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Evidence for release of free glucose from muscle during amylin-induced glycogenolysis in rats

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Amylin, a 37 amino acid product of pancreatic β-cells, inhibits insulin-stimulated radioglucose incorporation into muscle glycogen. In the current study, we exercised rats and then prelabelled the glycogen pool by infusing [3-^{3}H]glucose along with glucose and insulin. Subsequent amylin administration increased the rate of appearance of tritiated water 6.4-fold, consistent with stimulation of glycogenolysis and passage of the released moieties through the hexose → triose step of glycolysis. Further, there was an increase in plasma [3-^{3}H]glucose after amylin, consistent with the release of free glucose previously sequestered in muscle glycogen. Calcitonin gene-releated peptide (8–37), an amylin antagonist, prevented these actions.

Amylin; Muscle glucogenolysis; Cori cycle; Free glucose

1. INTRODUCTION

Amylin [1] is a 37 amino acid hormone synthesized in the pancreatic β-cells which is co-secreted with insulin in response to nutrient stimuli [2]. In vitro, amylin has been shown to inhibit insulin-stimulated incorporation of radiolabelled glucose into glycogen in isolated skeletal muscle [3,4]. Amylin was subsequently shown to both inhibit muscle glycogen synthase [5,6] and activate muscle glycogen phosphorylase. In contrast, the evidence from isolated perfused liver studies indicates that amylin does not have the glucagon-like effect of activating liver glycogen phosphorylase [7,8]. Amylin administration during euglycemic clamp procedures in intact rats acutely diminishes insulin-stimulated glucose disposal [9] and increases endogenous glucose production [10,11]. Free infusions of amylin in rats result in hyperlactemia and hyperglycemia. Calcitonin gene-related peptide (CGRP), which is ~50% homologous to amylin [1], shares amylin's metabolic actions on muscle, and the (8–37) fragment of CGRP has been shown to block these actions when amylin is used as an agonist [12].

Some authors have concluded that the acute in vivo insulin resistance induced by amylin (or CGRP) administration is due to inhibition of glycogen synthesis [13–15]. However, by measuring the incorporation of radiolabelled glucose into glycogen (as was done in those studies) it is not possible to distinguish between inhibition of glycogen synthesis and activation of glycogenolysis. Differences between (i) the rate of incorporation of labelled glucose into previously unlabelled glycogen and (ii) net glycogen formation may occur because the former is necessarily greater than or equal to zero, while the latter may be either positive or negative [4]. The present study examined the ability of amylin to activate glycogenolysis by prelabelling the muscle glycogen pool in rats with [3-^{3}H]glucose and by observing the rate of appearance of tritiated water in plasma. The appearance of this label in plasma reflects labelled glucosyl moieties being liberated from glycogen and passing through the hexose → triose step in glycolysis, where the ^{3}H is transferred to water. We also measured the appearance of tritiated glucose in plasma as a measure of the liberation of intact glucose molecules. Additionally, the effect of $^{8-37}$CGRP, which has been shown to be an effective amylin antagonist in vitro and in vivo [12], was tested for its ability to inhibit the observed effects of amylin.

2. MATERIALS AND METHODS

2.1. *Animals*

Male Harlan–Sprague–Dawley rats (weight 352 ± 85 g, age 87 ± 4 days) were housed at 22.7 ± 0.8°C in a 12:12 h light/dark cycle, experiments being performed during the light cycle. They were fed Teklad Diet LM-485 until the day before the experiments and were watered ad libitum.

2.2. *Procedures*

Rats fasted for 20.1 ± 0.2 h were vigorously exercised by one hour of swimming in tepid water to cause a general depletion of muscle glycogen. They were then anesthetized with halothane, tracheotomized, and cannulated via the femoral artery and saphenous vein. Body temperature was maintained by a heated operating table switched by a thermoregulator in response to colonic temperature. Mean arterial pressure was monitored and blood samples were taken via the arterial line. After a period of at least 90 min after surgery, at

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a time designated as $t = 0$ min, a 20 min infusion of a 3 ml solution containing 1 g (5.56 mmol) D-glucose, 0.5 mCi [3-^{3}H]glucose (New England Nuclear) and 300 mU of recombinant human insulin (Humulin-R, Eli Lilly, Indianapolis, IN) was begun. This regime was designed to rapidly replete muscle glycogen with the infused label. At $t = 20$ min, when the infusion stopped, the decay of labelled and total plasma glucose was followed until $t = 120$ min. At 120 min, rats received one of three treatments:

(1) An intravenous bolus of 0.1 ml of 0.15 M saline (controls) ($n = 5$).
(2) An intravenous bolus of 0.1 mg of synthetic rat amylin (Bachem, Torrance, CA) dissolved in 0.1 ml of saline ($n = 5$).
(3) An intravenous bolus of 0.1 mg of synthetic rat amylin dissolved in 0.1 ml of saline, as above, but preceeded by a 0.5 mg (in 0.25 ml) then 1.0 mg (per ml)/h primed/continuous infusion of $^{8\text{-}37}$hCGRP (Bachem, Torrance, CA; Lot ZH425) in saline, beginning at $t = 90$ min and continuing until $t = 240$ min.

2.3. *Analyses*

Arterial samples of 125 μl were collected at 30 min intervals for determination of total plasma glucose and lactate using immobilized enzyme chemistries (YSI 2300-Stat Analyzer, Yellow Springs, OH). Samples of 50 μl were collected at 30 min intervals for determination of plasma ^3H_2O and [3-^{3}H]glucose. Samples were deproteinized by perchloric acid precipitation then aliquots β-counted before and after evaporation to dryness [16]. Non-volatile counts remaining after evaporation were assumed to represent [3-^{3}H]glucose while the volatile counts lost during evaporation were assumed to represent ^3H_2O. At the end of the experiment, animals were sacrificed and livers were frozen for subsequent determination of glycogen content. Whole livers were powdered while frozen. The glycogen from ~ 200 mg of such powder was digested in hot KOH and precipitated in ethanol as previously described [4]. After drying, pellets were neutralized with 0.2 ml perchloric acid, resuspended into 1 ml of acetate buffer and the glycogen digested with 18.5 U/ml of amyloglucosidase (EC5 3.2.1.3, from *Aspergillus niger*, Sigma A7420, Sigma Chemical Company, St Louis, MO) for at least 15 min at 23°C. The supernatants were assayed for glucose in an analyser using D-glucose oxidase immobilized enzyme chemistry (Analyzer model 2300-STAT, YSI, Yellow Springs, OH). Purified rabbit liver glycogen (Sigma G4011) was used as a calibration standard. The assay was linear within the range of observed liver glycogen concentrations ($r = 0.9994$). All reagents were of analytical grade or better.

2.4. *Numerical Methods*

Rates of generation (or decay) of ^3H_2O or [3-^{3}H]glucose were derived over the 30 min sampling periods. Pairwise comparisons were performed using *t*-test routines contained within the STATS module of SYSTAT [17]. Measures of central tendency were means ± S.E.M., and unless otherwise stated, $P = 0.05$ is used as the level of significance.

3. RESULTS

Fig. 1 shows the change in total plasma glucose for the control and amylin treated groups. With the 20 min labelled glucose + insulin infusion, there was an increase in glucose to around 13–17 mM by the end of the infusion. Glucose then declined to normal levels (4–7 mM) by $t = 120$ min. When saline was infused at 120 min, plasma glucose rose slowly, commensurate with the responses observed previously in halothane anesthetized control animals [18]. When amylin was infused at 120 min, plasma glucose increased rapidly (within 30 min) and remained significantly elevated above control values for the next 2.5 h.

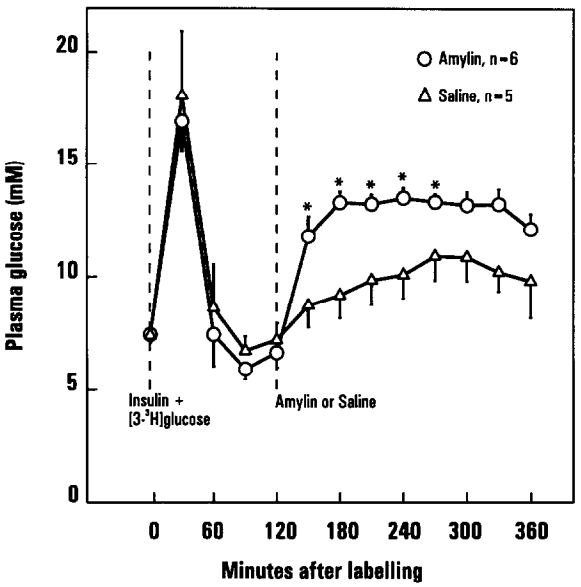

Fig. 1. Plasma glucose levels following the injection of insulin + labelled glucose ($t = 0$ min) and of amylin ($t = 120$ min). The restoration of normal plasma glucose levels by $t = 120$ min is consistent with the labelled glucose being sequestered, presumably partly in muscle glycogen. Symbols are means ± S.E.M. and asterisks indicate significant differences between treatment groups ($P < 0.05$).

Fig. 2 shows the plasma lactate response in amylin- and saline-treated rats to the above procedures. All groups had increases in lactate during the hyperglycemic hyperinsulinemic period from 0 → 60 min consistent with previously reported lactate responses [19]. The increase in lactate was likely to have been a consequence of increased glucose- and insulin-mediated entry of glu-

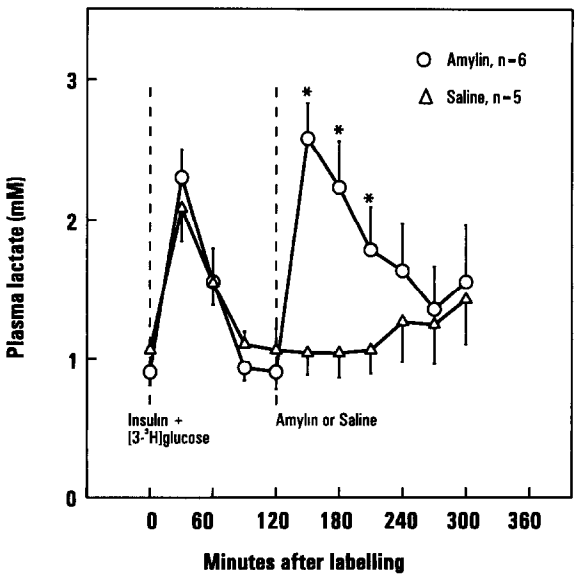

Fig. 2. Plasma lactate levels following the injection of insulin + labelled glucose ($t = 0$ min) and of amylin ($t = 120$ min). Lactate levels rose during hyperglycemia, consistent with insulin- and glucose-driven glycolysis, and again after amylin, consistent with a glycogenolytic drive of glycolysis. Symbols are as in Fig. 1.

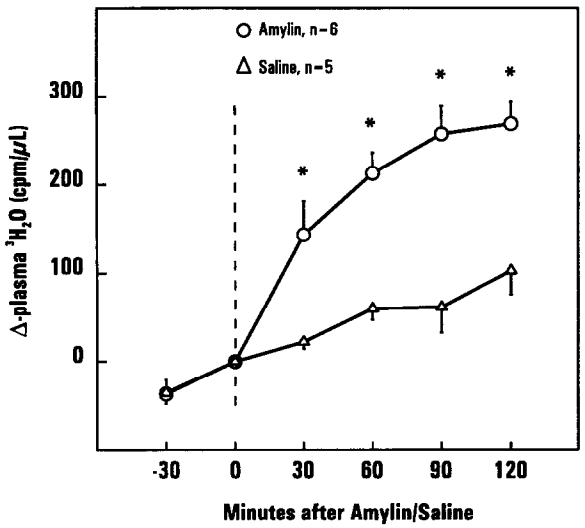

Fig. 3. Increment in plasma tritiated water after amylin injection. The increased appearance of tritiated water after amylin, resulting from a 6.4-fold increase in rate of its production, is consistent with amylin-mediated release of labelled glucosyls sequestered in muscle glycogen. Symbols are as in Fig. 1.

cose into cells, driving glycolysis to an extent that exceeded the rate of carbohydrate oxidation. Such an elevation of glycolysis is supported by the increased rate of tritiated water production during that period. Animals then injected with amylin at 120 min showed another increase in lactate, consistent with previous reports of amylin's separate effect on lactate [18,20].

The tritium counts assigned to plasma water are shown for amylin and saline injected rats in Fig. 3. Following the rapid generation of ^3H_2O during the period of hyperinsulinemia and hyperglycemia, rate of ^3H_2O production became more steady. At t = 120 min, saline injection did not appreciably change this rate. In contrast, amylin administration increased the rate of ^3H_2O generation 6.4-fold within 30 min of administration ($P < 0.02$).

The tritium counts assigned to [3-^{3}H]glucose are shown for amylin and saline injected rats in Fig. 4. As predicted, counts rose with the initial infusion and then declined, consistent with the labelled glucose either being metabolized through glycolysis, or being sequestered primarily as muscle glycogen. When amylin was administered at t = 120 min, there was a 35% increase in tritium assignable to plasma [3-^{3}H]glucose over the next 30 min. In the same period, plasma [3-^{3}H]glucose declined by 10% in control animals ($P < 0.02$).

Liver glycogen content was generally low (less than 5 mg/g), consistent with previous reports in fasted, glycogen depleted animals. Levels in amylin-treated rats were 2.8-fold higher than controls, but levels were highly variable and were not statistically different ($P = 0.1$).

$^{8-37}$hCGRP preinfusion inhibited all the observed actions of amylin. The amylin-induced change in plasma glucose and lactate was abrogated by $^{8-37}$hCGRP such that these responses were not measurably different from those of control animals, as previously reported [12]. Preinfusion of $^{8-37}$hCGRP reduced the amylin-induced acceleration in rate of appearance of plasma ^3H_2O by over 50% and suppressed the amylin-induced release of [3-^{3}H]glucose.

4. DISCUSSION

In the present study, we sought to follow the fate of labelled glucose moieties that could be incorporated into muscle glycogen. [3-^{3}H]glucose has the advantage that label is not lost during the futile cycling between glucose-6-phosphate and fructose-6-phosphate as occurs with [2-^{3}H]glucose [21]. It also has the advantage that the label is lost to water during the essentially irreversible hexose → triose step in glycolysis [21], so that the appearance of tritiated water is a measure of glycolytic rate [16]. Because the specific activity or distribution of label within the glycogen pool was unknown, it was not possible to quantitatively determine glycolytic or other fluxes. However, it was assumed that in groups of animals identically pretreated, the disposition of label within glycogen would be similar, so that qualitative inter-group comparisons could be made. When amylin was administered, there was a 6.4-fold increase in the rate of tritiated water production.

Glycogen synthesis and degradation appear to occur concurrently [22] so that net production or loss of glycogen reflects the balance of these two processes. Amylin-stimulation of cAMP in skeletal muscle [23,24] is likely

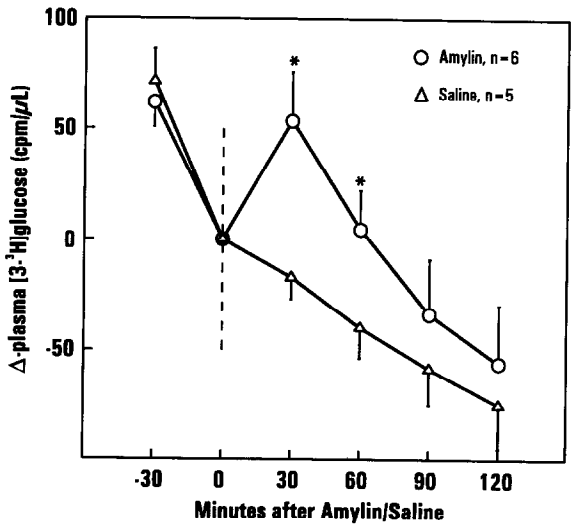

Fig. 4. Increment in plasma [3-^{3}H]glucose after amylin injection. Since it is irreversibly metabolized during glycolysis, the reappearance of [3-^{3}H]glucose in plasma after amylin injection is difficult to explain by a mechanism other than release from intracellular glycogen stores. Symbols are as in Fig. 1.

to underly its reciprocal inhibition of glycogen synthase [5] and activation of glycogen phosphorylase [6] and thus promote net loss of muscle glycogen. Inhibition of synthase, with consequent diversion of glucose-6-phosphate into glycolysis has previously been offered as an explanation of the increase in tritiated water evoked by CGRP in a preparation similarly preinfused with [3-^{3}H]glucose during hyperinsulinemia [15]. However, since amylin at the doses used in the present report inhibits glucose uptake by over 50%, and carbohydrate storage represents no more than about 70% of glucose disposal [25], it can be estimated that a diversion of glucose from carbohydrate storage into glycolysis could account for no more than a 1.7-fold increase in ^3H_2O generation; however, the observed amylin-evoked increase was 6.4-fold. On this basis we conclude that the increased generation of titriated water after amylin in these experiments was more a consequence of increased glycogenolysis providing labelled glucose to glycolysis than a consequence of decreased glycogen synthesis diverting incoming labelled glucose into the glycolytic pathway.

Other studies point to a dominant effect of amylin-stimulated glycolysis. [3-^{3}H]glucose is sequestered in muscle glycogen followed insulin stimulation [16], so amylin activation of phosphorylase (and inhibition of synthase) would flood the cell with labelled hexose monophosphates. Amylin-stimulation of cAMP would also activate phosphofructokinase via the fructose 2,6-bisphosphate regulatory mechanism, stimulating glycolysis and the observed generation of ^3H_2O and lactate. In regard to lactate production, isolated extensor digitorum longus muscles incubated with [U-^{14}C]glucose and exposed to CGRP (an amylin agonist) produced lactate whose specific activity was significantly diluted from that expected if it were derived from transported labelled glucose; but the dilution of specific activity fits with dilution of label by glucose moieties released from the endogenous glycogen pool [26]. Further, total enzymatic activity of glycogen phosphorylase is 12–85 times higher than that of glycogen synthase in muscle [27,28], so it is expected that changes in glycogenolytic flux will outweigh changes in glycogenic flux following near maximally effective stimuli (such as are likely to be present in the present experiments). These considerations do not preclude the possibility that in other conditions such as at lower amylin concentrations, the relative impact on glycogen synthesis and glycogen degradation could be different [15].

The data shown in Fig. 4 are surprising, in that they indicate amylin-stimulated release of free (labelled) glucose from a sequestered (non-plasma) pool. How could amylin administration produce free glucose? One possibility is that labelled glucose incorporated into liver glycogen could be released and pass through glucose-6-phosphatase to re-emerge from the liver as labelled glucose. However, amylin does not appear to have such action at the liver, as determined in isolated perfused preparations [7,8]. Indeed, liver glycogen measurements in the present study suggest the opposite – that there may have been a gain in liver glycogen during amylin administration. A similar gain in liver glycogen was observed by Cori et al. following peripheral glycogenolysis after epinephrine administration [29]. An alternative explanation is that the labelled glucose reappearing in plasma was derived from muscle glycogen. Release of free glucose from muscle has been proposed to occur through the following mechanism [30]. Although the glycogen chain consists mainly of α-1,4 glycosidic linkages, about 8–10% of glucosyls are represented as α-1,4 α-1,6 branches. While the straight chain linkages are broken by glycogen phosphorylase and phosphorylated, the branch glycosyl requires a separate debranching enzyme system [31]. The release of such a moiety consumes water and releases free glucose. Since intracellular glucose-6-phosphate is high during amylin-induced glycogenolysis [9], hexokinase is likely to be inhibited. Because its phosphorylation was inhibited and its rate of production was high, free intracellular glucose could accumulate. Glucose transporters are able to transport in either direction, so the intracellular glucose generated from glycogen debranching could result in net glucose efflux from muscle.

The contribution of muscle glycogenolysis, whether induced by amylin, exercise, catecholamines, hypoxia, or other mechanisms, to endogenous glucose production warrants further consideration.

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