *Biochem. J.*, **120**, 20P (1970).

(13) J. Bremer, Carnitine and its role in fatty acid metabolism, *Trends Biochem. Sci.,* **2,** 207–209 (1977).
(14) A. Deguercy, C. Garcia, and C. Chesne, Human adipocytes in suspension. An *in vitro* experimental model to test slimming products, *Facts and Illusions in Cosmetics,* IFSCC Congress, Montreux (1995).
(15) B. Zahorska-Markiewocz, C. Kucio, and D. Pikorska, Adrenergic control of lipolysis and metabolic responses in obesity, *Horm. Metab. Res.,* **18,** 693–697 (1986).
(16) D. Bylund, C. Ray-Prenger, and T. Murphy, *Alpha*-2A and *alpha*-2B adrenergic receptor subtypes: Antagonist binding in tissues and cell lines containing only one subtype, *J. Pharmacol. Exp. Ther.,* **245,** 600–607 (1988).
(17) C. Daly, J. McGrath, and V. Wilson, Evidence that the population of postjunctional-adrenoreceptors mediating contraction of smooth muscle in the rabbit isolated ear vein is predominantly alpha 2, *Br. J. Pharmacol.,* **94,** 1085–1090 (1988).
(18) B. Bonnekoh and G. Mahrle, Cutaneous administration of liposomes—A review of the literature with special reference to findings from keratinocyte cultures, animal experiments, and clinical studies, *Z. Hautkr.,* **65,** 99–105 (1990).
(19) P. Valet and J. Saulnier-Blache, Metabolic and trophic role of catecholamines in the development of white adipose tissue, *Ann. Endocrinol.,* **60,** 167–174 (1999).
(20) I. Castan, P. Valet, N. Quideau, T. Voisin, L. Ambid, M. Laburthe, M. Lafontan, and C. Carpene, Antilipolytic effect of α_2-adrenergic agonists, neuropeptide Y, adenosine, and PGE1 in mammal adipocytes, *Am. J. Physiol.* **266,** R1141–R1147 (1994).
(21) A. Gilman, G proteins: Transducers of receptor-generated signals, *Annu. Rev. Biochem.,* **56,** 615–649 (1987).
(22) A. Spiegel, Signal transduction by guanine nucleotide binding proteins, *Mol. Cell. Endocrinol.,* **49,** 1–16 (1987).