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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Received 29 September 1999

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 (IC50 360 nM and 6.8 nM, respectively) or in GSK-3β immunoprecipitates (IC50 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 25.1 ± 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.

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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 10000×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 (IPα). 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
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-32P]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 IC50 of 360 nM in total lysates and an IC50 of 190 nM in GSK-3β immunoprecipitates (Fig. 1A). Bisindolylmaleimide IX was much more potent in inhibiting GSK-3 activity in total lysates (IC50 = 6.8 nM) and in GSK-3β immunoprecipitates (IC50 = 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).

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 was subsequently measured in total lysates and in GSK-3β immunoprecipitates. Bisindolylmaleimide I inhibited GSK-3 activity with an IC50 of 360 nM in total lysates and an IC50 of 190 nM in GSK-3β immunoprecipitates (Fig. 1A). Bisindolylmaleimide IX was much more potent in inhibiting GSK-3 activity in total lysates (IC50 = 6.8 nM) and in GSK-3β immunoprecipitates (IC50 = 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).
activity, when subsequently assayed in total lysates (Fig. 2A).
In contrast, bisindolylmaleimide V had no effect. Insulin in-
hibited 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 immunopre-
cipitated 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 di-
rectly 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 im-
munoprecipitates. Bisindolylmaleimide IX was the more po-
tent inhibitor of GSK-3 activity, with an approximately 100
times lower IC50 value than bisindolylmaleimide I. Bisindol-
ymaleimide IX is an equally potent inhibitor of both GSK-3
and PKC (IC50 values for GSK-3 of 3-7 nM found in this
study compared to an IC50 of 5 nM for PKC [16]).

It is likely that bisindolylmaleimide I and IX compete re-
versibly 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 ex-
pected to potently inhibit PKC activity [1]. This strongly sug-
gests 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 bis-
indolylmaleimide IX, whereas Wingless-induced inactivation
was blocked [19].

Many of the recently reported stimulatory effects of bisin-
dolylmaleimide 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 activ-
ity [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, bisin-
dolylmaleimide IX was shown to stimulate glycogen synthase
activity in a phosphatidyl inositol-3 kinase (PI3 kinase)-in-
dependent manner [7], which is consistent with a direct inhibi-
tory 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 in-
sulin [20]. This contrasts with the effects of insulin and bisin-
dolylmaleimide IX on GS K-3 inhibition, which were not ad-
ditive (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, there-
fore, 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 hy-
pothesis is consistent with the observation that the bisindolyl-
maleimide 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 bisindolyl-
maleimide 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 phos-
phorylated 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 bisin-
dolylmaleimide IX [5]. Stimulation of AP-1 activity in re-
sponse 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 transcrip-
tional 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 bisindolylmale-
imide IX might therefore abolish this negative restraint, there-
by increasing c-Jun/AP-1 activity.

In summary, we have demonstrated that both bisindolyl-
maleimide 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.

Acknowledgements: This work was supported by grants from The
Medical Research Council and British Diabetic Association. J.M.T.
is a British Diabetic Association Senior Research Fellow.

References

[1] Toulec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Per-
ret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E. and
J. 294, 335-337.
Sedgwick, A.D., Wadsworth, J., Westmacott, D. and Wilkinson,
Keech, E., Kumar, M.K., Lawton, G., Nixon, J.S. and Wilkin-
Chem. 271, 27018-27024.
J. 333, 471-490.
709.