Polish Journal of Pharmacology
Pol. J. Pharmacol., 2004, 56, 223–231
ISSN 1230-6002

CENTRAL ROLE OF cAMP IN THE INHIBITION OF GLYCOGEN BREAKDOWN AND GLUCONEOGENESIS PROMOTED BY LEPTIN AND INSULIN IN PERFUSED RAT LIVER

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Central role of cAMP in the inhibition of glycogen breakdown and gluconeogenesis promoted by leptin and insulin in perfused rat liver. G.R. BORBA-MURAD, M. VARDANEGA-PEICHER, S.B. GALENDE, R. CURI, H.M. SOUZA, E.G. MARIO, B.K. BASSOLI, R.B. BAZOTTE. Pol. J. Pharmacol., 2004, 56, 223–231.

Leptin showed less prominent inhibiting effect on the activation of hepatic glycogen breakdown and gluconeogenesis promoted by cAMP. The role of cAMP in the inhibition of glycogen breakdown and gluconeogenesis induced by physiological levels of leptin (10 ng/ml) and insulin (20 μU/ml) in the perfused liver was investigated. Insulin but not leptin inhibited ($p < 0.05$) the activation of glycogen breakdown promoted by cAMP (3 μM). Contrary to cAMP, the activation of glycogen catabolism promoted by 8-Br-cAMP (0.3 μM), a cAMP analogue more resistant to hydrolysis by phosphodiesterase 3B (PD3B), was inhibited ($p < 0.05$) not only by insulin (20 μU/ml) but also by leptin (10 ng/ml). The effect of leptin, however, was less intense than that of insulin. To verify the participation of the intracellular levels of cAMP, the experiments were repeated with N^6-monobutyryl-cAMP (N^6-MB-cAMP), a cAMP analogue, which is not metabolized by PD3B. The activation of glycogen breakdown promoted by N^6-MB-cAMP (0.3 μM) was not affected by leptin or insulin. In agreement with the results regarding glycogen catabolism, insulin and leptin at 50 ng/ml but not leptin at 10 ng/ml inhibited ($p < 0.05$) the activation of gluconeogenesis promoted by cAMP (7.5 μM). Taken together, these results led us to postulate that the convergent signaling pathways of these two hormones causing the inhibition of glycogen catabolism and gluconeogenesis involve a reduction of intracellular cAMP. Thus, cAMP levels may play an important role in the cross talk between both hormones and for the insulin-like effects of leptin.

***Key words**: cAMP, leptin, insulin, liver, glycogen breakdown, gluconeogenesis*

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INTRODUCTION

Leptin, a 16-kDa protein produced by adipose tissue and secreted into the blood, has a pivotal role in the control of food intake, thermogenesis and glucose metabolism.

Leptin shares many similarities in physiological actions with insulin. The binding of leptin to its receptor in the hypothalamus induces appetite suppression while in the muscle, adipose tissue, pancreatic islets and hepatocytes, it influences glucose metabolism [21]. Both leptin and insulin induce a cascade of intracellular events that includes the activation of insulin receptor substrate 1 and 2 (IRS1 and IRS2) → phosphatidylinositol 3-kinase (PI3K) → protein kinase B (AKT) → phosphodiesterase 3B (PD3B). In agreement with trese findings, growing literature data suggest a cross talk between leptin and insulin receptor signaling. These investigations demonstrated that leptin and insulin could show additive [1, 13] or not additive [15, 19, 22] effects. However, these studies were performed using tissue lysates [22] culture of the isolated rat hepatocytes [1, 23], hepatoma cell line [13, 19] and hepatic cell line BRL [5].

On the other hand, the perfusion of liver *in situ* permits to study the effect of hormones with preserved physiologically relevant signaling pathways. Thus, by using the *in situ* perfused liver, we have shown that leptin at physiological concentrations inhibits glucagon-induced hepatic glycogen catabolism and gluconeogenesis [8]. In agreement with our results, Nemecz et al. [18] found that leptin also suppressed the activation of glycogen catabolism promoted by epinephrine.

Since a reduction of adenosine-3'-5'-cyclic monophosphate (cAMP) content is consistent with the above-described results [8, 18], we decided to compare the effect of leptin and insulin on the activation of hepatic glycogen breakdown and gluconeogenesis promoted by cAMP and analogues.

MATERIALS and METHODS

Experimental animals

Male Wistar rats weighing 200–220 g were used. The experiments on animals followed the Brazilian Law on the protection of animals. The rats were maintained at constant temperature (23°C) with automatically controlled photoperiod (12 h light/12 h dark). Standard laboratory diet (Nuvital®) was given to the animals.

The study of the glycogen catabolism, i.e. hepatic glucose production (HGP) and glycogenolysis was performed on the livers from rats with free access to food. On the other hand, gluconeogenesis was investigated on the livers from rats fasted for 24 h.

Materials

Recombinant mouse leptin (r-MuLeptin, purity > 95% by SDS-PAGE and HPLC analyses) was purchased from Peprotech Mexico S.A. Aliquots of the reconstituted leptin were stored in the deep-freeze at –70°C and thawed immediately before experiments. cAMP, 8-bromoadenosine-cAMP (8-Br-cAMP) and N^6-monobutyryl-cAMP (N^6-MB-cAMP) were obtained from Biolog. Life Science Institute, Germany. All others reagents were of the highest available purity.

Liver perfusion technique

Hemoglobin-free, monovascular, anterograde liver perfusion was performed. The major convenience of using the liver perfusion technique is that the effects of leptin and insulin can be determined immediately. In addition, this technique has the advantage of determining metabolite release rates directly from the effluent perfusate in the intact organ. The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (35 mg/kg) and submitted to laparotomy. The livers were perfused *in situ* using Krebs/Henseleit-bicarbonate buffer, pH 7.4, saturated with an oxygen/carbon dioxide mixture (95/5%). The perfusion fluid was pumped through a temperature regulated (37°C) membrane oxygenator prior to entering the liver *via* a cannula inserted into the portal vein. The perfusion was performed in an open system, without recirculation of the perfusate [20]. The viability of the liver during the perfusion was indicated by the absence of any leaking and/or tissue swelling. A constant flow rate in each individual experiment was adjusted according to the liver weight (4 ml/g of tissue fresh weight/min).

Hepatic glycogen catabolism

After 10 min of liver perfusion, 3 μM cAMP, 0.3 μM 8-Br-cAMP or 0.3 μM N^6-MB-cAMP were dissolved in the perfusion fluid and infused be-

tween the 10–30 min of the perfusion period, followed by a period of post-infusion (20 min) to allow the values to return to basal levels. Samples of the effluent perfusion fluid were collected at 2-min intervals and the levels of glucose [3], pyruvate [10] and L-lactate [11] were analyzed. Thus, the activation of glucose, L-lactate and pyruvate production were measured as the difference between the rates of release of these substances during (10–30 min) and before (0–10 min) the infusion of cAMP, 8-Br-cAMP or N^6-MB-cAMP. The differences permitted to obtain and compare the areas under the curves (AUC) for all investigated groups. The effect of these cyclic nucleotides was investigated in the presence of leptin (10 ng/ml) or insulin (20 μU/ml).

Glycogenolysis was calculated by the sum of glucose plus the half-sum of L-lactate and pyruvate [glucose + 1/2 (L-lactate + pyruvate)]. Since pyruvate oxidation, pentose-monophosphate shunt and recycling of pyruvate to glucose are minimal, the release of these metabolites provides the rate of glycogenolysis [14].

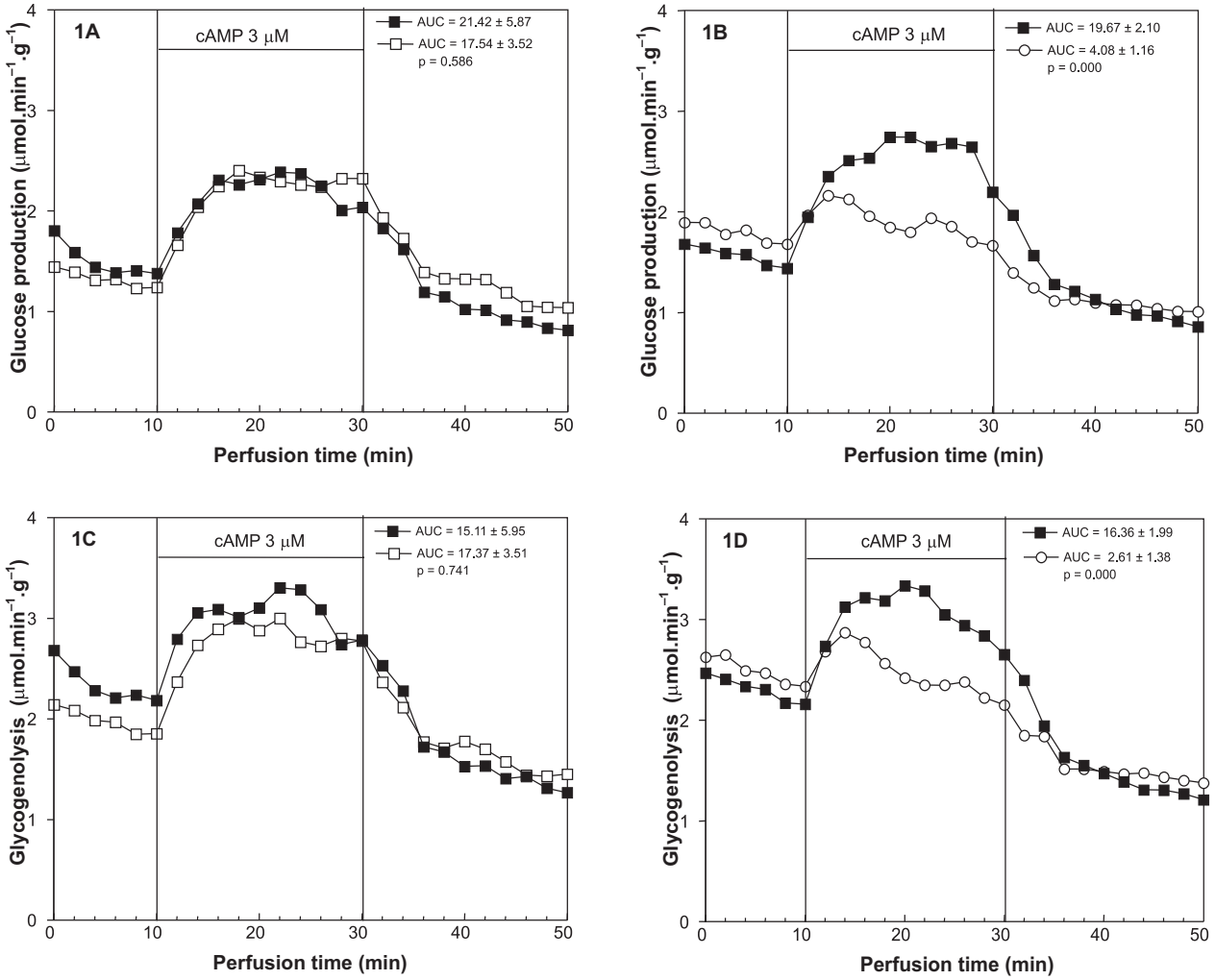

Fig. 1. Effect of insulin (20 μU/ml) or leptin (10 ng/ml) on the activation of hepatic glucose production (A and B) and glycogenolysis (C and D) promoted by adenosine-3'-5'-cyclic monophosphate (cAMP). Isolated perfused livers from fed rats were employed. All substances were infused between 10 and 30 min of the perfusion period. (■) Infusion of cAMP alone; (□) combined infusion of cAMP and leptin; (○) combined infusion of cAMP and insulin. The results were calculated as the means ± SEM of 4–6 individual liver perfusion experiments. The values of SEM are not shown for clarity. The livers were perfused as described in Materials and Methods

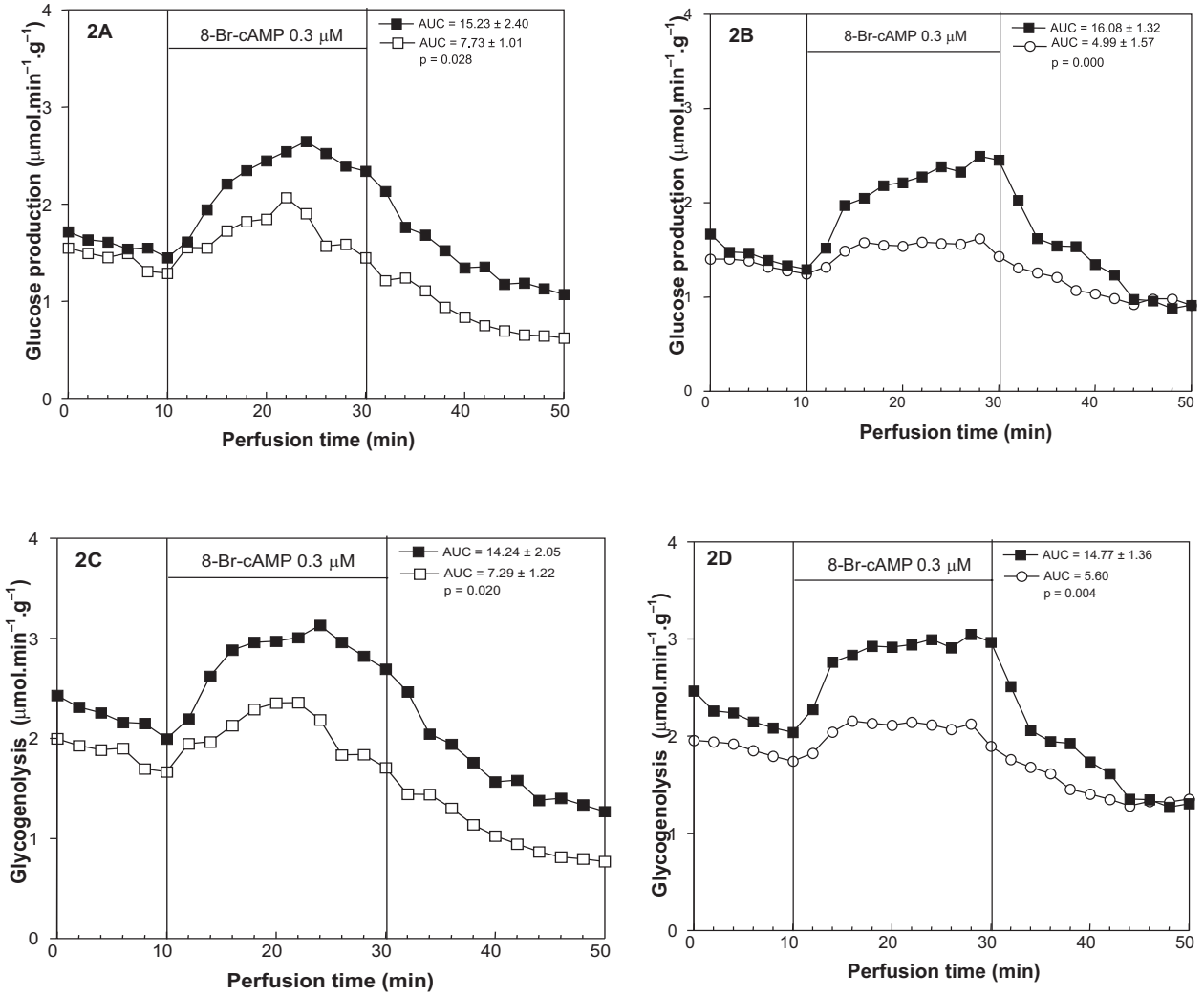

Fig. 2. Effect of insulin (20 μU/ml) or leptin (10 ng/ml) on the activation of hepatic glucose production (A and B) and glycogenolysis (C and D) promoted by 8-bromoadenosine-3'-5'-cyclic monophosphate (8-Br-cAMP). Isolated perfused livers from fed rats were employed. All substances were infused between 10 and 30 min of the perfusion period. (■) Infusion of 8-Br-cAMP alone; (□) combined infusion of 8-Br-cAMP and leptin; (○) combined infusion of 8-Br-cAMP and insulin. The results were calculated as the means ± SEM of 6–9 individual liver perfusion experiments. The values of SEM are not shown for clarity. The livers were perfused as described in Materials and Methods

Hepatic gluconeogenesis from L-alanine

In order to evaluate the effect of insulin and leptin on the activation of gluconeogenesis from L-alanine promoted by cAMP, the isolated livers from fasted rats were used. The minimal concentration of cAMP, i.e. 7.5 μM, which promoted stable activation of hepatic gluconeogenesis from L-alanine was employed. L-alanine (5 mM) was infused between 10th and 90th min of the perfusion period. cAMP, alone or combined with leptin (10 ng/ml or 50 ng/ml) or insulin (20 μU/ml), was infused between 30th and 90th min of the perfusion period. Samples of the effluent perfusion fluid were collected at 2-min intervals for a total collection period of 90 min. The differences in the glucose production during (30–90 min) and before (10–30 min) the infusion of cAMP permitted to obtain the rate of gluconeogenesis for all investigated groups.

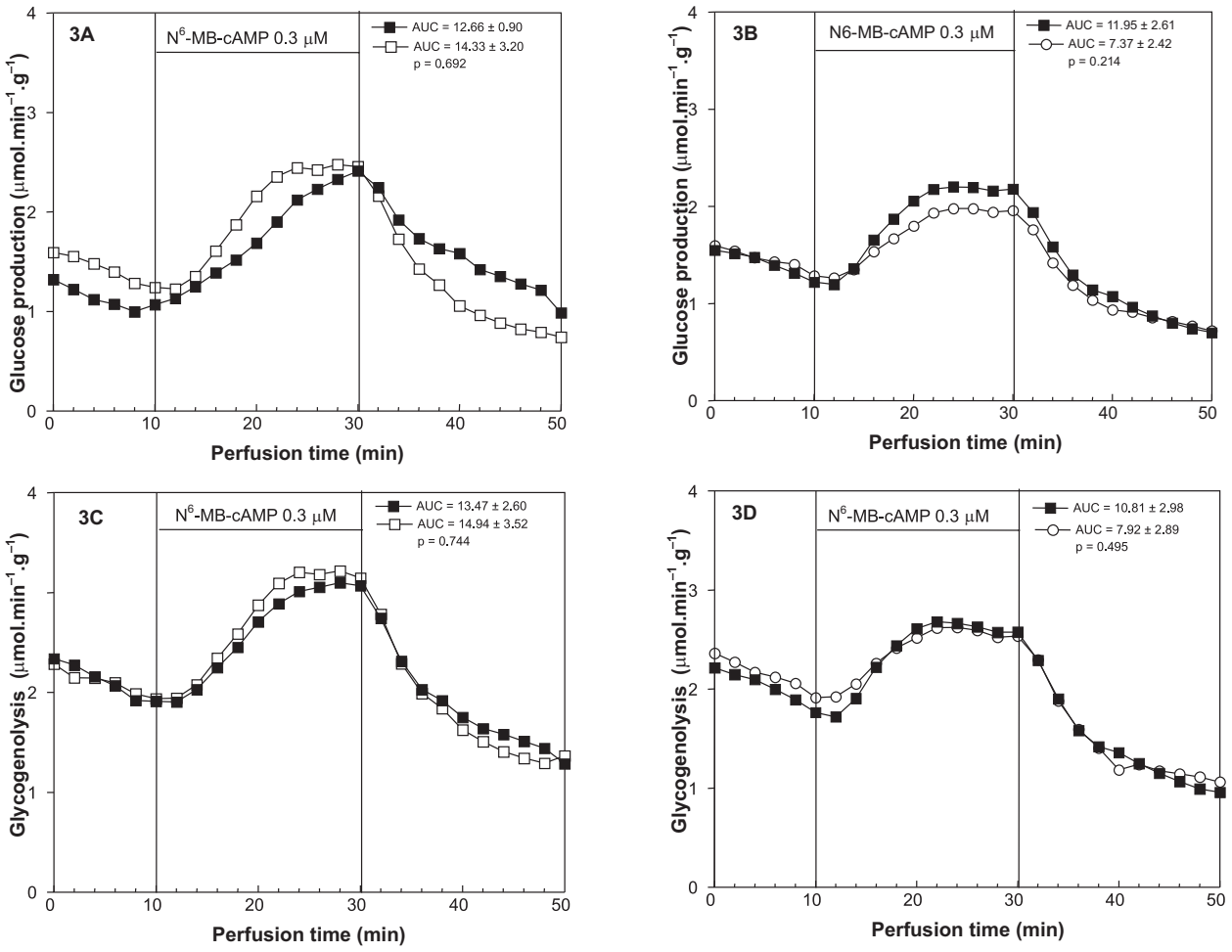

Fig. 3. Effect of insulin (20 μU/ml) and leptin (10 ng/ml) on the activation of hepatic glucose production (A and B) and glycogenolysis (C and D) promoted by N^6-monobutyryl-adenosine-3'-5'-cyclic monophosphate (N^6-MB-cAMP). Isolated perfused livers from fed rats were employed. All substances were infused between 10 and 30 min of the perfusion period. (■) Infusion of N^6-MB-cAMP alone; (□) combined infusion of N^6-MB-cAMP and leptin; (○) combined infusion of N^6-MB-cAMP and insulin. The results were calculated as the means ± SEM of 4–8 individual liver perfusion experiments. The values of SEM are not shown for clarity. The livers were perfused as described in Materials and Methods

Statistical analysis

All metabolic measurements were expressed as μmol/min/g of liver fresh weight and area under curves as μmol/g of liver fresh weight.

The program GraphPad Prism (version 2.0) was used to calculate the AUC. Data were analyzed statistically by the unpaired Student's *t*-test. The level of significance was set at $p < 0.05$. Results are reported as means ± SEM.

RESULTS

Since the intracellular levels of cAMP and analogues are proportional to their extracellular concentrations used in the liver perfusion experiment [20], the role played by cAMP was examined by using analogues that are metabolized at different rates.

The rates of HGP and glycogenolysis were increased by the rising cAMP concentrations as fol-

lows: 3 μM < 15 μM ≈ 30 μM ≈ 60 μM (results not shown). Thus, we employed the concentration of cAMP that promoted a minimal and stable activation of HGP and glycogenolysis, i.e. 3 μM. We observed lower ($p < 0.05$) cAMP-induced activation of HGP and glycogenolysis (Fig. 1B,D) in the presence of insulin (20 μU/ml). However, contrary to insulin, leptin (10 ng/ml) showed no effect on the activation of HGP and glycogenolysis induced by cAMP (Fig. 1A, C).

8-Br-cAMP is more resistant to hydrolysis by PDE3B than cAMP [4]. Thus, as expected, the level of 8-Br-cAMP that promoted a minimal and stable activation of HGP and glycogenolysis was lower (i.e. 0.3 μM) than that obtained with cAMP (results not shown). Nevertheless, differently than cAMP, activation of HGP and glycogenolysis was found to be lower ($p < 0.05$) when 8-Br-cAMP was infused not only with insulin (Fig. 2B and 2D) but also with leptin (Fig. 2A and 2C). However, it has

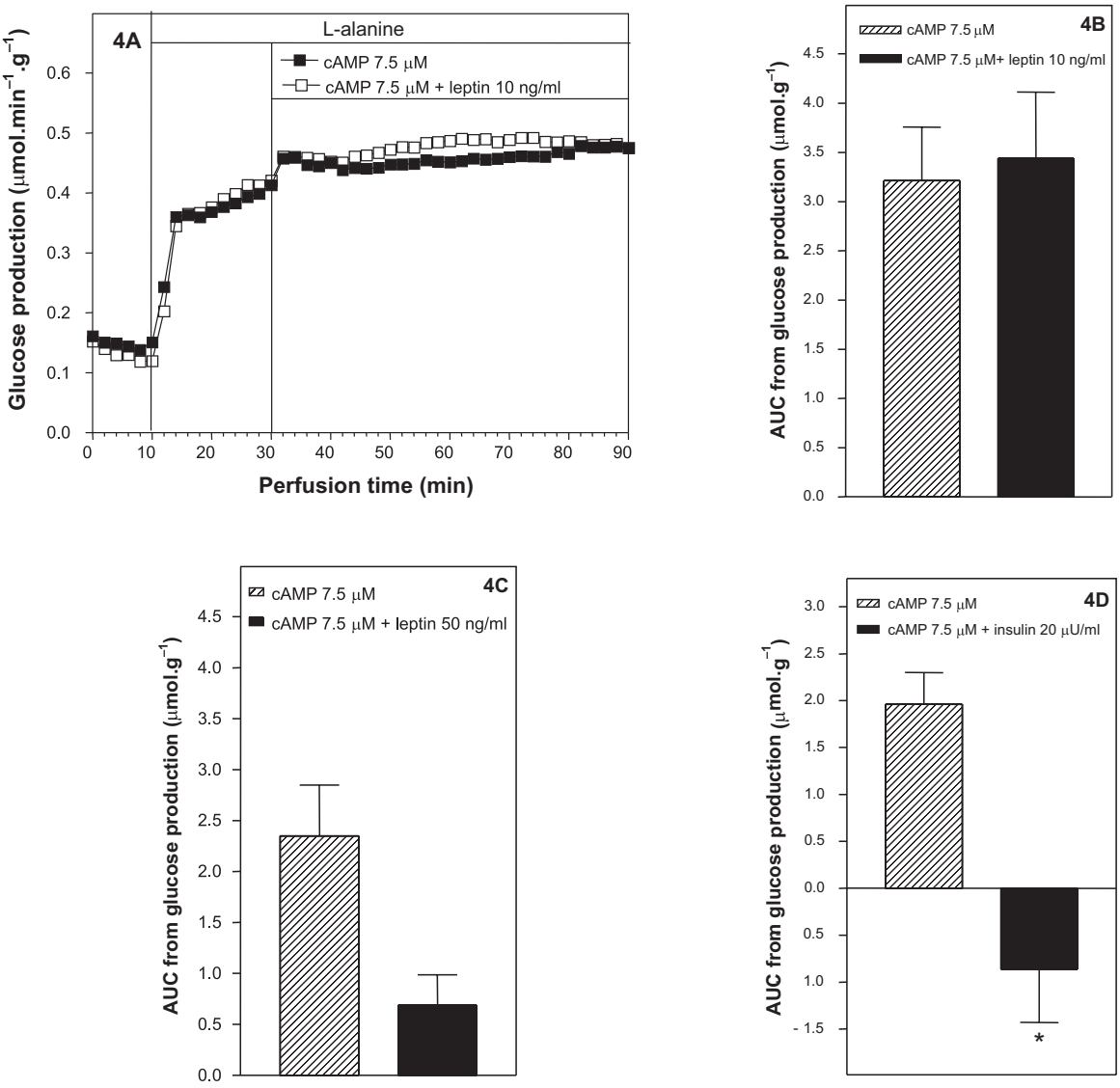

Fig. 4. Effect of insulin (20 μU/ml) and leptin (10 ng/ml or 50 ng/ml) on the activation of hepatic glucose production from L-alanine promoted by adenosine-3'-5'-cyclic monophosphate (cAMP). The isolated perfused livers from rats fasting for 24 h were employed. **4A** – Demonstrative experiment showing the activation of hepatic glucose production from L-alanine (5 mM) promoted by cAMP. **4B**, **4C**, **4D** – Areas under curves (AUC) calculated for the period between 30–90 min during the infusion of L-alanine + cAMP (4B,C,D); L-alanine + cAMP + leptin 10 ng/ml (4B), L-alanine + cAMP + leptin 50 ng/ml (4C) and L-alanine + cAMP + insulin 20 μU/ml. The results were calculated as the means ± SEM of 4–8 individual liver perfusion experiments. The values of SEM are not shown for clarity. The livers were perfused as described in Materials and Methods

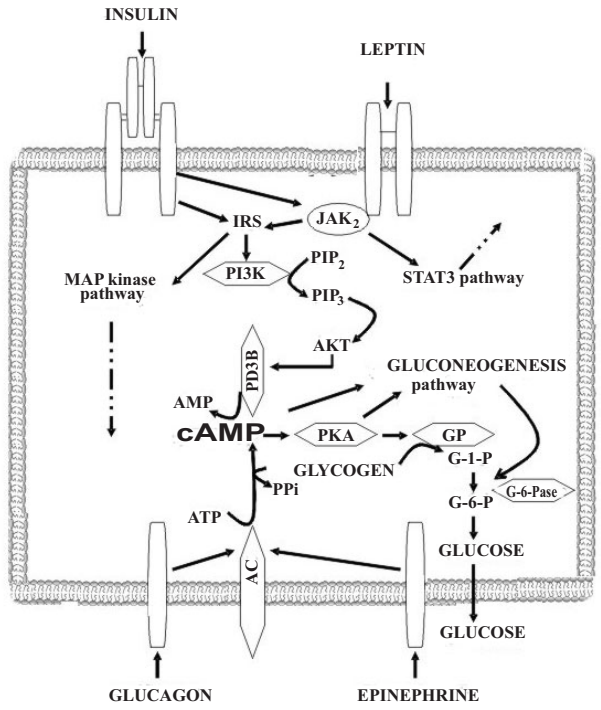

Fig. 5. Schematic representation of the interaction between leptin and insulin in the rat hepatocytes. Abbreviations: AC – adenyl cyclase; ATP – adenosine 5'-triphosphoric acid; AMP – adenosine monophosphate; cAMP – adenosine-3'-5'-cyclic monophosphate; GP – glycogen phosphorylase; G-1-P – glucose-1-phosphate; G-6-P - glucose-6-phosphate; G-6-Pase – glucose-6-phosphatase; IRS – insulin receptor substrate; MAP kinase – mitogen-activated protein kinase; JAK2 – janus kinase 2; PD3B – phosphodiesterase 3B; PKA – protein kinase A; AKT – protein kinase B; PI3K – phosphatidylinositol 3-kinase; PIP2 – phosphatidylinositol 4,5 biphosphate; PIP3 – phosphatidylinositol 3,4,5-triphosphate; STAT3 – activators of transcription-3

to be pointed out that the inhibition of the 8-Br-cAMP-induced activation of HGP and glycogenolysis was more intense in the presence of insulin (about 75%) than leptin (about 50%).

The participation of PDE3B in the effect of insulin and leptin on the activation of glycogen catabolism promoted by cyclic nucleotides was also investigated by replacing 8-Br-cAMP by N^6-MB--cAMP. Whereas 8-Br-cAMP is susceptible to hydrolysis by PDE3B, this enzyme does not degrade N^6-MB-cAMP. For comparative purposes, the same N^6-MB-cAMP concentration was used as in the experiments with 8-Br-cAMP, i.e. 0.3 μM. In contrast to 8-Br-cAMP, the activation of HGP and glycogenolysis promoted by N^6-MB-cAMP was not inhibited by leptin (Fig. 3A,C) or insulin (Fig. 3B, D).

The effect of physiological (10 ng/ml) and supraphysiological (50 ng/ml) levels of leptin and physiological levels of insulin (20 μU/ml) on the activation of gluconeogenesis from L-alanine (5 mM) promoted by cAMP (7.5 μM) was then investigated. Insulin at 20 μU/ml (Fig. 4D) and leptin at 50 ng/ml (Fig. 4C) but not leptin at 10 ng/ml (Fig. 4A, B) inhibited ($p < 0.05$) the cAMP-induced activation of hepatic gluconeogenesis from L-alanine.

DISCUSSION

In the liver, the binding of leptin to its receptor induces activation of janus kinase 2 (JAK2), receptor dimerization, and JAK2-mediated phosphorylation of the intracellular portion of the receptor. JAK2 activation triggers signaling *via* IRS-1 and IRS-2 which promotes activation of downstream targets including PI3K. Active PI3K phosphorylates phosphatidylinositol-4,5-diphosphate to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3, in turn, recruits and activates further downstream molecules, such as AKT and PDE3B, the main cAMP-degrading enzyme in the liver [6]. Therefore, although leptin and insulin receptors are structurally distinct, they share many similarities in their signal transduction pathways. In agreement with the molecular mechanism of action, several studies have shown an interaction between leptin and insulin effects [2, 6, 7, 9, 16, 17].

The results presented herein show that leptin is less effective than insulin to inhibit the activation of glycogen catabolism promoted by cAMP (Fig. 1) and 8-Br-cAMP (Fig. 2).

The metabolism of cAMP by PDE3B may explain the observed differences. N^6-MB-cAMP is a cAMP analogue in which one of the hydrogen atoms of the amino group in position 6 of the purine nucleobase is replaced by a butyrate group. This modification of the natural molecule of cAMP creates a membrane permeant activator of cAMP-dependent protein kinases that is not degraded by PDE3B [12]. Therefore, if the influence of the PDE3B is overcome, the difference obtained with cAMP (substrate to PDE3B) and 8-Br-cAMP (partially degraded by PDE3B) is abolished.

The pivotal role of cAMP levels in integrating signal transduction pathways from leptin and insulin receptors on the glycogen catabolism can be ex-

tended to gluconeogenesis. Thus, differently of insulin only supraphysiological levels of leptin were able to overcome the activation of gluconeogenesis from L-alanine promoted by cAMP (Fig. 4).

Considering that the effect of insulin to inhibit the activation of glycogen catabolism and gluconeogenesis promoted by cAMP was more pronounced than that of leptin, we can suggest that the process of activation of IRS-1 and IRS-2/PI3K which promotes activation of downstream targets, including PDE3B, is more sensitive to insulin than to leptin.

In agreement with our results, it has been shown that leptin is much less effective than insulin in stimulating IRS pathways. For example, PI3K activity associated with IRS-1 and IRS-2 and AKT activity in lysates from the liver, adipose tissue and muscles showed higher responsiveness to insulin than to leptin [22].

The results presented herein led us to postulate that the convergent signaling pathways of these two hormones causing the inhibition of glycogen catabolism and gluconeogenesis in the liver involve a reduction of intracellular cAMP. Thus, cAMP levels may play an important role in the cross talk between both hormones and for the insulin-like effects of leptin (Fig. 5). Further studies are, however, necessary to clarify the complex interplay and the potential points of cross talk between the intracellular signaling mechanisms stimulated by both leptin and insulin in the liver.

Acknowledgment. Research supported by CNPq, CAPES and PRONEX (168/97), Brazil.

REFERENCES

1. Aiston S, Agius L: Leptin enhances glycogen storage in hepatocytes by inhibition of phosphorylases and exerts an additive effect with insulin. Diabetes, 1999, 48, 15–20.
2. Barzilai N, She L, Liu BQ, Vuguin P, Cohen P, Wang J, Rossetti L: Surgical removal of visceral fat reverses hepatic insulin resistance. Diabetes, 1999, 48, 94–98.
3. Bergmeyer HU, Bernt E: Determination of glucose with glucose-oxidase and peroxidase. In: Methods of Enzymatic Analysis, Ed. Bergmeyer HU, New York, 1974, 1205–1215.
4. Campagne MMVL, Diaz FV, Jastorff B, Winkler E, Genieser HG, Kessin RH: Characterization of the yeast low Km cAMP-phosphodiesterase with cAMP analogues. Applications in mammalian cells that express the yeast PDE2 gene. J Biol Chem, 1990, 265, 5847–5854.
5. Cao X, Fu Z, Ming W, Yang R, Cheng H: Effects of acute or prolonged exposure to leptin on hepatic glucose oxidation. Chin Med J, 2001, 114, 592–595.
6. Carvalheira JB, Ribeiro EB, Folli F, Velloso LA, Saad MJ: Interaction between leptin and insulin signaling pathways differentially affects JAK-STAT and PI 3-kinase-mediated signaling in rat liver. Biol Chem, 2003, 384, 151–159.
7. Ceddia RB, Koistinen HÁ, Zierath JR, Sweeney G: Analysis of paradoxical observations on the association between leptin and insulin resistance. FASEB J, 2002, 16, 1163–1176.
8. Ceddia RB, Lopes G, Souza HM, Borba-Murad GR, Willian WNJr, Bazotte RB, Curi R: Acute effects of leptin on glucose metabolism of *in situ* rat perfused livers and isolated hepatocytes. Int J Obesity, 1999, 23, 1207–1212.
9. Cohen B, Novick D, Rubinstein M: Modulation of insulin activities by leptin. Science, 1996, 274, 1185–1188.
10. Czok R, Lamprecht W: Pyruvate, phosphoenolpyruvate and D-glycerate-2-phosphate. In: Methods of Enzymatic Analysis, Ed. Bergmeyer HU, New York, 1974, 1446–1448.
11. Gutmann I, Wahlefeld W: L-(+)-Lactate. Determination with lactate dehydrogenase and NAD. In: Methods of Enzymatic Analysis, Ed. Bergmeyer HU, New York, 1974, 1464–1472.
12. Hermsdorf T, Dettmer D: Combined effects of insulin and dexamethasone on cyclic AMP phosphodiesterase 3 and glycogen metabolism in cultured rat hepatocytes. Cell Signal, 1998, 10, 629–635.
13. Kaser S, Foger B, Ebenbichler CF, Kirchmair R, Gander R, Ritsch A, Sandhofer A et al.: Influence of leptin and insulin on lipid transfer proteins in human hepatoma cell line, HepG2. Int J Obesity, 2001, 25, 1633–1639.
14. Kimmig R, Mauch TJ, Kerzl W, Schwabe U, Scholz R: Actions of glucagon on flux rates in perfused rat liver. 1. Kinetics of the inhibitory effect on glycolysis and the stimulatory effect on glycogenolysis. Eur J Biochem, 1983, 136, 609–616.
15. Lamosova D, Zeman M: Effect of leptin and insulin on chick embryonic muscle cells and hepatocytes. Physiol Res, 2001, 50183–50189.
16. Li M, Zhan Z, Wu C, Yang J, Zhang K, Li X, Gan L: The relationship between serum leptin, proinsulin, true insulin levels and insulin resistance in non-diabetic subjects: a population-based study. Zhonghua Nei Ke Za Zhi, 2002, 41, 754–757.
17. Muoio DM, Lynis Dohm G: Peripheral metabolic actions of leptin. Best Pract Res Clin Endocrinol Metab, 2002, 16, 653–666.
18. Nemecz M, Preininger K, Englisch R, Furnsinn C, Schneider B, Waldhausl W, Roden M: Acute effect of leptin on hepatic glycogenolysis and gluconeogenesis in perfused rat liver. Hepatology, 1999, 29, 166–172.
19. Szanto I, Kahn CR: Selective interaction between leptin and insulin signalling pathways in a hepatic cell line. Proc Natl Acad Sci USA, 2000, 97, 2355–2360.

20. Vardanega-Peicher M, Curi R, Pagliarini e Silva S, Nascimento K.F, Bazotte R.B: Responsiveness of glycogen breakdown to cyclic AMP in perfused liver from rats with insulin-induced hypoglycemia. Braz J Med Biol Res, 2003, 36, 45–51.
21. William WN Jr, Ceddia RB, Curi R: Leptin controls the fate of fatty acids in isolated rat white adipocytes. J Endocrinol, 2002, 175, 735–44.
22. Young-Bum K, Uotani S, Pierroz DD, Flier JS, Kahn BB: In vivo administration of leptin activates signal transduction directly in insulin-sensitive tissues: overlapping but distinct pathways from insulin. Endocrinology, 2000, 141, 2328–2338.
23. Zhao AZ, Shinohara MM, Huang D, Shimizu M, Eldar-Finkelman H, Krebs EG et al.: Leptin induces insulin-like signalling that antagonizes cAMP elevation by glucagon in hepatocytes. J Biol Chem, 2000, 275, 11348–11354.

Received: October 14, 2003; in revised form: February 12, 2004.