The protein kinase C inhibitors bisindolylmaleimide I (GF 109203x) and IX (Ro 31-8220) are potent inhibitors of glycogen synthase kinase-3 activity

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Abstract Here we report that the widely used protein kinase C inhibitors, bisindolylmaleimide I and IX, are potent inhibitors of glycogen synthase kinase-3 (GSK-3). Bisindolylmaleimide I and IX inhibited GSK-3 in vitro, when assayed either in cell lysates (IC_{50} 360 nM and 6.8 nM, respectively) or in GSK-3β immunoprecipitates (IC_{50} 170 nM and 2.8 nM, respectively) derived from rat epididymal adipocytes. Pretreatment of adipocytes with bisindolylmaleimide I (5 μM) and IX (2 μM) reduced GSK-3 activity in total cell lysates, to 25.1 ± 4.3% and 12.9 ± 3.0% of control, respectively. By contrast, bisindolylmaleimide V (5 μM), which lacks the functional groups present on bisindolylmaleimide I and IX, had little apparent effect. We propose that bisindolylmaleimide I and IX can directly inhibit GSK-3, and that this may explain some of the previously reported insulin-like effects on glycogen synthase activity.

Key words: Insulin; Bisindolylmaleimide; Protein kinase C inhibitor; Glycogen synthase kinase-3; Adipocyte

1. Introduction.

The bisindolylmaleimide derivatives of staurosporine are widely used as specific inhibitors of protein kinase C (PKC) isoforms. Bisindolylmaleimide I (also known as GF 109203x) and IX (also known as Ro 31-8220) are the most commonly used PKC inhibitors [1–3]. In contrast, bisindolylmaleimide V does not inhibit PKC isoforms and is used as a negative control as it lacks the important functional groups present on bisindolylmaleimide I and IX [4].

Recently, several additional pharmacological effects of bisindolylmaleimide IX have been reported. Beltman et al. [5] showed that bisindolylmaleimide IX strongly stimulates the activity of c-Jun-N-terminal kinase (JNK) and the expression of c-*jun*, while it inhibits growth factor-stimulated mitogen-activated protein (MAP) phosphatase-1 (MKP-1) gene expression in Rat-1 fibroblasts in a PKC-independent manner. In contrast, bisindolylmaleimide I had no effect on these signalling components.

A later report by this group showed that bisindolylmaleimide IX induced the phosphorylation and possible activation of p38 MAPK, as well as phosphorylation of the activator protein-1 (AP-1) family member c-Jun, and a concomitant increase in AP-1 activity [6]. Subsequently, Standaert et al. [7] reported that bisindolylmaleimide IX activates JNK and increases glycogen synthase activity in primary rat adipocytes, independently of PKC inhibition. The authors concluded that JNK rather than PKC, protein kinase B or ERK1/2, was involved in the activation of glycogen synthase by bisindolylmaleimide IX.

Glycogen synthase is a key enzyme that catalyses the incorporation of the glycosyl residue of UDP-glucose into glycogen, and its activity is regulated by multisite phosphorylation. In particular, glycogen synthase kinase-3 (GSK-3) phosphorylates and inhibits the enzyme [8,9]. Insulin has been proposed to stimulate glycogen synthase by promoting an inhibition of GSK-3 [10] and/or activation of protein phosphatase-1 [11], resulting in a net dephosphorylation of glycogen synthase.

In our investigations of the mechanism by which insulin regulates glycogen synthase activity in rat adipocytes, we found that bisindolylmaleimide I and IX were potent inhibitors of GSK-3. We propose that this may be the likely mechanism by which bisindolylmaleimide IX stimulates glycogen synthesis.

2. Materials and methods

2.1. Materials

Male Wistar rats (160–210 g) were fed ad libitum on a stock diet (CRM; Bioshore, Manea, Cambs., UK). Bisindolylmaleimide I (GF 109203x) and V were from Calbiochem (Nottingham, UK), bisindolylmaleimide IX (Ro 31-8220) was from Alexis Corporation (UK) and the anti-GSK-3β monoclonal antibody was purchased from Transduction Laboratories (Becton Dickinson, UK). All other reagents were as described [12].

2.2. Preparation and incubation of epididymal fat cells

Adipocytes were isolated from the epididymal fat pads of Wistar rats as described previously [13]. Cells (150–250 mg dry cell weight) were extracted in 1 ml of ice-cold 50 mM Tris (pH 7.5) containing 1 mM EDTA, 120 mM NaCl, 50 mM NaF, 40 mM β-glycerophosphate, 1 mM benzamidine, 1% NP40, 1 μM microcystin and 1 μg/ml each of pepstatin, leupeptin and antipain. Lysates were centrifuged twice at 10 000 × *g* for 10 min at 4°C prior to use, and the infranatant was taken for measurement of protein kinase activity.

2.3. GSK-3 activity assay

GSK-3 activity was measured in cell lysates and in GSK-3β immunoprecipitates. GSK-3β was immunoprecipitated from cell lysates by tumbling with 4 μl of anti-GSK-3β monoclonal antibody and 3.75 mg protein A-Sepharose for 2 h at 4°C. The resulting immunoprecipitates were washed three times in kinase assay buffer (20 mM HEPES, pH 7.5, 20 mM β-glycerophosphate and 1 mM EDTA) and finally resuspended in 300 μl of kinase assay buffer containing 0.1% mercaptoethanol and 2.5 μM cAMP-dependent protein kinase inhibitor peptide (IP_{20}). The activity of GSK-3 was measured in duplicate in 20 μl of cell lysate or 20 μl of GSK-3β immunoprecipitate using the synthetic

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Abbreviations: GSK-3, glycogen synthase kinase-3; PKC, protein kinase C; JNK, Jun-N-terminal kinase; MAPKAP, mitogen-activated protein kinase-activated protein; AP-1, activator protein-1

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peptide substrate RRAAEELDSRAGS(P)PQL (0.71 mg/ml) [14] in the absence or in the presence of the GSK-3 inhibitor, lithium chloride (50 mM) [15]. The assay was terminated after 15 min incubation with [γ- ^{32}P]ATP by spotting onto P81 ion-exchange paper. The paper was washed four times in 0.6% phosphoric acid and bound radioactivity quantified by scintillation counting. Phosphorylation of peptide by adipocyte lysates and by GSK-3β immunoprecipitates was essentially completely inhibited by lithium chloride. The average activity of GSK-3 in the extracts was 1220 ± 144 pmol peptide phosphorylated/min/g dry weight of adipocytes (n = 11). The average activity of GSK-3β in immunoprecipitates was 276 ± 54 pmol peptide phosphorylated/min/g dry weight of adipocytes (n = 11).

3. Results

3.1. Direct effects of bisindolylmaleimide I and IX on GSK-3 activity

To investigate whether bisindolylmaleimide I and IX affect GSK-3 activity, freshly isolated primary adipocytes were extracted and GSK-3 activity was subsequently measured in total lysates and in GSK-3β immunoprecipitates. Bisindolylmaleimide I inhibited GSK-3 activity with an IC_{50} of 360 nM in total lysates and an IC_{50} of 190 nM in GSK-3β immunoprecipitates (Fig. 1A). Bisindolylmaleimide IX was much more potent in inhibiting GSK-3 activity in total lysates (IC_{50} = 6.8 nM) and in GSK-3β immunoprecipitates (IC_{50} = 2.8 nM, Fig. 1B). In contrast, bisindolylmaleimide V (5 μM) had no major effect on GSK-3 activity in total lysates (85.6 ± 9%, n = 7) and immunoprecipitates (98.3 ± 12.5%, n = 6).

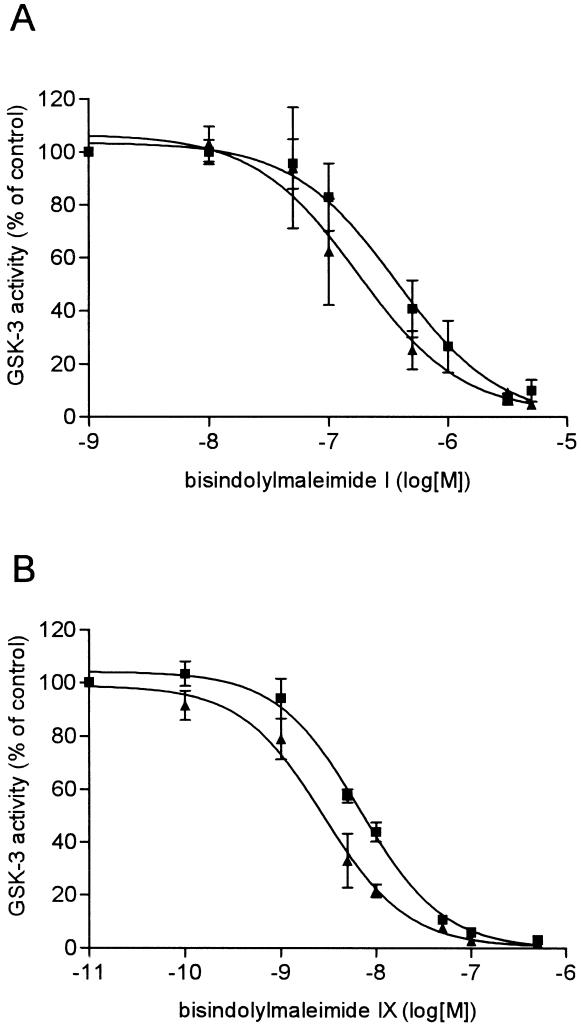

Fig. 1. Effect of bisindolylmaleimide I and IX on GSK-3 activity in total extracts and in immunoprecipitates. Total lysates (■) or GSK-3β immunoprecipitates (▲) from rat epididymal adipocytes were incubated for 5 min at 30°C with different concentrations of bisindolylmaleimide I (A) and bisindolylmaleimide IX (B) before addition of [γ-^{32}P]ATP and substrate peptide. The assay was terminated after 15 min incubation with [γ-^{32}P]ATP by spotting onto P81 ion-exchange paper. Data (mean ± S.E.M., 3–5 observations) are expressed as percentage of GSK-3 activity in the absence of inhibitor.

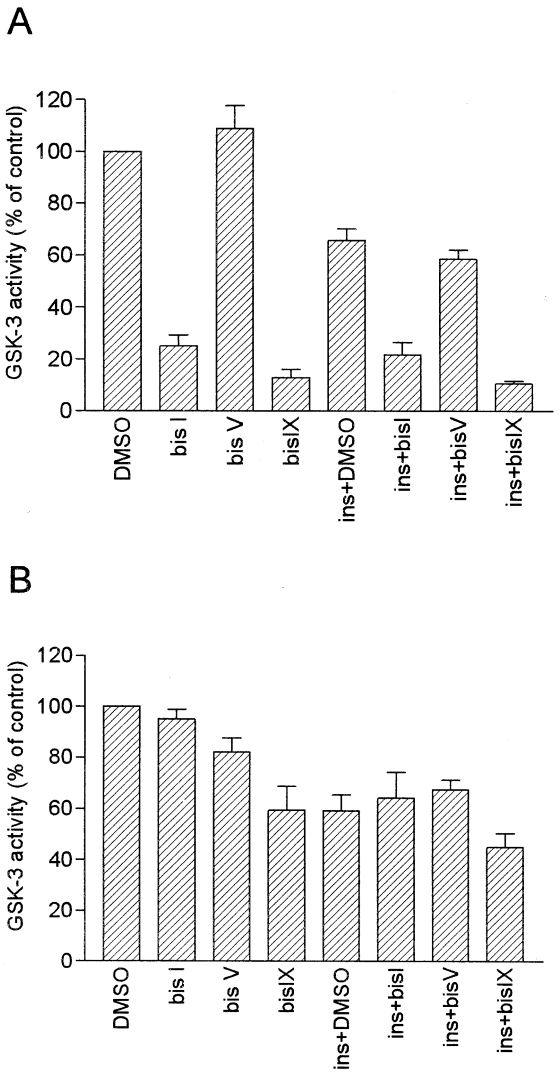

Fig. 2. Effect of various bisindolylmaleimides on GSK-3 activity in rat epididymal adipocytes. Cells were incubated with vehicle (DMSO), bisindolylmaleimide I (bis I, 5 μM), V (bis V, 5 μM) and IX (bis IX, 2 μM) for 5 min at 37°C prior to addition of insulin (ins, 83 nM). Cells were extracted after a 10 min incubation and GSK-3 activity was subsequently measured in total cell lysates (A) and in GSK-3β immunoprecipitates (B) as described in the legend to Fig. 1. Data (mean ± S.E.M., 3–5 observations) are expressed as percentage of GSK-3 activity in the absence of inhibitor.

3.2. Effect of bisindolylmaleimide I, V and IX on GSK-3 activity in primary adipocytes

As expected, incubation of intact adipocytes with the inhibitors bisindolylmaleimide I and IX strongly inhibited GSK-3

activity, when subsequently assayed in total lysates (Fig. 2A). In contrast, bisindolylmaleimide V had no effect. Insulin inhibited GSK-3 activity to 59 ± 6% of control and the effect was not additive to bisindolylmaleimide I- and IX-induced GSK-3 inhibition (Fig. 2A). When GSK-3β was immunoprecipitated following extraction of the cells, the inhibitory effect of bisindolylmaleimide I was lost (Fig. 2B). In contrast, the more potent inhibitor, bisindolylmaleimide IX, still reduced GSK-3 activity to 59 ± 9% of control.

4. Discussion

In this study we show that GSK-3, a key kinase in insulin-induced activation of glycogen synthase, is potently and directly inhibited by the PKC inhibitors bisindolylmaleimide I and IX. Both bisindolylmaleimides strongly inhibited GSK-3 activity when added directly to cell lysates and GSK-3β immunoprecipitates. Bisindolylmaleimide IX was the more potent inhibitor of GSK-3 activity, with an approximately 100 times lower IC_{50} value than bisindolylmaleimide I. Bisindolylmaleimide IX is an equally potent inhibitor of both GSK-3 and PKC (IC_{50} values for GSK-3 of 3–7 nM found in this study compared to an IC_{50} of 5 nM for PKC [16]).

It is likely that bisindolylmaleimide I and IX compete reversibly with ATP for binding to the nucleotide-binding site of GSK-3, as proposed for PKC [1]. The loss of the inhibitory effect of the agents during immunoprecipitation of GSK3β from cells previously exposed to them is compatible with this. Bisindolylmaleimide I had little effect on insulin-induced inhibition of GSK-3, under conditions where it would be expected to potently inhibit PKC activity [1]. This strongly suggests that PKC, although it has previously been implicated in GSK-3 regulation [17,18], is not involved in this process. This is consistent with observations that insulin-induced inhibition of GSK-3 in mouse 10T1/2 fibroblasts was unaffected by bisindolylmaleimide IX, whereas Wingless-induced inactivation was blocked [19].

Many of the recently reported stimulatory effects of bisindolylmaleimide IX [5–7] may be explained by its ability to inhibit GSK-3. Bisindolylmaleimide IX increases glycogen synthase activity in adipocytes. This effect was attributed to the ability of bisindolylmaleimide IX to stimulate JNK activity [7]. Given the observations in the present study, it is more simply explained by inhibition of GSK-3, since GSK-3, which has a high activity in resting cells, is able to phosphorylate and inactivate glycogen synthase. In contrast to insulin, bisindolylmaleimide IX was shown to stimulate glycogen synthase activity in a phosphatidyl inositol-3 kinase (PI3 kinase)-independent manner [7], which is consistent with a direct inhibitory effect on GSK-3.

Bisindolylmaleimide IX and insulin have additive effects on glycogen synthase activity [7]. Similar results have recently been found with lithium, a specific GSK-3 inhibitor, and insulin [20]. This contrasts with the effects of insulin and bisindolylmaleimide IX on GSK-3 inhibition, which were not additive (Fig. 2). This apparent discrepancy may be explained by the ability of insulin to activate protein phosphatase-1 [21], and hence the dephosphorylation of glycogen synthase by a mechanism independent of GSK-3.

Several groups have reported that bisindolylmaleimide IX activates JNK in cells in a PKC-independent manner [5,7]. Activation of JNK by insulin is blocked by wortmannin in CHO cells expressing the insulin receptor and is likely, therefore, to be downstream of PI3 kinase activation [22]. This raises the possibility that inhibition of GSK-3 activity may lead, presumably indirect, to the activation of JNK. This hypothesis is consistent with the observation that the bisindolylmaleimide IX- and insulin-stimulated JNK activation in rat adipocytes are not additive [7]. It requires rigorous testing, particularly as bisindolylmaleimide IX is known to inhibit other protein kinases, such as MAPKAP kinase and p70S6 kinase [16]. However, it should be noted that these particular kinases are unlikely to be involved as insulin and bisindolylmaleimide IX have opposite effects on their activity.

One of the substrates of JNK is c-Jun, which forms part of the activating protein-1 complex (AP-1 complex), and is phosphorylated by JNK on two regulatory sites Ser-63 and Ser-73. Phosphorylation of these sites transactivates c-Jun, and may also explain the increased c-*jun* expression induced by bisindolylmaleimide IX [5]. Stimulation of AP-1 activity in response to bisindolylmaleimide IX is likely, therefore, to be the result of increased c-Jun synthesis and/or phosphorylation of c-Jun on Ser-63 and Ser-73 by increased JNK activity [6]. However, GSK-3 phosphorylates c-Jun on three sites in a region proximal to the DNA-binding domain (residues 227–252), resulting in decreased c-Jun DNA binding and transcriptional activity [23]. Indeed, transfection experiments have shown that AP-1 activity is inhibited by co-expression of GSK-3 [24]. Inhibition of GSK-3 activity by bisindolylmaleimide IX might therefore abolish this negative restraint, thereby increasing c-Jun/AP-1 activity.

In summary, we have demonstrated that both bisindolylmaleimide I and IX are potent and direct inhibitors of GSK-3. Our results raise the possibility that some of the insulin-like effects of bisindolylmaleimide IX, in particular the activation of glycogen synthase, may be the result of the ability of these compounds to inhibit GSK-3.

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