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# INSULIN ALTERS THE SENSITIVITY OF GLYCOGEN SYNTHESIS TO INHIBITION BY OKADAIC ACID, A PROTEIN PHOSPHATASE INHIBITOR

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**Abstract**—We investigated the interactions of insulin and okadaic acid, an inhibitor of protein phosphatases type-2A and type-1, on glycogen synthesis in rat, guinea pig and rabbit hepatocytes. Insulin stimulated glycogen synthesis in rat and guinea pig but not in rabbit hepatocytes. In rat and guinea pig hepatocytes, the stimulation of glycogen synthesis by insulin was inhibited by low concentrations of okadaic acid (2.5–5 nM), which did not inhibit glycogen synthesis in the absence of insulin. In rabbit hepatocytes, insulin increased the sensitivity of glycogen synthesis to inhibition by low concentrations of okadaic acid even though it did not stimulate glycogen synthesis, and in the presence of insulin and okadaic acid (5 nM) glycogen synthesis was significantly lower than in the presence of okadaic acid alone. An increase in extracellular pH from 7.4 to 7.8 in a bicarbonate-free buffer, decreased the concentration of okadaic acid causing half-maximal inhibition of glycogen synthesis. It is suggested that an increase in cytosolic pH may be one mechanism by which insulin alters the sensitivity of glycogen synthesis to phosphatase inhibition.

Insulin alters the phosphorylation state of serine residues of several enzymes involved in the control of carbohydrate and fatty acid metabolism [1] and control of serine kinases and phosphatases by insulin is presumed to be an important mechanism in mediating the metabolic effects [1]. Four types of protein phosphatases designated types -1, -2A, -2B and -2C, account for most of the serine phosphatase activity in eukaryotic cells [2]. The involvement of protein phosphatases type-1 and type-2A in cellular events can be identified from their inhibition by okadaic acid [3] a potent inhibitor of protein phosphatases type-2A ($IC_{50}$ 0.05–2 nM) and type-1 ($IC_{50}$ 10–20 nM) [4].

Recent studies have reported that okadaic acid inhibits the effects of insulin on glucose transport [5] and fatty acid synthesis and activation of acetyl-CoA carboxylase and pyruvate dehydrogenase [6, 7] in rat adipocytes [6, 7] and on glycogen synthesis in hepatoma cells [7] and rat hepatocytes [8]. The inhibition by okadaic acid of the stimulation of glucose transport by insulin may be due to inhibition of a step prior to recruitment of glucose transporters to the cell surface [5], inhibition of fatty acid synthesis has been attributed to increased phosphorylation and inactivation of acetyl-CoA carboxylase [7] while inhibition of the activation of pyruvate dehydrogenase may be by an indirect mechanism [7]. In rat hepatocytes the stimulation of glycogen synthesis by insulin is blocked by low concentrations of okadaic acid to which type-2A phosphatases are sensitive, suggesting the involvement of these phosphatases in insulin action [8]. We demonstrate in this study that there are species differences in the extent by which insulin stimulates glycogen synthesis in hepatocyte monolayers from rat, guinea pig and rabbit liver, and that insulin increases the sensitivity of glycogen synthesis to inhibition by okadaic acid in situations where it does not increase glycogen synthesis. This increased inhibition by okadaic acid in the presence of insulin suggests that insulin alters the characteristics of the sensitivity to okadaic acid.

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† Abbreviations and enzymes: Glycogen synthase, EC 2.4.1.11; glycogen phosphorylase, EC 2.4.1.1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; MEM, Minimum Essential Medium.

## MATERIALS AND METHODS

*Materials.* Insulin and collagenases (type IV) were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Okadaic acid was from Dr Y. Tsukitani (Fujisawa, Chemical Co., Tokyo, Japan) through a generous gift from Professor P. Cohen (Department of Biochemistry, University of Dundee, U.K.). [U-$^{14}$C]Glucose was from Amersham International (Amersham, U.K.). Sources of other reagents were as reported previously [9].

*Preparation of hepatocyte monolayers.* Hepatocytes were isolated by collagenase perfusion of the liver [10] from male Wistar rats (body wt 200–260 g), Dunkin–Hartley guinea pigs (body wt 300–400 g) or Dutch strain rabbits (1–1.2 kg body wt), that were fed on standard chow *ad lib*. The hepatocytes were suspended in Minimum Essential Medium (MEM†) containing 5% (v/v) foetal bovine serum and inoculated in Corning multiwell plates at a density of $6.4 \times 10^4$ cells/cm$^2$. After cell attachment the hepatocyte monolayers were cultured for 14–18 hr in serum-free MEM containing 10 nM dexamethasone [9]. Incubations for determination of the interactions

of okadaic acid and insulin were performed in MEM without dexamethasone after the 14–18 hr pre-culture [9].

*Metabolic studies.* In the experiments in Tables 1 and 2, glycogen deposition and incorporation of [U-$^{14}$C]glucose into glycogen in hepatocyte monolayers were determined during a 3 hr incubation with MEM containing 15 mM [U-$^{14}$C]glucose (1.5 $\mu$Ci/mL) and the concentrations of okadaic acid indicated in the absence or presence of 1 nM-insulin. In the experiments in Fig. 1, hepatocyte monolayers were incubated in MEM without bicarbonate, containing 20 mM Na-HEPES buffered at either pH 7.4 or 7.8 and supplemented with 20 mM glucose. Incubations were terminated as described previously [9] and cellular glycogen was determined enzymically [9] and the incorporation of [$^{14}$C]glucose into glycogen was determined by ethanol precipitation of the glycogen [9]. Glycogen deposition represents the incremental change in glycogen content during the 3 hr incubation and is expressed as nmol of glucosyl units/3 hr/mg of cell protein. The incorporation of [$^{14}$C]glucose into glycogen is expressed as nmol of glucose incorporated/3 hr/mg of cell protein. Protein was determined by an automated Lowry method [11]. The concentration of okadaic acid that causes 50% inhibition ($IC_{50}$) of glycogen deposition or of [$^{14}$C]glucose incorporation into glycogen was determined from plots of glycogen deposition or synthesis in the presence of okadaic acid (mean data) expressed as a percentage of the control values in the absence of okadaic acid against the concentration of okadaic acid. For glycogen deposition, the effect of 100 nM okadaic acid was taken as 100% inhibition of glycogen deposition for all species, even though this concentration caused net glycogenolysis in guinea pig and rabbit. The concentrations of okadaic acid used in this study did not cause cell damage as assessed from the activity of lactate dehydrogenase in the medium determined on termination of the incubations [8]. Results are expressed as means ± SEM, for the number of hepatocyte preparations indicated. Statistical analysis was by the paired *t*-test.

## RESULTS

The cell glycogen content after pre-culture of hepatocyte monolayers in MEM containing 5 mM glucose was lowest in rat hepatocytes and highest in rabbit hepatocytes (Table 1). During incubation with 15 mM glucose, rates of glycogen deposition were similar in rat and guinea pig hepatocytes but 5-fold higher in rabbit hepatocytes (Table 1). Insulin caused a 2-fold increase in glycogen deposition in rat and guinea pig hepatocytes but did not further increase the high rates in rabbit hepatocytes (Table 1). The incorporation of [$^{14}$C]glucose into glycogen showed similar trends in the three species (Table 2) as glycogen deposition determined enzymically (Table 1), indicating that glucose is the main substrate for glycogen synthesis under these conditions.

In the absence of insulin, 10 nM okadaic acid caused significant inhibition of glycogen synthesis (Tables 1 and 2; 64–74% inhibition in rat and guinea pig and 31–37% inhibition in rabbit hepatocytes)

Table 1. Effects of okadaic acid on glycogen deposition in rat, guinea pig and rabbit hepatocytes

| Okadaic acid (nM) | Rat hepatocytes | | | Guinea pig hepatocytes | | | Rabbit hepatocytes | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | Insulin | [%Con/Ins] | Control | Insulin | [%Con/Ins] | Control | Insulin | [%Con/Ins] |
| | (nmol of glucosyl units deposited/3 hr/mg of protein) | | | | | | | | |
| 0 | 98 ± 12 | 186 ± 28‡ | [100/100] | 102 ± 13 | 219 ± 26§ | [100/100] | 541 ± 88 | 581 ± 82 | [100/100] |
| 1 | 110 ± 20 | 170 ± 36 | [112/91] | 120 ± 15 | 205 ± 28§ | [118/94] | 578 ± 76 | 587 ± 73 | [107/101] |
| 2.5 | 89 ± 13 | 96 ± 20† | [91/51] | 116 ± 16 | 148 ± 20*‡ | [114/68] | 537 ± 71 | 509 ± 90 | [99/88] |
| 5 | 72 ± 12 | 73 ± 10† | [74/39] | 91 ± 11 | 134 ± 17†§ | [89/61] | 568 ± 73 | 472 ± 84*‡ | [105/81] |
| 10 | 32 ± 8* | 25 ± 5† | [33/13] | 26 ± 13† | 35 ± 17† | [26/16] | 372 ± 61† | 298 ± 82† | [69/51] |
| 100 | −3 ± 8† | 2 ± 4† | [<0/1] | −56 ± 10† | −60 ± 6† | [<0/<0] | −182 ± 36† | −138 ± 69† | [<0/<0] |

Hepatocyte monolayers were incubated for 3 hr in MEM containing 15 mM [U-$^{14}$C]glucose with or without 1 nM insulin and the concentrations of okadaic acid indicated. The cellular glycogen content at the start of the incubation was 35 ± 6, 95 ± 12 and 636 ± 160 nmol of glucosyl units/mg of protein for rat, guinea pig and rabbit hepatocytes, respectively. The glycogen deposited during the incubation with substrates is expressed as nmol of glucose units deposited in 3 hr per mg of protein. Values are means ± SEM for either six (rat and guinea pig) or four (rabbit) hepatocyte preparations.

Statistical analysis was by the paired *t*-test: * $P < 0.05$, † $P < 0.005$ relative to the respective values without okadaic acid; ‡ $P < 0.05$, § $P < 0.005$ relative to the respective values without insulin.

Table 2. Effects of okadaic acid on incorporation of [$^{14}$C]glucose into glycogen in rat, guinea pig and rabbit hepatocytes

| Okadaic acid (nM) | Rat hepatocytes | | | Guinea pig hepatocytes | | | Rabbit hepatocytes | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | Insulin | [%Con/Ins] | Control | Insulin | [%Con/Ins] | Control | Insulin | [%Con/Ins] |
| | | | | (nmol of [$^{14}$C]glucose incorporated/3 hr/mg of protein) | | | | | |
| 0 | 47 ± 10 | 108 ± 29‡ | [100/100] | 43 ± 9 | 108 ± 21‡ | [100/100] | 538 ± 130 | 537 ± 109 | [100/100] |
| 1 | 55 ± 14 | 103 ± 21‡ | [117/95] | 45 ± 9 | 100 ± 21*‡ | [105/93] | 495 ± 116 | 529 ± 105 | [92/99] |
| 2.5 | 49 ± 12 | 54 ± 15* | [104/50] | 42 ± 9 | 73 ± 16*‡ | [98/68] | 469 ± 112 | 476 ± 105* | [87/89] |
| 5 | 35 ± 8 | 57 ± 22† | [75/53] | 33 ± 7 | 47 ± 11*‡ | [77/44] | 479 ± 113 | 420 ± 113*§ | [89/78] |
| 10 | 17 ± 4† | 21 ± 8† | [36/19] | 15 ± 4* | 19 ± 6† | [35/19] | 338 ± 100* | 315 ± 99† | [63/59] |
| 100 | 5 ± 1† | 5 ± 1† | [11/5] | 2 ± 1† | 2 ± 1† | [2/9] | 46 ± 22* | 29 ± 12† | [9/5] |

Experimental conditions were as in Table 1. The incorporation of [$^{14}$C]glucose into glycogen is expressed as nmol of glucose incorporated in 3 hr per mg cell protein. Values are means ± SEM for either six (rat and guinea pig) or four (rabbit) hepatocyte preparations.

Statistical analysis was by the paired *t*-test: * $P < 0.05$, † $P < 0.005$ relative to the respective values without okadaic acid; ‡ $P < 0.05$, § $P < 0.005$ relative to the respective values without insulin.

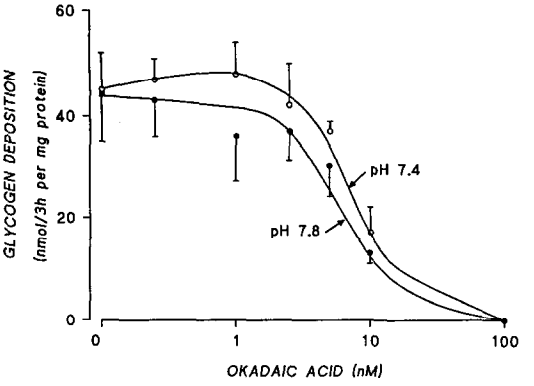


Fig. 1. Effects of pH on the sensitivity of glycogen synthesis to okadaic acid. Rat hepatocyte monolayers were incubated for 3 hr in MEM containing 20 mM Na-HEPES buffered at either pH 7.4 or 7.8 and supplemented with 20 mM glucose and the concentrations of okadaic acid indicated. Glycogen deposition was determined as described in Materials and Methods and is expressed as nmol of glucosyl units deposited/3 hr/mg of protein. Values are means ± SEM for three experiments.

and 100 nM okadaic acid caused complete inhibition of glycogen deposition in all three species and net glycogenolysis in guinea pig and rabbit hepatocytes.

In the presence of insulin, okadaic acid inhibited glycogen deposition and synthesis at lower concentrations than were effective in the absence of insulin (Tables 1 and 2). In rat and guinea pig hepatocytes, 2.5 nM okadaic acid suppressed the stimulation of glycogen synthesis by insulin, whilst this concentration had no effect in the absence of insulin. Although in rabbit hepatocytes there was no significant stimulation of glycogen deposition or synthesis by insulin, 5 nM okadaic acid significantly inhibited glycogen synthesis in the presence of insulin but not in its absence and at this concentration of okadaic acid, both glycogen deposition and synthesis were significantly lower in the presence of insulin than in its absence ($P < 0.05$ and $P < 0.005$, respectively, Tables 1 and 2). The concentrations of okadaic causing half-maximal inhibition of glycogen synthesis ($IC_{50}$) were lower in the presence of insulin (rat, 3 nM; guinea pig, 5 nM; rabbit, 20 nM) than in its absence (rat, 7 nM; guinea pig, 10 nM; rabbit, 35 nM, Tables 1 and 2), indicating that for all species the "sensitivity" (defined as the concentration causing half-maximal inhibition) to okadaic acid is greater in the presence of insulin.

Insulin increases cytosolic pH [12–14] by activation of $Na^+/H^+$ exchange [12–15]. It is, therefore possible that the increased inhibition by okadaic acid in the presence of insulin may be related to an increase in cytosolic pH. We determined the effects of an increase in pH from 7.4 to 7.8 in a bicarbonate-free buffer on the sensitivity of glycogen synthesis to inhibition by okadaic acid in rat hepatocytes (Fig. 1). The concentration of okadaic acid causing half-maximal inhibition of glycogen synthesis was lower at pH 7.8 ($IC_{50}$ 7 nM) than at pH 7.4 ($IC_{50}$ 9 nM, Fig.

1). Glycogen deposition at 1 nM and at 5 nM okadaic acid was significantly higher ($P < 0.05$) at pH 7.4 than at pH 7.8 suggesting that an increase in pH alters the characteristics of glycogen synthesis to inhibition by okadaic acid.

## DISCUSSION

Okadaic acid exerts a wide range of cellular effects as a result of increased protein phosphorylation [3]. The inhibition of glycogen deposition and of incorporation of glucose into glycogen by okadaic acid (Tables 1 and 2) probably reflects activation of glycogen phosphorylase as well as inactivation of glycogen synthase [8] since 100 nM okadaic acid caused net glycogen breakdown in guinea pig and rabbit hepatocytes incubated with glucose despite some residual incorporation of glucose into glycogen. Inactivation of glycogen synthase by okadaic may occur by a variety of mechanisms including: direct inhibition of synthase and phosphorylase phosphatases; inactivation of synthase phosphatase by phosphorylation and dissociation of either or the glycogen-binding sub-units of protein phosphatase-G; inhibition of synthase phosphatase by inhibitor protein-1 which is activated by phosphorylation and inactivated by type-2A phosphatases [2]. Inactivation of these phosphatases by okadaic acid would lead to phosphorylation and activation of the inhibitor protein. Synthase phosphatase may also be inhibited by the active phosphorylated form of glycogen phosphorylase which would be formed when phosphorylase phosphatase is inhibited. Inhibitor-1 is present in guinea pig but not in rat liver. In view of the similarity in the effects of okadaic on glycogen synthesis in hepatocytes from these two species (Tables 1 and 2), it seems unlikely that this protein has a major role in mediating the effects of okadaic acid on glycogen synthesis in guinea pig hepatocytes.

In cell-free systems okadaic acid inhibits protein phosphatases (Type-2A, $IC_{50}$ 0.05–2 nM; Type 1, $IC_{50}$ 10–20 nM) with little effect on protein kinases within the concentration range that is effective at inhibiting protein phosphatases [4, 16]. In studies with whole cells, higher concentrations of okadaic acid are frequently necessary (in the micromolar range), to inhibit or activate specific processes that are regulated by protein phosphorylation than are effective at inhibiting protein phosphatases in cell-free systems (picomolar to nanomolar concentrations). This is particularly evident in studies on adipocytes, muscle and hepatoma cells which have used okadaic acid concentrations ranging from 1–10 $\mu$M [5–7, 17], although studies on hepatocytes have used lower concentrations [8, 18]. Various arguments have been advanced to explain the requirement for micromolar concentrations of okadaic acid in studies with whole cells [3, 19]. It is unlikely that tissue differences in sensitivity to okadaic acid reflect differences in plasma membrane permeability since okadaic acid equilibrates freely across artificial lipid membranes [20]. It is possible that the inhibitory characteristics of okadaic acid on phosphatases in intact cells differ from those determined on the purified enzymes if these are assayed with different concentrations of monovalent and divalent cations than are present in the cytosol [4]. Furthermore, in intact cells activities of protein phosphatases and kinases and their regulatory characteristics may be influenced by the macromolecular environment and by their association with the cytoskeleton or other cellular structures and this may also affect their sensitivity to okadaic acid.

The present study demonstrates that in hepatocytes incubated with insulin lower concentrations of okadaic acid are effective at inhibiting glycogen synthesis than in the absence of hormone. The data from rat and guinea pig hepatocytes could be interpreted in terms of specific inhibition by low concentrations of okadaic acid (1–2 nM) of the stimulation caused by insulin. Thus, the inhibition by okadaic acid in the absence of insulin ($IC_{50}$ 7–35 nM) could be explained by inhibition of type-1 phosphatases, whereas the inhibition of the 2-fold stimulation of glycogen synthesis by insulin by 1–2 nM okadaic acid could be explained by the involvement of type-2A phosphatases (which are more sensitive to okadaic acid than type-1 phosphatases) in insulin action [8]. However, this explanation cannot account for the observations in rabbit hepatocytes. The inhibition of glycogen synthesis by low concentrations of okadaic acid in the presence of insulin cannot be explained by inhibition of the insulin effect, since insulin does not stimulate glycogen synthesis in these cells. It appears therefore that insulin alters the characteristics of the sensitivity to okadaic acid.

From the kinetic analysis of the effects of okadaic acid on protein phosphatases in cell-free systems [4], Cardenas and Cornish-Bowden [19] have estimated that inhibition of protein phosphatases by okadaic acid involves non-competitive (catalytic) as well as competitive (specific) inhibition and furthermore they have suggested that the requirement for high concentrations of okadaic acid in studies with whole cells is compatible with concomitant weak stimulation of protein kinases and potent inhibition of protein phosphatases. Thus, the inhibitory characteristics of okadaic acid in the whole cell on the activity of a process that is regulated by protein phosphatases and kinases would be a function of the intrinsic activities of the protein phosphatase and kinase and on their sensitivity to okadaic acid [19]. Insulin may therefore alter the characteristics of glycogen synthesis to inhibition by okadaic acid by altering the activities of the kinases and phosphatases that determine the phosphorylation state of glycogen synthase and glycogen phosphorylase. Since an increase in pH also lowers the concentration of okadaic acid that causes half-maximal inhibition of glycogen synthesis (Fig. 1), it is possible that the effects of insulin on the characteristics of inhibition of glycogen synthesis by okadaic acid may be in part mediated by an increase in cytosolic pH [12–14] as a result of activation of $Na^+/H^+$ exchange [15]. It is noteworthy however, that insulin increases cell volume [21] as well as cell pH [12] and cell swelling stimulates glycogen synthesis and activates glycogen synthase [21–24], probably via changes in the activities of protein kinases and phosphatases that regulate glycogen synthase [23]. The link between cell swelling and changes in enzyme activities may

involve either a cytoskeletal mechanism [25] or changes in the regulatory properties of enzymes via macromolecular crowding [26, 27]. The mechanism by which insulin alters the characteristics of the response of glycogen synthesis to inhibition by okadaic acid in hepatocytes warrants further study.

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