PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
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Pakistan Journal of Nutrition 9 (8): 776-780, 2010
ISSN 1680-5194

Investigating the Metabolic Effects of the Cyclic Nucleotide Phosphodiesterase Inhibitors on Immature *balb/c Mice*

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Abstract: We have investigated the metabolic effects of IBMX (3-isobutyl-1-methyl xanthine, a non-selective PDE inhibitor), amrinone, MC7 (6-(4-(tetrahydro-2H-pyran-4-yl)-4-oxobutoxy)-4-methylquinolin-2(1H)-one) and MC9 (6-(4-(1-ethylpiperidin-4-yl)-4-oxobutoxy)-4-methylquinolin-2(1H)-one) (selective PDE3 inhibitors) on immature *balb/c mice*. Controls included groups of mice that received no intervention (control 1) and a group injected with 1mg/kg of drug carrier (control 2). The four experimental groups were weighed and injected separately with IBMX, amrinone, MC7 and MC9 (1mg/kg) daily for seven days after which they were killed and 59 blood and tissue samples were collected. The administration of drug carrier into the male mice, decreased the growth pattern during the second ($P<0.05$) and third ($P<0.001$) day. After the injection of drug carrier, there wasn't any significant difference between the growth pattern, and the concentrations of biochemical factors in the male, comparing with female mice. Therefore, a group of mice, including both male and female, was used. MC7 decreased the growth pattern on the day two ($P<0.01$). IBMX increased glucose ($P<0.05$), triglyceride ($P<0.05$) and glycogen ($P<0.01$) concentrations, whereas amrinone decreased the glucose ($P<0.05$) comparing with control 2. The ability of stress tolerating in immature female mice was more than that in the male mice. In spite of the similar inhibition influence, the experimental drugs used in this study, had different metabolic effects.

Key words: Cyclic nucleotide phosphodiesterases, growth pattern, cAMP, cGMP

INTRODUCTION

cAMP is an important second messenger, known to control many cellular processes that include: triglyceride hydrolysis, glycogenolysis, gluconeogenesis and insulin secretion (Collins *et al*., 2001). Phosphodiesterase enzymes (PDEs) play an important role in the regulation of intracellular levels of cAMP and cGMP. They have been divided into 11 families. These enzymes are often referred to as class I cyclic nucleotide PDEs (Bender *et al*., 2006).

PDE3 is often referred to as the cGMP-inhibited PDE. Two PDE3 genes, PDE3A and PDE3B, have been identified (Bender *et al*., 2006). PDE3A is relatively more abundant in the cardiovascular system and PDE3B in cells involved in energy metabolism, including adipocytes, hepatocytes and pancreatic ß-cells (Shakur *et al*., 2001; Zhao *et al*., 1997). A general characteristic of PDE3s includes their phosphorylation and short-term activation in response to insulin as well as to agents that increase cAMP (Degerman *et al*., 1996). Activation of PDE3B plays a major role in the antilipolytic action of insulin in adipose tissue (Hagstrom-Toft *et al*., 1995) and may be important in the inhibition of cAMP-induced glycogenolysis in hepatocytes (Zhao *et al*., 2000). PDE3B may also be involved in the regulation of insulin-induced glucose uptake, glucose transporter-4 (GLUT-4) translocation, and lipogenesis (Zmuda-Trzebiatowska *et al*., 2006, Eriksson *et al*., 1994).

PDE3 selective inhibitors induce lipolysis and may have effects on glucose homeostasis because they stimulate glucose-induced insulin release (Snyder, 1999; Cheung *et al*., 2003). These compounds have been used in the treatment of congestive heart failure (CHF). They produced an improvement in haemodynamic parameters (Arnold, 1993). However, long-term, oral therapy with PDE3 inhibitors increases mortality in CHF patients (Cruickshank, 1993).

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PDE3 inhibitors may also be useful for treating obese patients because they promote adipocyte lipolysis, thus mobilizing stored fat. Although, their effect on the cardiovascular system may be a major limitation to their use. So, development of isoform-specific agents that inhibit PDE3B but not PDE3A may provide a way around here this obstacle (Snyder, 1999). Since, PDE3 isoform selective inhibitors do not have the long-term side effects of non-specific PDE3 inhibitors (Movsesian, 2003), they may be useful for obese individuals (Snyder, 1999) and developing such drugs is currently being investigated.

Many obese individuals also suffer from non-insulin-dependent diabetes (NIDDM). These patients show peripheral insulin resistance and their glucose stimulated insulin release is attenuated. Since PDE3 inhibitors should elevate ß-cell cAMP, they may augment glucose-stimulated insulin release and thus have benefit for type II diabetics also (Snyder, 1999). The aim of this current study was to investigate the metabolic effects of a non-selective PDE inhibitor, such as IBMX (3-isobutyl-1-methyl xanthine), PDE3 selective inhibitors, such as amrinone (5-amino- [3, 4- bipyridin]- 6 [1H]-one), MC7 (6-(4-(tetrahydro-2H-pyran-4-yl)-4-oxobutoxy)-4-methylquinolin-2(1H)-one) and MC9 (6-(4-(1-ethylpiperidin-4-yl)-4-oxobutoxy)-4-methylquinolin-2(1H)-one), on immature male and female *balb/c mice*. To do this, the effect of these drugs on growth, serum glucose; triglycerides, and cholesterol concentrations and the hepatic glycogen content were measured.

MATERIALS AND METHODS

Male and female immature *balb/c mice* (10-12 g) were selected randomly from the animal house of the Pharmacology Department at Mashhad University of Medical Sciences. The sample size with regard to reference 11 was considered to be at least 4, with the power calculation of 90%.

The mice were provided with standard laboratory food and water. All mice, during the experiment, were maintained at 55% relative humidity and 22°C in a 12-h day/night rhythm. They had free access to water and food and weighed individually every day for a period of 1 week. This project was approved by the Ethical Committee of Alzahra University, Tehran, Iran.

The mice were divided into control (n = 25) and experimental groups (n = 34). The control 1 group (C1) was left with no treatment (male control 1, n = 7; female control 1, n = 6). The control two group (C2) (male control 2, n = 6; female control 2, n = 6) was injected with 1 mg/kg of drug carrier (normal saline solution containing dimethyl sulfoxide) using the subcutaneous procedure. The four experimental groups were weighed using Sartorius scale and injected separately, every day for 1 week with IBMX (FLUKA), amrinone (SIGMA), MC7 or MC9. Administration of drugs was carried out using the subcutaneous procedure. MC7 and MC9 were synthesized in the chemistry department, faculty of basic sciences, Ferdosi University in Mashhad. Dimethyl sulfoxide (DMSO, FLUKA) used as drug solvent. Then on day 7, after 1.5-2 hrs of injection of the drug, the mice were anesthetized using the thiopental sodium, (80 mg/kg, ip.), followed by taking a sample of blood from their heart. The serum of each sample was separated and frozen at -18°C for the subsequent measurement of glucose, cholesterol and triglyceride concentrations. Automatic enzymatic spectrophotometery (Alcyon Autoanalyser instrument) using the Iranian Man Company kits. In order to measure the glycogen concentration, the liver of each mouse was separated, weighed and frozen at -18°C. At the time of measuring, each liver sample was divided into small pieces and homogenized. Then, liver glycogen was hydrolyzed to glucose by hydrochloric acid (HCL, MERCK). Finally the glucose concentration was measured using a glucose kit (Zahedi-Asl *et al*., 2000).

Data were expressed as mean ± SEM. Both C1 male with female, C2 male with female, C1 male with C2 male and finally C1 female along with C2 female groups were analyzed using the Student t-test. C2 group and four experimental groups were evaluated using one-way analysis of variance with Dunnett's multiple comparison test. P-values less than 0.05 were considered to be significant.

RESULTS

The effect of PDE inhibitors on the growth: In C1 mice, the rate of growth in male mice was more than female mice, particularly on the 3rd ($P<0.01$) and 6th ($P<0.05$) days (Fig. 1). Total growth percentage of male mice (68%±2.6) was more than female mice (57%±1.6), over the 1 week period ($P<0.01$). However, in C2 immature mice, there was no difference between the growth pattern of male and female mice that were injected with drug carrier. Moreover, there was no significant difference between the total growth percentage of the C2 male (44%±3.9) and the female (47%±2.8) mice. Therefore, to study the effect of drugs on growth, a group of mice, including both male and female was used. In C2 male mice as compared with C1 male mice, daily administration of drug carrier decreased the growth pattern during the second ($P<0.05$) and the third ($P<0.001$) day, whereas it did not produce any significant effect on C2 female compared to C1 female mice (Fig. 1).

In the presence of amrinone, the growth pattern was at first similar to C2 and then increased by day 7 ($P<0.01$). Although, low increasing effect of the amrinone on the growth rate, during the day 2 was not significant ($P>0.05$). On the 2nd day, MC7 decreased the growth pattern sharply ($P<0.01$), and then after an initial increase, returned back to the C2 level (Fig. 2). The effect

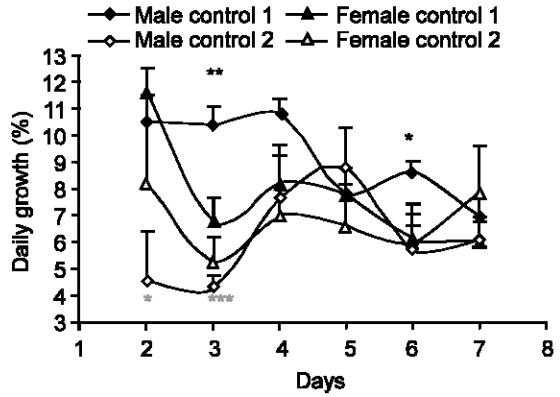

Fig. 1: The effect of drug carrier on growth pattern. Male control 1, n = 7; female control 1, n = 6; male control 2, n = 6; female control 2, n = 6. (*) is comparison of the male and female control 1 groups (C1). (*) is comparison of the male C1 group with the male control 2 group (C2). * P< 0.05, ** P<0.01, *** P<0.001. Both C1 male with female, C2 male with female, C1 male with C2 male and finally C1 female along with C2 female groups were analyzed using the Student t-test.

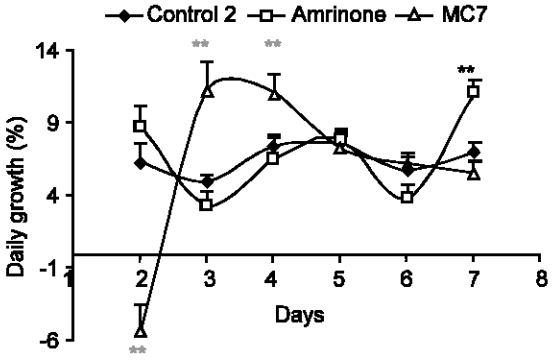

Fig. 2: The effect of PDE3 inhibitors (amrinone and MC7) on growth pattern. Control 2, n = 12; MC7, n = 8; amrinone, n = 9. (*) is comparison of amrinone group with the C2 group. (*) is comparison of the MC7 group with the C2 group, ** P< 0.01.

of MC9 and IBMX on growth pattern was similar to the effect of drug carrier in C2 group.

There was no significant effect on the total growth percentage, over the period of 1 week, and all groups grew up to 41-51% during this time.

The effect of PDE inhibitors on the serum biochemistry: There was no significant difference between the concentration of biochemical factors in the serum and liver glycogen of male comparing with female immature mice of the control group, which were administered with the drug carrier. Therefore, a group of mice, containing both male and female was used.

Serum cholesterol, triglyceride, glucose and liver glycogen concentrations for control group were 103 ± 5.2 mg/dl, 91.25 ± 11.54 mg/dl, 193.5±8.1 mg/dl and 11.5±1 mg/g respectively. IBMX increased the serum concentration of cholesterol (118.2±6 mg/dl, P>0.05), triglyceride (132.5±9 mg/dl, P<0.05) , glucose (247.8±11 mg/dl, P<0.01) and liver glycogen concentrations (24.4±4 mg/g, P<0.01) whereas amrinone decreased these concentrations to 87.6±6 mg/dl (P>0.05), 71.4±8 mg/dl (P>0.05), 158.9±12 mg/dl (P<0.05) and 6.5±2 mg/g (P>0.05) respectively.

MC7 and MC9 produced no significant effect on the above factors (P>0.05). MC7 decreased cholesterol and glucose concentrations to 96±4 mg/dl and 169.9±10 mg/dl respectively, whereas it did not change serum triglyceride (90.6±7 mg/dl) and the liver glycogen content (14.8±3 mg/g).

MC9 decreased serum cholesterol, glucose and triglyceride concentrations to 93.6±6 mg/dl; 184±8 mg/dl and 83±6 mg/dl respectively, however there was an increase in the liver glycogen storage amount (18.8±2 mg/g), (Fig. 3-4).

DISCUSSION

The effect of drugs on growth: With regard to Fig. 1, it was shown that the stress was associated with subcutaneous injection of drug carrier diminished the rate of growth on the immature male *balb/c mice*, however it did not have any effect on the female mice. In order to investigate the effect of MC7 on the growth on the 2nd day, another group (n = 8), that was injected with drug carrier, during the first 3 days, and with MC7, on the day 4, was studied. In this group, MC7 produced no significant effect on growth. This finding suggests that MC7 may have augmented the stress effect (Fig. 2). Hence, increasing the rate of growth by amrinone on the day 2 may be due to the diminishing effect of stress.

The effect of drugs on the serum biochemical factors: It is known that PDE3 inhibitors increase cAMP levels (Degerman *et al*., 1996) and stimulate glucose-induced insulin secretion. Insulin increases HMG-COA redoctase activity that regulates the synthesis of cholesterol, however increase of cAMP levels can phosphorylates and decreases its activity (Robert *et al*., 2003). The absence of any difference between the serum cholesterol concentration of four experimental groups comparing with the control group, indicates that probably these two effects have counteracted one another.

PDE3 inhibitors elevate beta cell cAMP and activate the cAMP-dependent protein kinase (PKA), and phosphorylates hormone sensitive lipase (HSL), which hydrolyzes stored triglyceride (White *et al*., 1994). PDE3

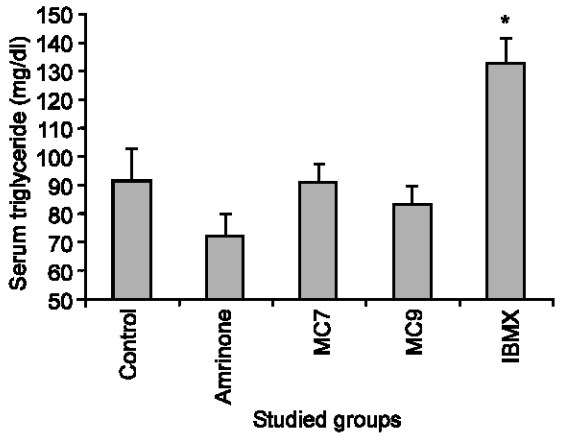

Fig. 3: The effect of PDE inhibitors on the serum triglyceride concentration. Control, n = 12; amrinone, n = 9; MC7, n = 8; MC9, n = 10; IBMX, n = 7. (*) is comparison of the four experimental groups with the control group,* P< 0.05.

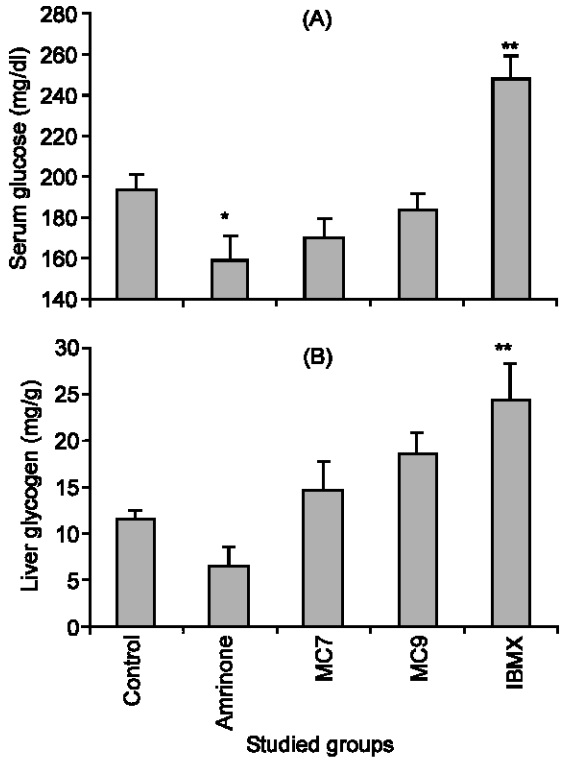

Fig. 4: The effect of PDE inhibitors on the serum glucose and liver glycogen concentrations. A, The effect of PDE inhibitors on the serum glucose concentration. Control, n =12; amrinone, n = 9; MC7, n = 8; MC9, n = 10; IBMX, n = 7. B, The effect of PDE inhibitors on the liver glycogen concentration. Control, n = 12; amrinone, n = 9; MC7, n = 8; MC9, n = 10; IBMX, n = 7. (*) is comparison of the four experimental groups with the control group, * P< 0.05, ** P< 0.01.

inhibitors promote continued breakdown of triglyceride even in the presence of an increase in the level of circulating insulin (Snyder, 1999). By the use of microdialysis technique, it has been shown that amrinone increases lipolysis in a dose-dependent manner (Arner *et al*., 1993). It has been demonstrated that iv. administration of amrinone increased blood levels of glycerol and FFA, the breakdown products of triglyceride (Wilmshurst *et al*., 1984; Ruttimann *et al*., 1994). In this study, amrinone acted as indicated above whereas IBMX showed an effect opposite to amrinone. In fact the effect of insulin on the adipose tissue, has been demonstrated by IBMX. It can be said that MC7 and MC9 have counteracted the effect of cAMP and insulin signaling on adypocytes (Fig. 3).

Present results indicated that amrinone decreased the serum glucose concentration, probably by the stimulation of insulin secretion. However, the serum glucose level was increased by IBMX (Fig. 4-A). It is necessary to mention that the effect of IBMX and amrinone on appetite was not investigated in previous studies.

One study suggest that PDE3 inhibitors stimulate hepatocyte glycogenolysis and antagonize the effect of insulin to suppress hepatocyte glycogenolysis (Snyder, 1999). In the current work, amrinone acted as above, in partially bringing down the concentration of liver glycogen. It seems that MC7 and MC9 have counteracted the effect of cAMP and insulin signaling on hepatocytes too (Fig. 4-B).

With regards to the fact that IBMX has increased both the liver glycogen content and serum glucose concentration, it could be suggested that IBMX prevented the influx of glucose into the cells of peripheral tissues. However, amrinone had an opposite action to IBMX in the immature mice.

It seems that the ability of stress toleration in immature female mice was more than that in the immature male mice. The growth effect of MC7 may be related to the augmentation of stress effect. Presumably, through an unknown mechanism, IBMX decreased the catabolism of glucose in the immature mice.

If the effect of MC7 and MC9 on the PDE activity is assessed and if these agents augment may cardiac contractility, then they might well be useful drugs for the treatment of CHF. These drugs probably are important to use, since they have less metabolic side effects than amrinone.

ACKNOWLEDGMENTS

We would like to appreciate Pharmacology and Biochemistry-Nutrition departments of Mashhad University of Medical Sciences for their help and support, and Dr. A.R. Moosavi for his edition cooperation in this paper.

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