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EARLY EFFECT OF BCNU ON RAT ASTROCYTES

INHIBITION OF S6 KINASE ACTIVATION BY GROWTH FACTORS

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Abstract—In primary cultures of astrocytes, methylmethane, 2-*N*-methyl 9-hydroxy-ellepticinium acetate, ditercalinium, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea and 1,3 bis (2-chloroethyl)-1-nitrosourea (BCNU) blocked to various extents the activation of S6 kinase by acidic fibroblast growth factor and insulin [or insulin-like growth factor 1 (IGF1)]. The effects of the most active agent, BCNU, were time and concentration dependent. Pretreatment of cells with 50 μM BCNU for 1 hr completely prevented S6 kinase activation by growth factors for at least 2 days. The S6 kinase activity of unstimulated cells was slightly affected. S6 kinase activation by 12-*O*-tetradecanoylphorbol 13 acetate was also strongly impaired by treating cells with BCNU whereas activation by 8-bromo-cyclic AMP was slightly reduced. Cyclic AMP-dependent protein kinase and phospholipid and Ca^{2+}-dependent protein kinase were unaffected. BCNU had no direct effect on IGF1 binding to cell surface receptors or on the S6 kinase activity of cell cytosols.

Some drugs have been reported to prevent the action of growth factor for example, it has been shown that erbstatin inhibits epidermal growth factor (EGF‡) tyrosine kinase, reduces *in vivo* the autophosphorylation of the tyrosine kinase $P60^{src}$ [1], and inhibits proliferation of L1210 leukemia cells and A431 cells [2]. Suramin, previously known as an antimalaria drug prevents interaction between some growth factors and their receptors [3]. It is now tested against different human tumors. The antitumor agent Adriamycin® at 10^{-6} M has been reported to inhibit some cytosolic tyrosine kinases [4]. Herbimycin reduces the level of the $P60^{src}$ tyrosine kinase, by accelerating its degradation, and reverses the phenotype promoted by *src* transformation [5]. Genistein has first been found to be an inhibitor *in vitro* of EGF, $P60^{src}$ and $P110^{gagfes}$ tyrosine kinases [6]. At lower concentrations, sufficient to block cell proliferation, genistein inhibits other steps of transduction [7]. Tyrphostin blocks EGF-tyrosine kinase and EGF dependent proliferation of clone 15 of A431 cells [8]. Flavonoids and other drugs are inhibitors of phosphatidyl inositol kinase [9], an enzyme enhanced by PDGF and transformation by oncogenes. *In vivo* effects of cancerogens, such nitrosamines, on tyrosine kinase activities [10] seem to be due to indirect mechanisms.

At this time, little work has been performed to look for pharmacological agents able to block serine kinases activated by growth factors. S6 kinases are cytosolic protein (serine) kinases that phosphorylate S6, a protein from the small ribosomal subunit. This phosphorylation is believed to play a crucial role in control of mRNA translation [11]. These enzymes are growth factor-stimulated kinases [12–17].

In this work, we studied the effects of intercalating (NMHE, ditercalinium) and alkylating (MMS, CCNU, BCNU) agents, capable of inhibiting cell proliferation [18, 19], on the growth factor-stimulated S6 kinase in astrocytes [16, 20–22], probably S6 kinase P70 [23] since S6 kinase II activity (90^{rsk} kinase) is not easily detectable in mammals with the current S6 kinase assay [17]. We found that BCNU, an agent used in the treatment of various tumors including brain tumors [24], is a particularly effective inhibitor of S6 kinase activation and we analysed the parameters of this inhibition.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP, [^{125}I]IGF1 and IGF1 were obtained from Amersham, France. Insulin was from Novo (Denmark). 12.*O*-Tetradecanoylphorbol 13 acetate (TPA) was from PL Biochemicals (F.R.G.), methylmethane sulfonate (MMS), Br-cAMP, histones, heat-stable inhibitor of PKA, aprotinin, leupeptin, phosphatidyl serine and diolein were all from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

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‡ Abbreviations: aFGF, acidic fibroblast growth factor; BCNU, 1,3 bis (2-chloroethyl)-1-nitrosourea; CCNU, 1-2(chloroethyl)-3-cyclohexyl-1-nitrosourea; Ditercalinium, 2-2′{4,4′-bipiperidine)-1,1′ bis 2,1 (ethane diyl)] bis (10-methoxy-7-*H*-pyrido[4,3-C] carbazolium tetramethane sulfonate; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; F12, Ham F12; IGF1, insulin-like growth factor 1; MMS, methylmethane sulfonate; NMHE, 2-*N*-methyl 9-hydroxy-ellepticinium acetate; PKA, cyclic AMP-dependent protein kinase; PKC, phospholipid and Ca^{2+}-dependent protein kinase; TPA, 12.*O*-tetradecanoylphorbol 13 acetate; Br-cAMP, 8-bromo-cyclic AMP.

Table 1. Effect of alkylating and intercalating compounds on the S6 kinase activation by aFGF and insulin

Compound	S6 kinase activity (cpm ^{32}P incorporated)		
	Basal	Insulin stimulated	aFGF stimulated
None	1434	3675	3738
MMS	937	2754	2626
NMHE	1130	3144	2637
Ditercalinium	1141	2609	2653
CCNU	1173	2293	ND
BCNU	998	1384	1057

After pretreatment for 1 hr with 200 μM MMS, 0.5 μM NMHE, 0.5 μM ditercalinium, 50 μM CCNU or 50 μM BCNU, astrocytes were washed with medium and stimulated with 1 μM insulin or 3 nM aFGF for 45 min. S6 kinase activity was measured as described in the legend of Fig. 1. The typical experiment which is presented reflects three independent experiments.

aFGF, purified from bovine brain, was a generous gift from Dr Barritault (University Paris XII, Créteil, France). BCNU was from Laboratoires Bristol (France), NMHE from Pasteur Vaccins (France), ditercalinium was from Roger Bellon (France) and CCNU was from Aldrich (France).

All other chemicals were reagent grade.

Culture media were purchased from Gibco (France). Fetal calf serum (FCS) was from Seromed (F.R.G.). Newborn rats were supplied by Iffa-Credo (France).

Cell culture. Primary cultures of rat astrocytes were prepared according to Aizenman and de Vellis [25]. Briefly, cerebral hemispheres from 2-day-old Sprague–Dawley rats were cleaned of meninges and dissociated into cell suspension by passage through a nylon mesh (48 μm pore size). The cells obtained from one hemisphere were suspended in 20 mL DMEM supplemented with 10% FCS. The cell suspension (3 mL) was placed in 60 mm Petri dishes and the cells were grown to confluence (7–8 days). These cells were placed in a chemically defined medium DMEM/F12 (1/1) containing 5.1 g/L glucose and 2.7 g/L sodium bicarbonate and cultured for 3–5 days, with daily changes of medium.

Cell treatment. Drugs were added to the cell cultures at the indicated concentrations and for the times indicated. The medium was then removed, replaced with fresh medium and the cells were incubated for 45 min with one of the following agents: 10 nM IGF1 (or, in some experiments, pharmacological concentration of insulin, 1 μM), 3 nM aFGF, 1 mM Br-cAMP and 100 nM TPA. At the end of incubation, the medium was removed and the cells were washed with 3 mL/dish 80 mM β-glycerophosphate, 20 mM EGTA and 15 mM $MgCl_2$, pH 7.4 (buffer A). The cells were scraped off in 0.3 mL/dish buffer A containing 1 mM PMSF, 1 mM Benzamidin, 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, aprotinin (50 μg/mL), leupeptin (4 μg/mL), antipaïn (4 μg/mL), trypsin inhibitor (1 μg/mL) and disrupted by sonication for 3 sec. The homogenates were centrifuged at 100,000 *g* for 1 hr and the resulting supernatants were stored at −70°.

Protein kinase assays. Ribosomal subunits (40S) were isolated from rat liver according to Martin and Wool [26]. S6 kinase was assayed by incubating aliquots of the supernatants (5–10 μg protein) for 20 min at 30° in a final volume of 50 μL containing 20 mM Hepes, 3 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol (pH 7.4), 15–25 μg (40S ribosomal proteins and 50 μM [$\gamma^{32}P$]ATP (0.5–2 μCi/nmol). When assays were performed with supernatants from cells treated with Br-cAMP, the thermostable inhibitor of PKA was added. The reaction was initiated by adding supernatant and stopped by adding concentrated electrophoresis sample buffer. Reaction products were separated by SDS-electrophoresis on 15% polyacrylamide gels [27]. The gells were stained with Coomassie blue, dried and autoradiographed with Kodak XAR5 films. ^{32}P was incorporated into a single band of ribosomal protein, the S6 band [16]. No phosphorylation was seen at this level in the absence of 40S as currently controlled. This band was excised from the dried gels and ^{32}P was quantified by counting. Protein kinases A and C were assayed as in Ref. 28.

IGF1 binding. IGF1 binding was measured as previously described [29]. Briefly, cells were washed with culture medium (DMEM/F12) and incubated with DMEM/F12 containing 0.1 nM [^{125}I]IGF1 for 5 hr at 4°. Cells were washed five times with ice-cold PBS and the bound radioactivity was released using 1 N NaOH. Non specific binding (NS) was defined as the radioactivity bound in the presence of 10 μM unlabelled insulin.

RESULTS

Effect of various inhibitors of cell proliferation on S6 kinase activation by aFGF and insulin

All agents tested significantly reduced the stimulation of S6 kinase by insulin or aFGF (Table 1). MMS gave reductions of 42% (insulin) and 44% (aFGF). NMHE gave reductions of 23% (insulin) and 44% (aFGF). Ditercalinium reduced stimulation by 47% (insulin) and 36% (aFGF). CCNU reduced stimulation by 60% (insulin). BCNU reduced stimulation by 105% (insulin) and 115% (aFGF).

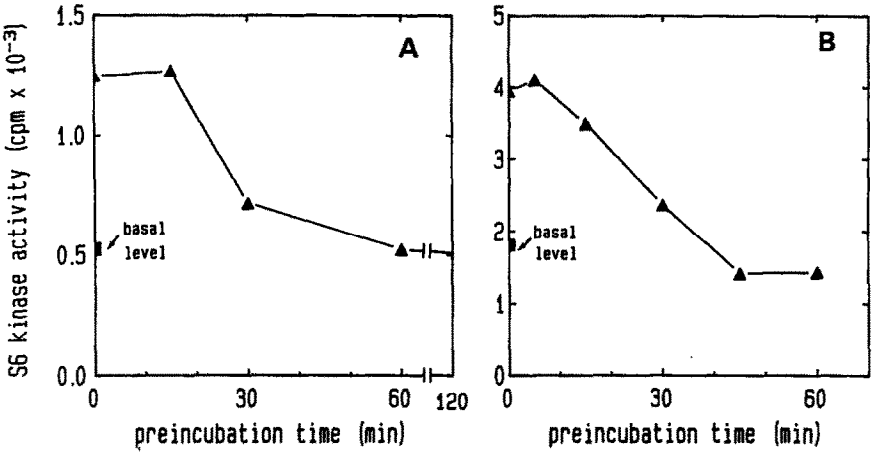

Fig. 1. Time dependence of the BCNU effect on insulin (A) or aFGF (B) stimulated S6 kinase. Astrocytes were preincubated with 50 μM BCNU for various times, washed with medium, stimulated with 1 μM insulin or 3 nM aFGF for 45 min and homogenized. S6 kinase activity was assayed on the post-microsomal fraction. Enzyme activity was determined by analyzing the ^{32}P-incorporation into the S6 band of rat liver 40S ribosomal subunit.

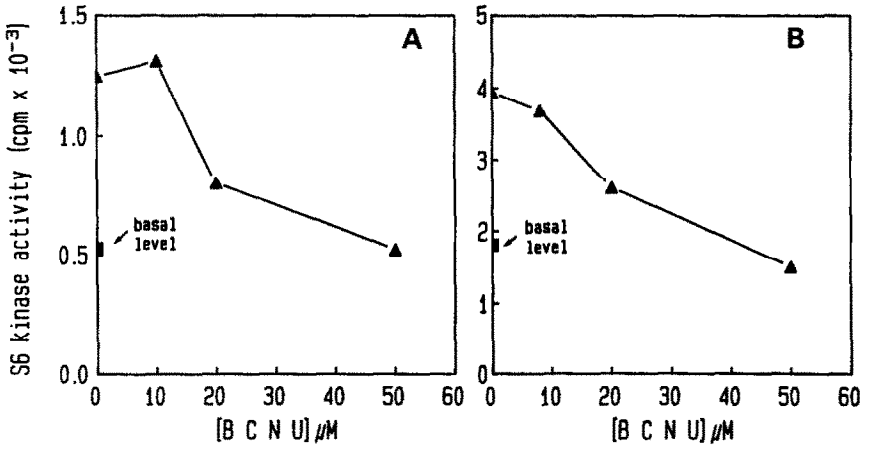

Fig. 2. Concentration dependence of the BCNU effect on insulin (A) or aFGF (B) stimulated S6 kinase. Astrocytes were preincubated with 0–50 μM BCNU for 60 min, washed with medium, stimulated with 1 μM insulin or 3 nM aFGF and extracted for S6 kinase assay. For further details see legend to Fig. 1.

BCNU (50 μm) therefore completely prevented S6 kinase activation by insulin or aFGF. BCNU also reduced the S6 kinase activity measured in the absence of growth factor by 30%. BCNU had similar effects when S6 kinase was stimulated by 10 nM IGF1 instead of 1 μM insulin (not shown).

Time and concentration dependence of the effect of BCNU on the activation of S6 kinase by insulin and aFGF

As effects of BCNU on cell proliferation vary with the duration of treatment and BCNU concentration, we examined their influence on S6 kinase activity.

Effect of the duration of the treatment. Figure 1 shows the time course of inhibitory action of 50 μM BCNU on the stimulation of S6 kinase by growth factors. Pretreatment for 15 min had no effect; 30-min pretreatment reduced stimulation of S6 kinase by 83% (insulin) or 78% (aFGF). Stimulation by insulin or aFGF was completely blocked by 60-min pretreatment with BCNU. Pretreatment for 60 min with 50 μM BCNU also prevented the response to growth factors when the stimulation of S6 kinase by these factors was examined 24 or 48 hr after the pretreatment (not shown). Cell viability was checked by trypan blue exclusion. There was no difference between test and control cells 24 hr after BCNU treatment. But 30% of the BCNU-treated cells were dead 48 hr after BCNU treatment.

Effect of BCNU concentration. Astrocytes were pretreated for 60 min with concentrations of BCNU between 0 and 50 μM and stimulated with insulin or

Table 2. Effect of BCNU on S6 kinase activation by TPA or Br-cAMP

Pretreatment with BCNU	S6 kinase activity (cpm ^{32}P incorporated)		
	Basal	Br-cAMP stimulated	TPA stimulated
–	890	2200	3700
+	810	1760	1460

ND, not determined.

Astrocytes were incubated with 50 μM BCNU for 60 min, washed with medium and treated with 100 nM TPA or 1 mM Br-cAMP for 45 min. The cells were extracted and S6 kinase activity was assayed as in Fig. 1. The typical experiment which is presented reflects three independent experiments.

aFGF. Figure 2 shows growth factor-stimulated S6 kinase activity as a function of BCNU concentration. Up to 10 μM, BCNU had little or no effect, 20 μM reduced insulin stimulation by 75% (Fig. 2A) and aFGF stimulation by 63% (Fig. 2B). BCNU (50 μM), as shown above, completely prevented S6 kinase activation by either growth factor.

Effect of BCNU on S6 kinase activation by TPA and cAMP. As we have previously shown that astrocyte S6 kinase can be activated by treating the cells with TPA or Br-cAMP, the effect of BCNU on S6 kinase activation by these agents was examined. Table 2 shows that 50 μM BCNU strongly inhibited S6 kinase activation by TPA and had less effect on Br-cAMP activation. Treating cells with 50 μM BCNU had no effect on PKA and PKC (not shown).

Absence of direct BCNU interaction with IGF1 receptors or S6 kinase. We attempted to identify the steps in the mechanism of S6 kinase activation impaired by BCNU by examining the direct effect of BCNU on IGF1 binding to cell surface receptors and S6 kinase. Figure 3 shows that IFG1 binding was not affected by BCNU pretreatment.

Otherwise, there was no change in S6 kinase activity when cytosol from unstimulated cells or from insulin-stimulated cells was incubated with 50 μM BCNU for 10–45 min (not shown).

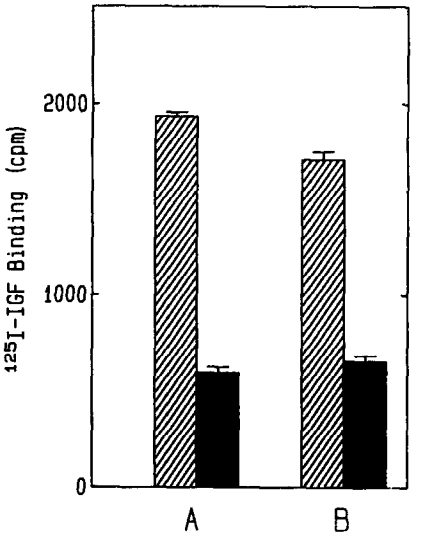

Fig. 3. Effect of BCNU on IGF1 binding to astrocyte surface receptors. Cells were incubated with (A) or without (B) 50 μM BCNU for 60 min. The cells were washed with medium and IGF1 binding was assayed. The typical experiment which is presented reflects three independent experiments. Values are the means of three dishes ± SEM (▨) total binding, (■) non specific binding.

DISCUSSION

The results of this study indicate that MMS, NMHE, ditercalinium and BCNU all inhibit S6 kinase activation promoted via aFGF and IGF1 receptors. These compounds were used at concentrations known to inhibit cell proliferation [21, 22, 24]. In these conditions, BCNU was the most effective inhibitor of S6 kinase activation. But the other compounds might be active at higher concentrations than those tested.

Pretreatment of astrocytes with 50 μM BCNU for 60 min completely prevented S6 kinase activation by the growth factors without immediately killing the cells. This effect of BCNU is probably not due to an inhibition of macromolecular synthesis since S6 kinase activation by growth factors is not suppressed when macromolecular synthesis is blocked [2–5] and since the time required to observe this inhibition of S6 kinase by BCNU is short. BCNU, probably, acts at a step between the growth factor receptors and the S6 kinase enzyme, since BCNU had no effect on either the binding of IGF1 to its cell surface receptors or on the S6 kinase activity of cytosols incubated with BCNU. In cells stimulated via IGF1 receptors, the steps impaired by BCNU may be the tyrosine kinase associated with the IGF1 receptors or some substrates of this kinase leading to the S6 kinase activation. This hypothesis is being explored. The effects obtained with IGF1 (insulin), aFGF, TPA and cAMP may be due to an action of BCNU on a common step in their S6 kinase activation pathway but we cannot exclude that BCNU acts on different steps in each pathway. At least, the inhibition of the effect of Br-cAMP and TPA on S6 kinase activation is not due to a direct action of BCNU on PKA or PKC, respectively.

It is also possible that BCNU impairs other transduction mechanisms such as c-*fos* induction.

Future work should be performed to clarify the early effects of BCNU on transduction.

The effect of BCNU on S6 kinase activation might contribute to the effect of BCNU on cell multiplication. The time and concentration dependence of the action of BCNU on S6 kinase activation are consistent with those for its action on cloning efficiency and the rates of proliferation of various cells [24]. Most of the authors have until now considered DNA as the only target for the antiproliferative effect of BCNU [24, 30], but the action of BCNU on DNA *in situ* (alkylation and cross-linking) does not seem to explain completely the cytoxicity of this drug [30]. Targets other than DNA are perhaps implicated in this phenomenon. BCNU also interacts with both RNA and proteins. It promotes carbamylation of glutathione reductase [31, 32], chymotrypsin [33] and tubulin [34] and may also interact with nuclear matrix proteins [35]. Studies on resistant cells might clarify the role of inhibition of transduction mechanisms leading to S6 kinase activation in the antiproliferating effect of BCNU.

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