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The Effect of Carbonic Anhydrase Inhibition on Leptin Secretion by Rat Adipose Tissue

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It is well known that the role of leptin in the body is to regulate food intake and energy expenditure but the process of leptin secretion by adipose tissue and the components involved in this process are still obscure. Carbonic anhydrase III (CA III) is the most abundant protein of the rat adipose tissue and its amount decreases with obesity. The effect of the inhibition of CA III on leptin secretion by rat epididymal adipose tissue was examined. Dorzolamide, a CA inhibitor, caused a decrease in dexamethasone and insulin-induced leptin secretion suggesting a possible role for CA III in the mechanism of leptin secretion.

Keywords: Leptin; Insulin; Dexamethasone; Carbonic anhydrase III; Dorzolamide; Rat adipose tissue

INTRODUCTION

Leptin, the *ob* gene product, is produced primarily in adipose tissue and acts on the hypothalamus to regulate appetite and energy expenditure. The plasma content of leptin in rats and human reflects the adipose tissue mass.[1] Although the leptin receptor is highly expressed in the hypothalamus, it is also found in peripheral tissues such as white adipose tissue, pancreatic β-cells, liver, skeletal muscle and kidney. Leptin regulates glucose and fatty acid metabolism in these tissues by influencing the expression of some related enzymes. Adipocyte leptin production and secretion are probably maintained through direct (autocrine and/or paracrine) negative feedback signals, as well as by other hormones, many of which are also regulated by leptin.[2,3] For example, glucocorticoids such as dexamethazone are potent stimulators of leptin secretion,[4] however, the role of insulin is unclear.[5–7] Although some investigators reported that leptin secretion was decreased by insulin others[1,3,8] mentioned opposite results. In addition, the intracellular factors involved in leptin secretion by adipose tissue are poorly understood.

One of the abundant proteins in rat adipose tissue is carbonic anhydrase III (CA III).[9] Carbonic anhydrases (EC 4.2.1.1) catalyse the reversible hydration of CO_2 and generate HCO_3^- and H^+. So far, fourteen different CA isoenzymes have been identified in mammals.[10] These isoenzymes are involved in important physiological processes connected with respiration and transport of CO_2/HCO_3^- between metabolising tissue and lungs, pH homeostasis, electrolyte secretion in various tissue/organs and biosynthetic reactions, such as gluconeogenesis, lipogenesis and ureagenesis.[11] CA III, predominantly found in adipose tissue, skeletal muscle and liver, represents 24% of the cytosolic protein content in the rat white adipose tissue.[9] In addition, CA II is found in very small amount in this tissue. Expression of CA III decreases with obesity in adipose tissue and the liver of rats. The reason for this decrease and the function of CA III in adipose tissue are not clear.[12] In addition it was reported that inhibition of CA V by acetazolamide decreased glucose-stimulated insulin secretion by pancreatic islets cell culture.[13] This event suggests that CA isoenzymes and reaction products might affect secretion of other peptide hormones.

Here, we examined the effect of dorzolamide, a CA inhibitor, on leptin secretion by rat adipose tissue

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culture. Strong inhibition was observed, suggesting the involvement CA isoenzymes in the mechanism of leptin secretion.

MATERIALS AND METHODS

Materials

Tissue culture media and ingredients, bovine insulin, dexamethasone and general chemicals were purchased from Sigma (St. Louis, MO, USA) and dorzolamide (Trusopt) from Merck Sharp & Dohme.

Animals and Tissue Culture

Following euthanasia by ether anesthesia at between 9 and 10 am adipose tissue was taken from the epididymal fat pads of 6–7 (150–250 g) Sprague-Dawley rats. The tissue was excised and placed in a sterile Iscove's modified Dulbecco's medium (IMDM) containing 25 mM HEPES, pH 7.2, 25 mM glucose, 1% (w/v) bovine serum albumin (BSA), 100 IU/ml penicillin and 100 μg/mL streptomycin. The total tissue was trimmed of visible vessels and blood clots, minced into 2–4 mm^3 pieces, mixed gently and distributed into plastic culture wells such that each well contained 100 mg tissue in 1 ml of the above IMDM. Besides the control group, five other groups were formed by addition of 1) 1 mM dorzolamide, 2) 100 nM dexamethasone, 3) 100 nM insulin, 4) 1 mM dorzolamide and 100 nM dexamethasone, 5) 1 mM dorzolamide and 100 nM insulin. The samples were then incubated at 37°C under 5% CO_2 for 24 h and 100 μL aliquots of incubation medium were removed for measurement of leptin.

Inhibition of CA and Quantitative Protein Assay

In order to see if dorzolamide passed into the cytoplasm of fat cells and inhibited CA activity, epididymal adipose tissue explants were incubated in the medium mentioned above with 0,0.1,1,10 and 100 mM dorzolamide for 24 h. After incubation, these explants were washed three times with 0.9% NaCl, homogenized with 0.05 M Tris–SO_4 (pH = 7.2) and CA activity measured by Maren's phenol red technique.[14] The protein content of the homogenate was quantified by the method of Lowry *et al*.[15]

Leptin Secretion and Statistics

Leptin was measured with a commercial rat leptin enzyme immunometric assay (EIA) kit (DRG International, Inc. U.S.A.). The sensitivity of this assay kit is 41.4-pg/mL leptin. The intra-assay coefficient of variation was 5.6%.

Results are given as a mean ± S.D. The paired Student's *t*-test was used for statistical analysis.

RESULTS

The results of the tests conducted to see if dorzolamide passed into the cytoplasm of fat cells and inhibited CA activity are shown in Figure 1. From these results, 1 mM dorzolamide concentration, which is the lowest concentration giving a reasonable inhibition, was selected for use in the experiments, so avoiding the possible hazards of using higher concentrations of the inhibitor.

In the control group, the rates of leptin secretion by rat epididymal adipose tissue were found to be 4.13 ± 0.67 ng leptin/100 mg tissue/24 h. The addition of 1 mM dorzolamide caused no significant change in the leptin secretion (4.03 ± 0.68 ng leptin/100 mg tissue/24 h). The pharmacological amount of dexamethasone (100 nM) and the hyperinsulinemic dose of insulin (100 nM) increased leptin secretion 160% ($p < 0.001$) and 30% (10.75 ± 2.96 and 5.42 ± 1.39 ng leptin/100 mg tissue/24 h), respectively. Dorzolamide inhibited dexamethasone-stimulated and insulin-stimulated leptin secretion 73% ($p < 0.001$) and 46% ($p < 0.05$) (2.82 ± 0.84 ng leptin/100 mg tissue/24 h and 2.91 ± 0.98 ng leptin/100 mg tissue/24h), respectively, compared to their respective values without dorzolamide (Figure 2).

DISCUSSION

In this study the effect of CA inhibition on leptin secretion by adipose tissue was investigated

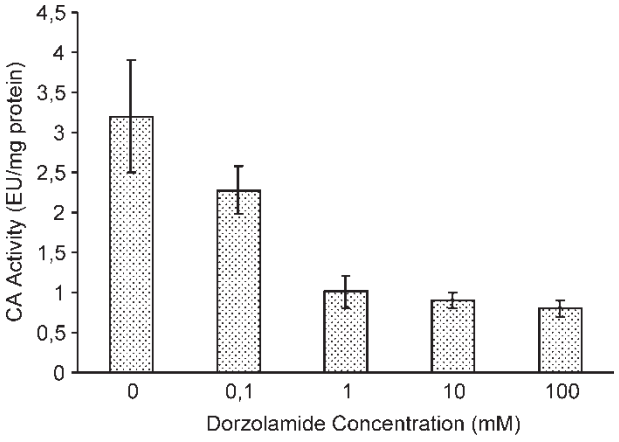

FIGURE 1 Effect of dorzolamide on CA activity in rat epididymal adipose tissue. 0.1 mM (2.28 ± 0.3 EU/mg protein), 1 mM (1.01 ± 0.2 EU/mg protein), 10 mM (0.9 ± 0.1 EU/mg protein), 100 mM (0.8 ± 0.1 EU/mg protein) dorzolamide inhibited CA activity 29%, 69%, 72%, 75%, respectively. Results are expressed as mean ± S.D. (n = 3).

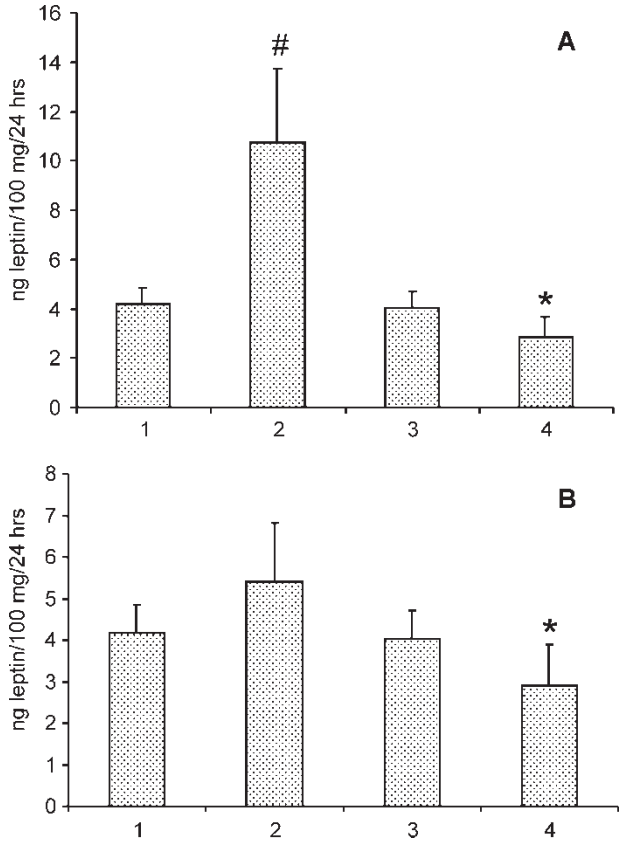

FIGURE 2 The change in leptin secretion in rat epididymal adipose tissue by CA inhibition. (**A**) 1) control (no dexamethasone or dorzolamide), 2) dexamethasone (100 nM), 3) dorzolamide (1 mM), 4) dexamethasone and dorzolamide (100 nM and 1 mM respectively). (**B**) 1) control (no insulin or dorzolamide), 2) insulin (100 nM), 3) dorzolamide (1 mM), 4) insulin and dorzolamide (100 nM and 1 mM respectively). Results are expressed as mean ± S.D. (n = 6). #, ($p < 0.001$), *, ($p < 0.05$) compared with control.

in cultured epididymal fat explants. The amount of peptide hormone secretion mainly depends on two processes: 1) the rate of synthesis, and 2) the rate of secretion. From this point of view, little information is present for leptin. The abundance of CA III in adipose tissue, where leptin production is very active, and the reduction of glucose-stimulated insulin secretion of pancreatic islets cells by inhibition of CA V by acetazolamide were the starting points for this research. For this purpose, besides measuring the extent of the CA inhibition effect on adipose tissue leptin secretion, dexamethasone and insulin stimulated leptin secretion are also considered.

We observed that dexamethasone and insulin caused an increase of 160% and 30%, respectively, in leptin secretion in adipose tissue explants (Figure 2A). This effect of dexamethasone has been shown in many other *in vitro* and *in vivo* studies.[6,7,16] In these researches, both leptin synthesis and leptin release were shown to increase.[17,18] Dexamethasone, mostly, regulates transcription through the glucocorticoid response element, which is present in the *ob* gene. But, after a deletion in the leptin promoter, which caused a break at the binding site for glucocorticoid response element, the effect of dexamethasone is reported be seen. This shows that the dexamethasone effect on leptin transcription does not solely rely on the classical molecular mechanism of glucocorticoid receptor action and that cis-elements may be involved in this process.[19]

We found that insulin increased leptin secretion by 30% with respect to the control group (Figure 2B). These results are consistent with the results from some previous studies.[1,18] Different data from the studies about the effect of insulin on leptin release by adipose tissue have also been reported.[6] These could arise from differences in experimental approach, animals of different strains, race and age, and also from the adipose pads which have different localisation and metabolism such as epididymal, subcutaneous, omental, prerenal. In addition cell culture media and incubation time might have caused the different results.[5,20] Although, the mechanism of insulin action on leptin secretion is poorly understood, it was suggested that the increased rate of glucose utilization of the fat tissue exerts an increase in leptin secretion by post-transcriptional mechanisms.[1] In fact, a decrease in leptin secretion was shown after inhibition of glucose transport and catabolism by using 2-deoxy-D-glucose and sodium fluoride.[1]

In order to be able to attribute a role to CA in leptin synthesis and/or secretion, the use of a CA inhibitor was necessary. In previous studies with CA, the most used inhibitor was acetazolamide, but its penetration into the cell is difficult.[21] For this purpose, we used dorzolamide because of its fast penetration through the cell membrane.[22] In addition, dorzolamide is a stronger inhibitor than acetazolamide ($K_i = 8 \times 10^3$ nM, $K_i = 3 \times 10^5$ nM, respectively).[23]

Although, no significant changes in the amount of leptin released by inhibition of CA in rat adipose tissue explants was observed, dexamethasone and insulin-stimulated leptin secretion decreased. Recently, a reduced glucose-stimulated insulin secretion of pancreatic islets cell culture after inhibition of CA V by acetazolamide was reported.[13] Since, little is known about the leptin secretion mechanism and the components involved in this process in adipose tissue, it is difficult to explain this effect of CA inhibition on leptin secretion. However, it was shown that the CO_2 formed should be removed very fast in order for glucose utilization to continue in a study with yeast cells.[24] Accumulation of CO_2 by inhibition of CA may cause a reduction in leptin secretion. In addition, dexamethasone stimulated lipolysis decreases intracellular pH and this

cannot be compensated for when CA is inhibited.[9] The disturbance of the metabolic pathways such as glycolysis, lipolysis and *de novo* lipogenesis, which are regulated according to the needs of the cell and easily affected by intracellular pH, might have caused a reduction in leptin secretion.

The fact that CA III is found in very high concentrations in three specific tissues controlling energy metabolism (adipose tissue, skeletal muscle and liver) makes it a good candidate for a potential role as a metabolic modulator.[25] CA III may have a role in dexamethasone and insulin-stimulated leptin secretion mechanism of rat adipose tissue so that CA III inhibition may lead to decreased leptin secretion. When, these hormones and dorzolamide were used together, leptin levels are lower than the control and dorzolamide group levels. This situation may be a result of the increased metabolic rate and decreased intracellular pH when CA is inhibited.

In summary, CA inhibition in rat adipose tissue causes a decrease in dexamethasone- and insulin-stimulated leptin secretion. Since, CA III is found in high concentration in this tissue, it may play a role in a hormone stimulated leptin secretion mechanism.

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