Effects of Skyrin, a Receptor-Selective Glucagon Antagonist, in Rat and Human Hepatocytes

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Peptidic glucagon antagonists have been shown to lower blood glucose levels in diabetic models (1–3), but attempts to identify small molecular weight glucagon receptor–binding antagonists have met with little success. Skyrin, a fungal bisanthroquinone, exhibits functional glucagon antagonism by uncoupling the glucagon receptor from adenylate cyclase activation in rat liver membranes (1). We have examined the effects of skyrin on cells transfected with the human glucagon receptor and on isolated rat and human hepatocytes. The skyrin used was isolated from *Talaromyces wortmanni* American *Type Culture* Collection 10517. In rat hepatocytes, skyrin (30 µmol/l) inhibited glucagon-stimulated cAMP production (53%) and glucose output (IC_{50} 56 µmol/l). There was no detectable effect on epinephrine or glucagon-like peptide 1 (GLP-1) stimulation of these parameters, which demonstrates skyrin's selective activity. Skyrin was also evaluated in primary cultures of human hepatocytes. Unlike cell lines, which are largely unresponsive to glucagon, primary human hepatocytes exhibited glucagon-dependent cAMP production for 14 days in culture (EC_{50} 10 nmol/l). Skyrin (10 µmol/l) markedly reduced glucagon-stimulated cAMP production (55%) and glycogenolysis (27%) in human hepatocytes. The inhibition of glucagon stimulation was a specific property displayed by skyrin and oxyskyrin but not shared by other bisanthroquinones. Skyrin is the first small molecular weight nonpeptidic agent demonstrated to interfere with the coupling of glucagon to adenylate cyclase independent of binding to the glucagon receptor. The data presented in this study indicate that functional uncoupling of the human glucagon receptor from cAMP production results in metabolic effects that could reduce hepatocyte glucose production and hence alleviate diabetic hyperglycemia.
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ATCC, American *Type Culture* Collection; BSA, bovine serum albumin; DMEM, Dulbucco's modified Eagle's medium; GLP-1, glucagon-like peptide 1; KIU, kallikrein inhibitor units; PBS, phosphate-buffered saline; RIA, radioimmunoassay.

Glucagon is a 29–amino acid polypeptide hormone that contributes to the regulation of blood glucose levels. Counterregulatory to insulin, glucagon produces an increase in blood glucose by stimulating glycogenolysis and gluconeogenesis in the liver. Glucagon action in the liver is mediated via glucagon binding and signaling through the glucagon receptor, which has now been cloned from both rodent and human tissue (2,3). This receptor is a member of the family of seven transmembrane domain G-protein–coupled receptors. Intracellular signaling by the glucagon receptor involves the activation of adenylate cyclase and a consequent increase in intracellular cAMP concentrations. The glucagon receptor has also been reported to be independently coupled to a Ca^{2+}-mediated signaling pathway (4). In both type 1 and type 2 diabetes, inadequate insulin production results in a relative hyperglucagonemic state. This condition promotes increased liver glucose output and contributes to the hyperglycemia that characterizes both forms of diabetes. An agent that could reduce the response of the liver to glucagon would be beneficial for the treatment of diabetic patients, which has been demonstrated experimentally with peptide antagonists that bind to the glucagon receptor (5) and with antiglucagon antibodies (6,7).

The fungal bisanthroquinone skyrin has been shown to inhibit glucagon-stimulated cAMP production from rat liver plasma membranes (1). This antagonistic effect of skyrin is not caused by competition with glucagon for binding to its receptor but instead to a functional antagonism that appears to specifically uncouple the glucagon receptor from adenylate cyclase activation.

To date, skyrin's effects have been demonstrated only in preparations of rat liver membranes. Therefore, we examined the effects of skyrin on glucagon-stimulated cAMP production and on consequent biological responses in Chinese hamster ovary (CHO) cells transfected with the cloned human glucagon receptor in freshly isolated rat hepatocytes and in primary cultures of human hepatocytes.

METHODS

Isolation of skyrin. Several potential producers of skyrin were identified from the American *Type Culture* Collection (ATCC) and tested for skyrin production. *Talaromyces wortmanni* (ATCC 10517) was grown in potato dextrose broth (Difco). The cell paste from a 500-ml culture was extracted with 5 × 100 ml acetone and filtered. The filtrate was evaporated to dryness, reconstituted with 90% methanol, and extracted with 2 × 150 ml hexane. The methanol/water layer was diluted with water and further extracted with 2 × 200 ml CH_2Cl_2. The CH_2Cl_2 layer was applied to a 100-ml SiO_2 column and eluted with hexane:acetone (6:4,

vol:vol). The eluate was applied to a preparative high-performance liquid chromotography column (YMC ODS-AM [20 × 250 mm]) with a solvent phase of 70% CH_2CN/0.05% TFA, yielding a single peak of skyrin (40 mg).

Cloning and expression of human glucagon receptor in CHO cells. A single cDNA clone was isolated from the Stratagene human liver cDNA library. This clone was sequenced and determined to contain the glucagon receptor sequence from +631 to the polyadenylation site at +1789 (8). Reverse transcription–polymerase chain reaction (RT-PCR) of Clontech human liver RNA for isolation of the 5′ end of the gene yielded the PCR sequence comprising nucleotide bases –76 to +872. The two fragments were ligated using the Sac II site at +684 and subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). CHO cells were transfected with the glucagon receptor as follows: CHO cells (10^5 in 2 ml Dulbucco's modified Eagle's medium (DMEM)-Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum [Gibco BRL, Gaithersburg, MD] were cultured overnight in a 35-mm culture dish at 37°C in 95% air and 5% CO_2. The DNA encoding the glucagon receptor sequence was cloned into pcDNA3 and 0.2 µg, mixed with 100 µl LipofectAMINE and 100 µl Optimem (Gibco BRL), and incubated 30 min at room temperature. After incubation, an additional 800 µl Optimem was added to the DNA complex and mixed. The incubation medium was removed from the cells which were then washed with phosphate-buffered saline before the addition of the DNA. Cells and DNA were incubated at 37° for 6 h after which 0.9 ml DMEM-Ham's F12 medium + 20% heat-inactivated serum was added and the incubation continued overnight. The medium was replaced on Day 2, and on Day 3, the cells were split 1:10 into 100-mm dishes in culture medium containing 400 µg/ml G418 (Gibco BRL). The cells were maintained in the culture for 14 days, replacing the medium every 2–4 days as needed, after which colonies were picked and tested for expression of the glucagon receptor.

[^{125}I]-glucagon binding to CHO cells expressing the human glucagon receptor (hGGNR cells). Cells (5×10^4) were plated overnight in 2-cm^2 wells, then incubated for 60 min at room temperature in HEPES-buffered saline containing 0.25 nmol/l [^{125}I]-glucagon with or without unlabeled glucagon (1 nmol/l to 10 µmol/l). Nonspecific binding was determined in the presence of 10 µmol/l unlabeled glucagon. At the end of the incubation, the medium was removed by aspiration and the cells washed twice with 0.1-ml ice-cold buffered saline (150 mmol/l NaCl, 50 mmol/l HEPES, pH 7.4), after which 150 µl 0.1mol/l NaOH was added to each well. The culture plates were placed on a rotating platform for 20 min at room temperature, and the contents of the wells were collected and the radioactivity counted.

Preparation of membranes from rat liver and hGGNR cells. Livers from adult male Sprague-Dawley rats were homogenized and diluted to 50 vol in cold buffer (50 mmol/l Tris, 0.1% bovine serum albumin (BSA), pH 7.4). The homogenate was centrifuged at 30,000*g* for 15 min at 4°C. The supernatant was discarded, and the pellets were resuspended to the original volume and centrifuged again (30,000*g* for 15 min at 4°C.). The supernatant was discarded and the pellets weighed and stored frozen at –80°C until resuspended for use.

Cells were grown to confluence and harvested by washing the monolayers with cold Dulbecco's calcium-magnesium-free phosphate-buffered saline (PBS). The cells were then scraped into cold PBS containing aprotinin (100 kallikrein inhibitor units [KIU]/ml). The cells were centrifuged at 180g to sediment the cells without breaking. The cell pellets were resuspended in cold PBS with aprotinin, homogenized for 20 s with the PT-DA 1212/2 probe of a Polytron PT1200, then centrifuged (30,000g) for 10 min. The supernatant was discarded and the membrane-containing pellets were resuspended and recentrifuged. The final pellet(s) were weighed and stored frozen at –80°C until resuspended for use.

[^{125}I]-glucagon binding to membranes from rat liver and hGGNR cells. Membranes were resuspended to 20 mg/ml in buffer (50 mmol/l Tris, 5 mmol/l $MgCl_2$, 0.01% bacitracin, and 100 KIU/ml aprotinin). Binding assays were conducted in 96-well plates. The binding reaction mixture comprised 50 pmol/l [^{125}-I]-labeled glucagon and 1 mg wet weight of membranes in a total volume of 200 µl of buffer containing 50 mmol/l Tris pH 7.4, 5mmol/l $MgCl_2$, 0.1% BSA, and 0.01% bacitracin. Plates were incubated for 1 h at room temperature, then 150 µl of each reaction mixture was transferred to the corresponding well in a millipore multiscreen glass-fiber filter plate, which had been pretreated by addition of 200 µl/well of 0.2% polyethyleneimine, incubated at 4°C for 2 h, washed twice with 50 mmol/l Tris, pH 7.4, then filled with 50 mmol/l Tris, pH 7.4, 3 mg/ml BSA and incubated for 10 min at room temperature. The pretreated filter plates were drained by aspiration before use. After the addition of the binding reaction mixture, the filter plate was aspirated and each well washed five times with 50 mmol/l Tris buffer, pH 7.4 containing 0.1% BSA. The filter plate was then air-dried, 25µl/well of scintillation cocktail was added, and the plate was counted on a microbeta plate reader (Wallac, Gaithersburg, MD).

Hepatocyte experiments. Rat hepatocytes were isolated by collagenase digestion of livers from fed male Sprague-Dawley rats, as described previously (9). Cells were suspended in Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 2.5 mmol/l $CaCl_2$ and 1% (wt/vol) gelatin. Hepatocyte suspensions at a concentration of ~6.0×10^6 cells/ml were incubated in 25-ml flasks in a 37°C shaking water bath and continuously gassed with 95% O_2/5% CO_2. In experiments in which glucose output was measured, cells were preincubated for 5 min in the absence or presence of skyrin and glucagon (0.3 nmol/l final concentration) or epinephrine (10 µmol/l) was added for 20 min. Aliquots of cell suspension were removed after the 5 min preincubation and after the 30 min of incubation. In experiments in which glucose output was measured, after centrifugation to sediment the cells, the glucose concentration of the supernatant buffer was measured. In experiments in which cAMP production was measured, cells were preincubated for 15 min in the absence or presence of skyrin, after which glucagon (3 nmol/l) was added for a further 5-min incubation. To terminate the experiment, cells were pelleted by centrifugation and then extracted with 50% trichloroacetic acid. The extract was stored at –20°C until analyzed for cAMP concentration by radioimmunoassay (RIA) (NEN, Boston, MA).

Primary cultures of human hepatocytes (Clonetics, San Diego, CA) were maintained on six-well collagen-coated plates in Williams Medium E supplemented as described previously (10) for 4–14 days before use. Production of cAMP was measured as described above for rat hepatocytes, except that the assay was scaled to six-well assay format (total volume 1 ml/well). The assay was terminated by aspirating the media and directly extracting the cell monolayer with 10% trichloroacetic acid for 10 min at 37°C, followed by scraping and titration. The extract was then recovered by transfer of the material to a microfuge tube, centrifugation, and storage of the supernatant at –20°C until analyzed for cAMP concentration, as described above.

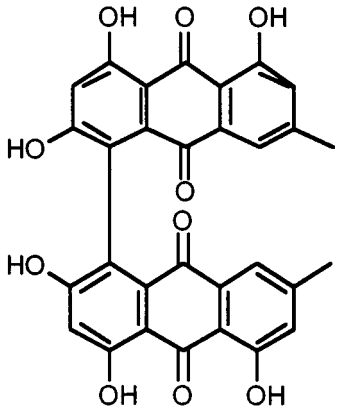


FIG. 1. Structure of skyrin.

RESULTS

CHO cells, which were stably transfected with the human glucagon receptor, exhibited specific binding of [^{125}I]-glucagon (Fig. 2). Scatchard analysis of binding data indicated two classes of site with K_d values of 1.6 and 24.2 nmol/l. There were ~6.4×10^6 receptors per cell. Binding of [^{125}I]-glucagon to membranes prepared from these cells was unaffected by the inclusion of 30 µmol/l skyrin (Fig. 1) in the incubation (Fig. 3*A*). Similarly, skyrin did not affect binding of [^{125}I]-glucagon to rat liver membranes (Fig. 3*B*). Skyrin (50 µmol/l) did not affect the ability of a range of glucagon concentrations (1–100 nmol/l) to displace [^{125}I]-glucagon either from membranes prepared from CHO cells transfected with the human glucagon receptor (Fig. 4*A*) or from rat liver membranes (Fig. 4*B*). The IC_{50} (calculated using Deltagraph software) for displacement of [^{125}I]-glucagon from the receptor expressed in hGGNR cells was 3.5 nmol/l in the absence of skyrin and 3.2 nmol/l when 50 µmol/l skyrin was present. The corresponding values for the rat liver receptor were 4.3 and 5.5 nmol/l, respectively.

The binding of glucagon to the receptor expressed in hGGNR cells was functionally coupled to cAMP production.

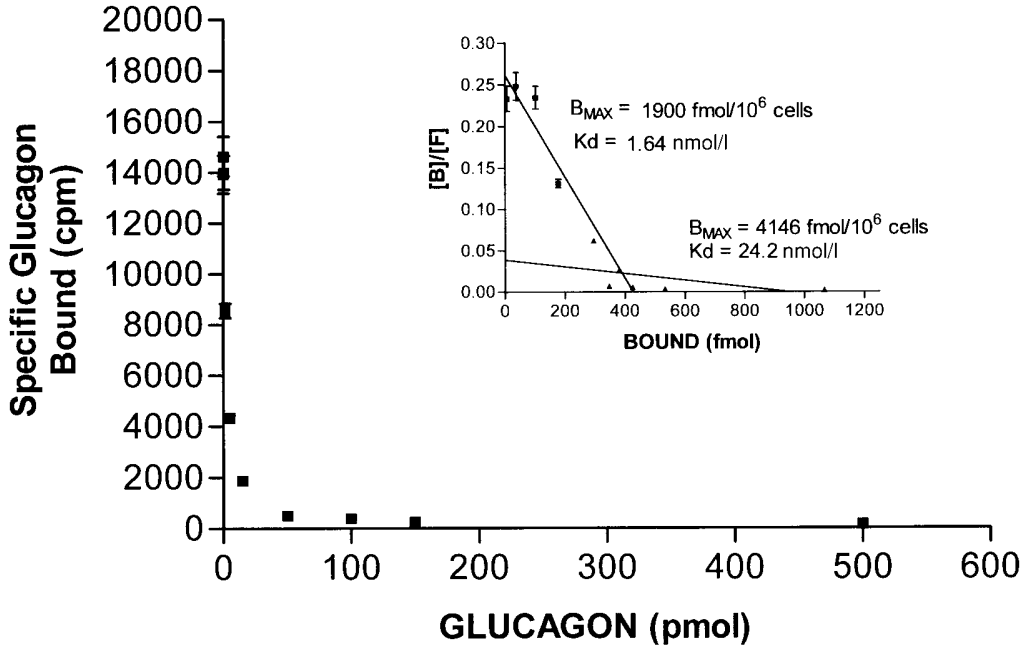


FIG. 2. Specific binding of [^{125}-I]-labeled glucagon to hGGNR cells. CHO cells stably transfected with the human glucagon receptor were treated for 60 min with 0.25 nmol/l [^{125}I]-glucagon and the indicated concentrations of unlabeled glucagon. Each data point represents the means ± SE of quadruplicate measurements. Where no error bar is visible, the size of the symbol exceeds that of the error.

Incubation of hGGNR cells with glucagon (3 nmol/l for 5 min) stimulated cAMP production >10-fold, from 2.2 ± 0.2 µmol/l to 50.2 ± 3.4 µmol/l. A 15-min preincubation with skyrin (30 µmol/l) before the addition of glucagon reduced the subsequent production of glucagon by ~30%, so that the cAMP concentration after 5 min of glucagon stimulation was 35.0 ± 4.7 µmol/l (data are means ± SE for four measurements and the effect of skyrin was significant at the $P < 0.01$ level). Comparable effects were observed in isolated rat hepatocytes (Fig. 5) in which skyrin (30 µmol/l) inhibited glucagon-stimulated cAMP production by 50% but had no effect on basal levels of cAMP. This inhibition of glucagon-stimulated cAMP production translated into a reduction in glucose output; skyrin inhibited glucagon-stimulated glucose output from isolated rat hepatocytes in a concentration-dependent manner (Fig. 6) but was without effect on either basal or epinephrine-stimulated rates of glucose output. The EC_{50} of skyrin for inhibition of glucagon-stimulated glucose output from rat hepatocytes was calculated to be 57 µmol/l. Cultured human hepatocytes were responsive to glucagon, although the concentration-response relationship for effects on cAMP production differed from that observed in rat hepatocytes. Glucagon-stimulated cAMP production from human hepatocytes in a concentration-dependent manner over the range 1–100 nmol/l (Fig. 7), with a concentration of 25 nmol/l giving a stimulation ~30% of maximal. An equivalent percentage of stimulation was achieved by 3 nmol/l glucagon in rat hepatocytes. The magnitude of the effect of glucagon on cAMP production also differed between the two systems; a 30% maximal stimulation represented a 20-fold increase in cAMP production in human hepatocytes and only a 2- to 3-fold increase in rat hepatocytes. Because the two types of hepatocytes were prepared under very different conditions, it is not possible to assess the degree to which these differences reflect actual species differences as opposed to experimental artifact. Skyrin inhibited the glucagon stimulation of cAMP production in human hepatocytes (Table 1) as it did in rat hepatocytes. A concentration of 10 µmol/l skyrin inhibited 3 nmol/l glucagon stimulation by 75%, but human cell availability did not permit a more extensive concentration-response analysis.

The inhibition of glucagon stimulation was a specific property displayed by skyrin and oxyskyrin but not shared by other bisanthroquinones tested. The effects on glucagon stimulation of rat hepatocyte glucose output by a series of structural analogs of skyrin are shown in Table 2. Skyrin's oxygenated derivative oxyskyrin (entry 2) produced an effect similar to that of skyrin itself, with an IC_{50} = 106 µmol/l. Oxyskyrin also showed similar selectivity to skyrin, inhibiting glucagon-stimulation of glucose output, but having no effect on epinephrine stimulation (data not shown.) The bis-anthraquinone ring system appears to be necessary for the effect because related mono-anthraquinones (entries 3 and 4) failed to inhibit glucagon stimulation of glucose output at a concentration of 100 µmol/l. In particular, emodin (entry 3), the anthraquinone directly related to the skyrin dimer, was almost entirely inactive at this concentration. Another bis-anthraquinone, rugulosin (entry 5), was tested, as were a series of analogous naphthopyrones (entries 6–10). None of these compounds inhibited glucagon stimulation of glucose output at 100 µmol/l, including cephalochromin (entry 6), a close analog of skyrin, which weakly inhibited glucagon receptor binding (IC_{50} 39 µmol/l). These results indicate that both the overall structure and the functionality pattern of skyrin and oxyskyrin are important for producing the glucagon-antagonistic effects observed.

DISCUSSION

The bihormonal hypothesis for the pathogenesis of diabetes (11,12) proposes that insulin deficiency causes impaired glucose utilization, whereas elevated circulating levels of

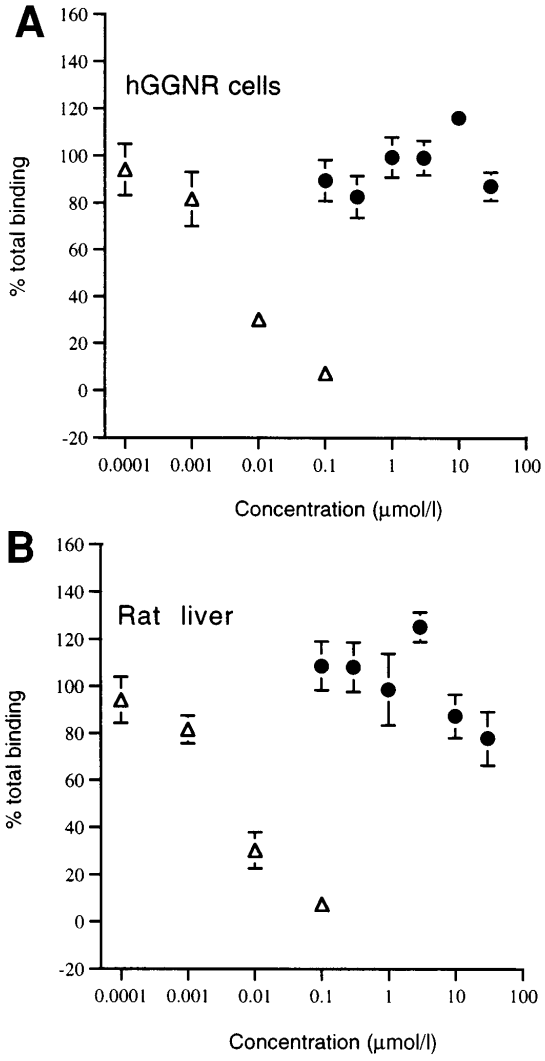


FIG. 3. Effect of skyrin on specific binding of [^{125}-I]-labeled glucagon to membranes prepared from hGGNR cells and from rat liver. Membranes were prepared and incubated as described in RESEARCH DESIGN AND METHODS in the presence of the specified concentrations of glucagon (▲) or skyrin (●). Results are expressed as a percentage of total binding (no glucagon or skyrin present). Total binding was 1.6 ± 0.1 fmol/mg wet weight for hGGNR cell membranes and 0.7 ± 0.1 fmol/mg wet weight for rat liver membranes. Data are means ± SE of 4–8 measurements obtained in a total of four independent experiments. Where no error bar is visible, the size of the symbol exceeds that of the error.

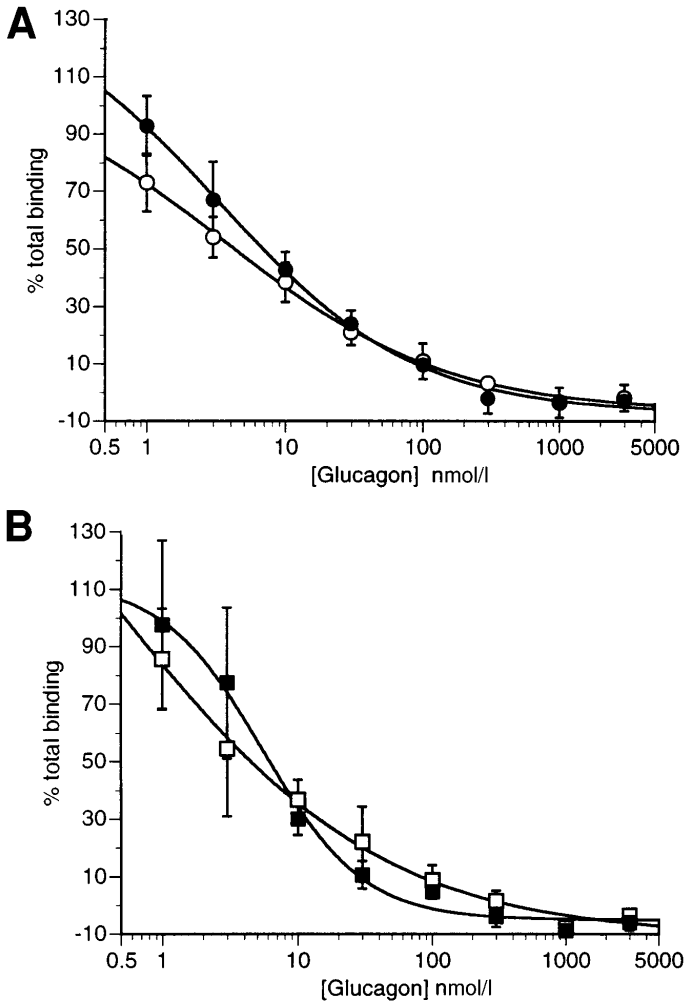


FIG. 4. Effect of skyrin on displacement by glucagon of [^{125}-I]-labeled glucagon from membranes prepared from hGGNR cells (panel *A*) and from rat liver (panel *B*). Membranes were prepared as described in RESEARCH DESIGN AND METHODS and incubated in the presence of the specified concentrations of glucagon alone (○, □) or glucagon plus 50 μmol/l skyrin (●, ■). Results are expressed as a percentage of total binding measured in the absence of unlabeled glucagon. Total binding was 1.2 ± 0.1 fmol/mg wet weight for hGGNR cell membranes and 0.6 ± 0.1 fmol/mg wet weight for rat liver membranes. Data are means ± SD of quadruplicate measurements from one of three similar experiments. Where no error bar is visible, the size of the symbol exceeds that of the error.

glucagon stimulate overproduction of glucose (13). It follows that an agent that inhibits the action of glucagon would have considerable therapeutic potential for the treatment of diabetes. A number of peptide analogs of glucagon that incorporate amino acid substitutions and/or modifications have been synthesized and tested for activity in a variety of in vitro and in vivo systems. In vivo data obtained using these peptides support the hypothesis that a glucagon receptor antagonist can effectively lower circulating blood glucose levels (5,14,15), whereas in vitro data indicate that it is possible to design pure glucagon antagonists by modifying the amino acid sequence of native glucagon (16). These peptides are classified as antagonists because they bind to the glucagon receptor without activating the associated signal transduction pathway. Extensive efforts to design or otherwise identify nonpeptide glucagon antagonists that meet this criterion have met with limited success, and only comparatively recently have compounds have been reported that act in vitro as receptor-binding antagonists (17–21). A recent scientific meeting report described antagonism by one of these compounds of the hyperglycemic effect of glucagon in human subjects, but the structure of the agent was not disclosed (22).

Skyrin is a bisanthroquinone that was identified as an active constituent of a fungal fermentation of a Penicillium sp. that inhibited glucagon stimulation of cAMP production from a preparation of rat liver plasma membranes (1). Skyrin acts as a functional antagonist with respect to glucagon action.

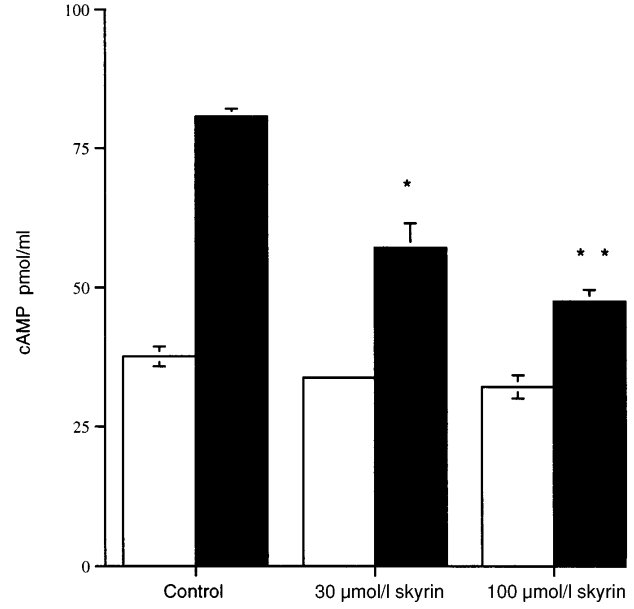


FIG. 5. Effect of skyrin on cAMP production in rat hepatocytes. Hepatocytes were preincubated with the specified concentration of skyrin or with an equivalent volume of DMSO (0.1%, vol/vol) for 15 min, followed by a 5-min incubation with glucagon (3 nmol/l). The concentration of cAMP in cells + medium was measured by RIA and the results expressed as a percentage of the basal cAMP production (no glucagon and no skyrin). Basal cAMP production under these conditions was 32.2 ± pmol/ml. Data are means ± SE for two to four separate measurements for each condition. *P < 0.05 vs. glucagon alone. □, without glucagon; ■, with 3 nmol/l glucagon.

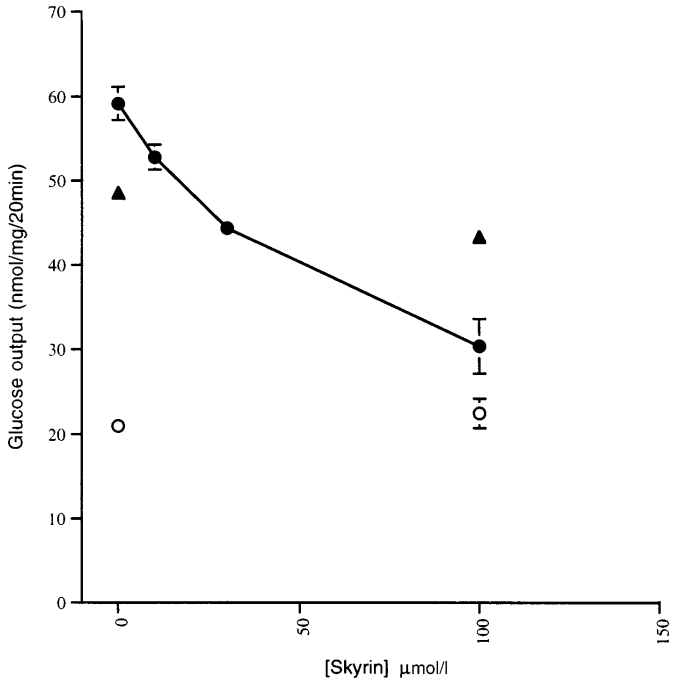


FIG. 6. Effect of skyrin on glucose output by rat hepatocytes. Hepatocytes were preincubated for 5 min with the specified concentration of skyrin or with an equivalent volume of DMSO (0.1%, vol/vol), then incubated for 20 min with 0.3 nmol/l glucagon (●) or 10 µmol/l epinephrine (▲) or without further additions (○), at which time glucose was measured in the medium. Data are means ± SE for quadruplicate measurements. *P < 0.05 vs. corresponding glucose output in the absence of skyrin.

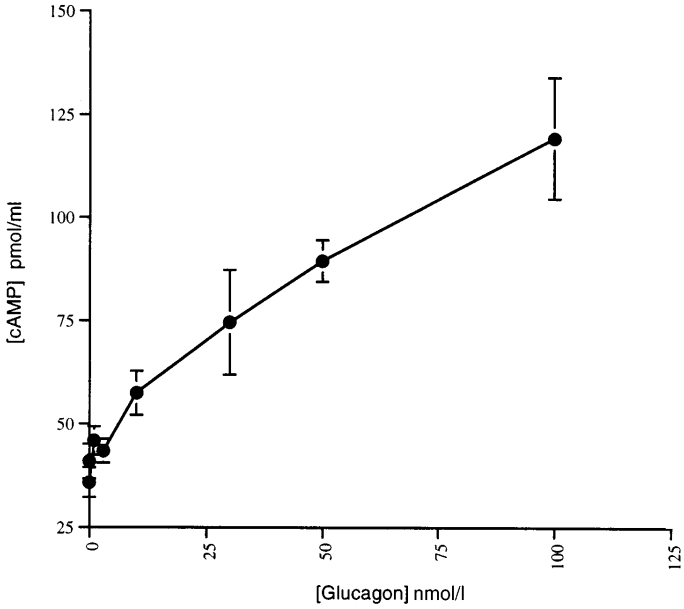


FIG. 7. Effect of glucagon on cAMP production in primary human hepatocytes. Hepatocytes were incubated with glucagon at the concentrations specified for 5 min. Data are means ± SE for 3–10 measurements made in three independent experiments.

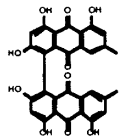
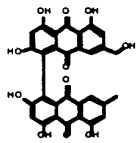
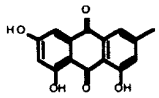
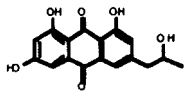
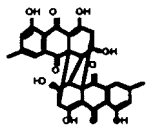
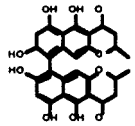
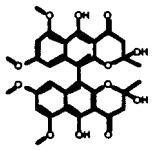
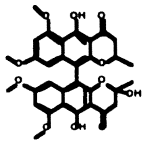
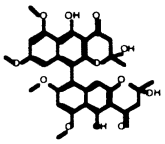
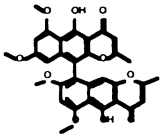
Unlike the peptidic antagonists that have been shown to lower blood glucose in animal models of diabetes (5,14,15), skyrin does not inhibit the binding of glucagon to its receptor but instead seems able to uncouple the receptor from adenylate cyclase activation (1). In the liver, binding of glucagon to its receptor on the hepatocyte plasma membrane serves, via interaction with a stimulatory G-protein, Gs, to activate adenylate cyclase. The cAMP produced as a result activates protein kinase A, with a consequent increase in both glycogen breakdown and gluconeogenesis, leading to an increase in glucose output. We have demonstrated that in hepatocytes from rat and from human sources, skyrin can block this signal transduction sequence, such that the interaction of glucagon with its receptor does not result in an increase in cAMP production. A similar separation between receptor binding and signal transduction was observed in hGGNR cells. In this artificial system, glucagon binding to the transfected human recep-

TABLE 1
Effect of skyrin on basal and glucagon-stimulated cAMP production in primary human hepatocytes

	cAMP µmol/l
Control	25.0 ± 3.5 (2)
Glucagon (25 nmol/l)	422.0 ± 24.8 (2)
Skyrin (10 µmol/l)	41.0 ± 2.8 (2)
Glucagon (25 nmol/l) + skyrin (10 µmol/l)	168.0 ± 27.6 (2)*

Data are means ± SE (n) measurements from a representative of two independent experiments. Hepatocytes were preincubated 15 min with skryin (10 µmol/l) or an equivalent volume of vehicle (DMSO 0.1%, vol/vol), followed by a 5-min incubation with glucagon (25 nmol/l). The total cAMP content of cells + medium was measured by RIA. *P < 0.05 vs. glucagon alone.

TABLE 2
Structure-activity relationship of skyrin and structurally related compounds

Entry	Compound name	Structure	Rat liver membranes Binding IC_{50}	Rat hepatocyte glucose output EC_{50}	Rat hepatocyte glucose output % Inhibition (concentration tested)
1	Skyrin		>300	42	
2	Oxyskyrin		144	106	
3	Emodin				3 (100)
4	Isorhodoptilometrin		>300		6 (100)
5	Rugulosin		42		0 (690)
6	Cephalochromin		39		6 (827)
7			299		8 (1,649)
8			340		5 (1,699)
9			>165		1 (100)
10			>175		7 (100)

Data are expressed as micromoles per liter. EC_{50}, concentration of compound that inhibited by 50% the effect of 3 nmol/l glucagon glucose output.

tor stimulated cAMP production. This response was specifically blocked by skyrin in a concentration-dependent manner. The effect of skyrin was highly specific for the glucagon receptor, because skyrin had no effect on glucagon-like peptide 1 (GLP-1)–stimulation of cAMP production by CHO cells transfected with the GLP-1 receptor, which belongs to the same subfamily of G-protein–coupled receptors as the glucagon receptor (data not shown).

In hepatocytes, which contain the full complement of enzymes, substrates, and cofactors necessary for glucose production, the consequence of this skyrin blockade of cAMP production was coupled to a reduction in net glucose production. The effect of skyrin appears to be specific for the glucagon-stimulated portion of glucose production from hepatocytes, since it was without effect on basal glucose output and did not significantly reduce the stimulation of glucose output by epinephrine. While the mechanism of skyrin's effect is unknown, this latter observation suggests that skyrin acts either by affecting the communication of the glucagon receptor with its specific G-proteins or by modulating the interaction of those G-proteins with adenylate cyclase. It is possible that skyrin interacts with a secondary site, possibly within the transmembrane region of the receptor to exert an allosteric effect that lowers the affinity for glucagon at the primary binding sites located in the N-terminal and extracellular loops. Alternatively, skyrin may act to interfere with transmission of the signal from the extracellular binding domains to the transmembrane and cytoplasmic domains. The present study does not provide data that would enable us to distinguish between these possibilities.

The inhibition of glucagon stimulation was an activity displayed by skyrin and oxyskyrin but not shared by other bisanthroquinones tested. Indeed, the structure-activity relationship of the glucagon-antagonist activity is fairly narrow, with a number of structural analogs being functionally inactive. This argues that skyrin has a specific site of action, rather than acting, for example, via widespread nonspecific membrane-modulating effects.

Anthraquinones, bisanthroquinones, and other mycotoxins have been demonstrated to have a number of biological activities, including mutagenic, cytotoxic, and antibacterial activity in whole-cell assays (23,24). We observed no evidence of cytotoxicity by skyrin in the experiments reported here. Emodin, but not skyrin, has been shown to inhibit protein kinases and phospahatidylinositol-3-kinase (25,26). In none of these studies does skyrin distinguish itself as having a pattern of activity distinct from, or not shared by, other members of these chemical classes. Thus, these studies provide little insight into possible molecular mechanisms underlying the specific glucagon antagonistic activity of skyrin, a question that warrants further investigation and one that will need to be resolved if the therapeutic potential of skyrin, or agents like it, is to be exploited.

Skyrin is the first small molecular weight nonpeptidic agent demonstrated to interfere with the coupling of glucagon to adenylate cyclase independently of binding to the glucagon receptor. The effective consequence of this is that skyrin specifically blocks the metabolic sequelae of glucagon action in hepatocytes. Whereas the modest potency of skyrin limits its own therapeutic usefulness, it is likely to be a useful tool to explore the potential of a mechanistically novel class of antidiabetic agents. It seems possible that specific inhibition of glucagon stimulation of hepatocyte glucose output has the potential to improve glycemic control in the diabetic state.

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