The number of obese people has been increasing over recent decades, and now more than one billion adults and children are overweight. Obesity has a crucial role in developing many diseases such as diabetes mellitus, hyperlipidemia, cardiovascular diseases, and cancer [1, 2]. Obesity can be overcome by increase in energy expenditure in the form of increased physical activity or heat production (thermogenesis) or by reduction of energy intake from diet. Weight loss induced by dieting has been shown to be successful in reducing body weight and the health consequences of obesity, but unfortunately >90% of individuals who lose weight through dietary control eventually return to their original weight [2, 3]. Pharmacological treatment may therefore be desirable for those patients with associated comorbid conditions who have been unable to control their obesity through diet and exercise.

Mitochondrial uncoupling agents were proposed to be a potential treatment for obesity [1, 4, 5]. The artificial uncoupler 2,4-dinitrophenol (DNP) was used for this purpose for many years in the 1930s [6, 7], but then was discontinued due to high toxicity. DNP is a weak acid, which acts as a protonophore: it crosses the inner mitochondrial membrane in neutral protonated state, loses its proton, and returns as the anion driven by membrane potential. In this way, it increases the proton conductance of mitochondria and energy expenditure of the whole organism [8-10].

DNP is now extensively studied in mice as a model compound to elucidate the effects of mitochondrial uncoupling on animal energy homeostasis, mitochondrial adaptations, mechanisms of oxidative stress, and aging [9-13]. Long-term treatment with a very low dose of DNP reduced body weight, improved serological glucose, triglyceride, and insulin levels, as well as extended the life span of mice [11]. Another study confirmed the positive effects of DNP on body weight and metabolic parameters of mice but reported a negative effect on exercise capacity [9]. In experiments with isolated heart, a low concentration of DNP caused profound QT shortening on the elec-
trocardiogram and triggered ventricular fibrillation [14]. Such inappropriate activities in critical tissues in combination with a narrow therapeutic range (small difference between the effective and the fatal doses) of DNP [6, 15] excluded its therapeutic application against obesity [16].

Alternative cationic uncoupling agents [17, 18], including derivatives of rhodamine 19 [19, 20], have been recently designed. A positive charge on these compounds moves them into mitochondria. Moreover, their uncoupling activity linearly depends on membrane potential, thus, to be self-regulating [21]. Due to the self-regulation as well as an ability to accumulate specifically in mitochondria and probable low toxicity, novel cationic uncouplers could be considered as promising anti-obesity agents.

A long-chain alkyl derivative of rhodamine, C₁₂R₁, increases proton conductance of artificial bilayer lipid membranes as well as possessing uncoupling activity in mitochondria and intact cells [19, 20]. A short-chain alkyl derivative of rhodamine 19, C₄R₁, exhibits low protonophoric activity in artificial bilayer lipid membranes; however, it uncouples mitochondria even more effectively than C₁₂R₁ did [20].

The aim of the present investigation was to study effects of the novel cationic uncoupler C₄R₁ in vivo in mice. To humanize our research, it was performed in C57BI/6 mice prone to obesity, the animals being kept on high fat diet and at thermoneutrality (30°C). It is well known that mice living at low ambient temperature produces a lot of heat to maintain their body temperature [22, 23], and this facultative thermogenesis masks uncoupling effect of studied compounds [10, 24]. Quite often anti-obesity drugs decrease food intake. Ignoring this fact leads to overestimation of the role of increased energy expenditure in loss of weight [25, 26]. To distinguish food intake-dependent and independent effects of C₄R₁, a pair-fed group of mice was included in the study. Another task of our study was to compare effects of C₄R₁ with effects of the classical uncoupler DNP. DNP is known to uncouple mitochondria at 10 times higher concentration than C₄R₁ (20] and [27] for comparison). However, data about DNP doses effective in vivo are quite contradictory (effective doses differ 1000 times in [11] and [9]). In our work, we studied effects of DNP at a dose similar to the C₄R₁ dose.

MATERIALS AND METHODS

Animals housing. Thirty male C57BI/6J mice, 7-8-week-old in the beginning of the experiment, were used. The mice were kept at thermoneutrality (30°C) and on high fat diet (45% fat content; D12451; Research Diets Inc., USA) eight weeks prior to the experiment to induce obesity and during the experiment. The mice were housed at 8 a.m., and the dark period started at 8 p.m. The mice were single caged. Cages were enriched with tissue paper, tissue tube as a shelter, and sawdust for welfare. The entire experiment was approved by the ethical committee of North Stockholm.

C₄R₁ and DNP treatments and monitoring of physiological parameters. Prior to treatment, the mice were divided into three groups according to their body weight and fat mass: 5 control, 5 pair-fed, and 5 treated mice. Treated mice received food ad libitum and C₄R₁ in drinking water. C₄R₁ was initially dissolved in 95% ethanol to concentration 0.25 M and then added in estimated amount (according to body weight of mice) to 30 ml tap water in small drinking bottles. The concentration of the drug in the drinking water varied during the experiment: 0.39-0.41 mM (depending on body weight) for the first six days, 0.19-0.21 mM for the next 16 days. On the 22nd day, the experiment was discontinued for six days and then started again with 0.19-0.21 mM C₄R₁ for 8 days. The control group received food ad libitum and drinking water with corresponding concentration of sodium bromide and ethanol (0.08-0.16%), since C₄R₁ was a bromide initially dissolved in ethanol. Pair-fed mice received drinking water with the same supplemenations as the control group and the same amount of food as treated mice ate in the previous 1-2 days.

The water was changed every 4-5 days. Water and food intakes were measured every 1-2 days. Actual dose of C₄R₁ (in μmol/kg daily) was calculated based on water intake. The pair-fed group received a daily meal every day at 8 p.m., when active night period starts, to avoid disturbing circadian rhythms of the animals. For this reason, water and food intakes as well as body weight were measured also at 8 p.m. Body composition (fat and lean masses) was measured twice before the treatment as well as directly prior to the treatment (day 0) and on the 2nd, 6th, and 15th days of the treatment using in vivo magnetic resonance imaging (MRI) with an EchoMRI-100 TM instrument (EchoMRI, USA). Note that fat mass measured by MRI is a much broader concept than mass of adipose tissues. It is sum of all lipids in all tissues of the body. Similar to this, lean mass is a broader concept than mass of muscles and includes proteins and carbohydrates of all tissues in the body, cytoplasm of all cells and extracellular fluid, blood plasma (except for lipids of plasma), etc. In other words, lean mass is the body mass with the exception of all lipids.

The experiment with DNP was performed separately during eight days. Groups of mice were formed similarly to the C₄R₁ experiment. DNP was initially dissolved in 95% ethanol to concentration 0.5 M and then diluted in 30 ml tap water to concentration 0.39-0.41 mM (according to body weight of the mice). Bottles with water containing DNP were protected from light. Control and pair-fed mice received ethanol (0.07-0.1%) in tap water. Other conditions were like in the C₄R₁ experiment.