Original Research

A Hydroxychalcone Derived from Cinnamon Functions as a Mimetic for Insulin in 3T3-L1 Adipocytes

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Objectives: These studies investigated the ability of a hydroxychalcone from cinnamon to function as an insulin mimetic in 3T3-L1 adipocytes.

Methods: Comparative experiments were performed with the cinnamon methylhydroxychalcone polymer and insulin with regard to glucose uptake, glycogen synthesis, phosphatidylinositol-3-kinase dependency, glycogen synthase activation and glycogen synthase kinase-3β activity. The phosphorylation state of the insulin receptor was also investigated.

Results: MHCP treatment stimulated glucose uptake and glycogen synthesis to a similar level as insulin. Glycogen synthesis was inhibited by both wortmannin and LY294002, inhibitors directed against the PI-3-kinase. In addition, MHCP treatment activated glycogen synthase and inhibited glycogen synthase kinase-3β activities, known effects of insulin treatment. Analysis of the insulin receptor demonstrated that the receptor was phosphorylated upon exposure to the MHCP. This supports that the insulin cascade was triggered by MHCP. Along with comparing MHCP to insulin, experiments were done with MHCP and insulin combined. The responses observed using the dual treatment were greater than additive, indicating synergism between the two compounds.

Conclusion: Together, these results demonstrate that the MHCP is an effective mimetic of insulin. MHCP may be useful in the treatment of insulin resistance and in the study of the pathways leading to glucose utilization in cells.

INTRODUCTION

Diabetes mellitus is the most common metabolic disease worldwide, with an estimated 1700 new cases diagnosed daily [1]. Of these, 85% to 90% of the patients have type 2 diabetes mellitus (type 2DM) [2,3], with insulin resistance playing a key role in the development of the disease [3]. Symptoms of insulin resistance include a decreased stimulation of muscle glycogen synthesis, defects in glycogen synthase activity, hexokinase activity and glucose uptake [3]. Insulin resistance in a subset of the patients with type 2DM is due to clear defects in their insulin signaling. Over 50 different mutations have been shown in the insulin receptor [4]. In addition, altered enzymatic activities, such as an increased phosphatase activity and/or seryl phosphorylation of the insulin receptor substrate by glycogen synthase kinase 3 (GSK-3) [5–7], have also been shown to be involved in some cases of type 2DM.

Compounds which augment the action of insulin or bypass the insulin receptor may be beneficial in developing long-term treatments for type 2DM. One such compound is the mineral chromium. Chromium plays a key role in the regulation of glucose metabolism and a dietary deficiency of chromium may increase the risk of developing diabetes [8]. Initial research established that chromium complexes potentiated the action of insulin in rats [9], as determined by an increase in glucose oxidation. To further study the importance of chromium in the

Abbreviations: DMEM = Dulbecco's modified Eagle's medium, G6P = glucose-6-phosphate, GS = glycogen synthase, GSK-3β = glycogen synthase kinase-3β, IBMX = 1-isobutyl-3-methylxanthine, IRS = insulin receptor substrate, MAP = mitogen-activated protein, MHCP = methylhydroxychalcone polymer, PI-3-K = phosphatidylinositol-3-kinase, PTP-1 = protein tyrosine phosphatase I, type 2DM = type 2 diabetes mellitus.

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diet and insulin signaling, various types of foods and spices were analyzed for the level of chromium present and insulin potentiating abilities. The data were also evaluated to determine if there was a correlation between chromium levels and the insulin potentiating activities. However, no correlation was observed. The extract from cinnamon had a very low level of chromium present, yet elicited a strong insulin-like response in stimulating glucose oxidation [10,11]. Incorporation of wortmannin, a PI-3-kinase inhibitor, into the glucose oxidation assay inhibited this response [12]. This suggested that there was activation upstream of PI-3-kinase, perhaps on the insulin receptor itself. To test the effect on the insulin receptor, the cinnamon extract was incubated with a truncated version of the β subunit of the insulin receptor, containing the intact kinase domain. The subunit was quickly phosphorylated, within the first minute of treatment, and the receptor was completely dephosphorylated within 30 minutes [12]. As this assay contained only the cinnamon extract, the purified truncated kinase domain and radiolabelled ATP, it can be assumed that there was direct interaction between a component in the cinnamon extract and the kinase domain. It is hypothesized that the cinnamon compound enters the cells, interacts with the intracellular kinase domain and triggers an insulin-like response. The bioactive compound from cinnamon was purified and analyzed by nuclear magnetic resonance spectroscopy. It is classified as a methylhydroxychalcone polymer (MHCP) [11]. The purified polymer of the hydroxychalcone is capable of stimulating glucose oxidation.

Many cellular responses to insulin, with numerous enzymes and regulatory proteins, have been identified over the years. The initial critical step is insulin binding to its receptor and the subsequent transphosphorylation of the kinase domains. Phosphorylation continues on toward the insulin receptor substrate 1-4 (IRS 1-4) proteins and SHC. Once phosphorylated, these proteins interact with Src homology-2 domain-containing proteins, such as the regulatory phosphatidylinositol-3-kinase (PI-3-K), the adapter proteins Grb2, Syr, Crk and Nck and other proteins [13,14]. The binding of Grb2 to SHC activates the Ras/Raf/Mitogen-activated protein (MAP) kinase kinase/MAP kinase cascade and results in nuclear activity, mitogenesis and possibly in an increase in glycogen synthesis [13,14]. Inhibition of this pathway does not block the insulin-stimulated upregulation of glucose utilization [15]. Activation of PI-3-K, by the interaction with the phosphorylated IRS proteins, is so extensive that this kinase has been termed the key switch mechanism in insulin signaling [14]. The lipids phosphorylated by PI-3-K, located at the cytoplasmic face of the cell membrane, bind to pleckstrin homology domains, such as those in phosphoinositide-dependent protein kinase and protein kinase B [14]. Activation of protein kinase B has been shown to be required for activation of glycogen synthase [16] and is thought to lead to both the translocation and activation of GLUT4 and the inactivation of glycogen synthase kinase. Although much research has been completed, the precise events linking the enzymes and regulatory proteins to the physiological responses continue to be under investigation.

The goal of this study was to investigate the ability of MHCP to stimulate insulin-like responses in 3T3-L1 adipocytes and evaluate whether MHCP could affect protein phosphorylation. The 3T3-L1 cells are routinely used in the signaling studies and are regarded as demonstrating all of the features of adipocytes. It was also of interest to investigate whether the MHCP could further stimulate insulin treated cells. MHCP was compared to the insulin response in a glucose uptake assay, glycogen synthesis, inhibition by PI-3-kinase inhibitors, glycogen synthase, GSK-3β activity and the phosphorylation state of the insulin receptor.

MATERIALS AND METHODS

Materials

The purified hydroxychalcone from cinnamon (MHCP) was prepared as described [11]. Insulin, dexamethasone, 1-isobutyl-3-methylxanthine and bovine serum albumin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). 2-deoxy-D-[1,2-^{3}H]glucose, D-[^{14}C]glucose, uridine diphosphate D-[^{14}C]glucose and [γ-^{32}P] adenosine 5-triphosphate were obtained from ICN Radiochemicals (Costa Mesa, CA). Antibodies against the insulin receptor β subunit and GSK-3β were purchased from Upstate Biotechnology (Lake Placid, NY). Dulbecco's modified Eagle's medium (DMEM), donor calf serum, sodium pyruvate, glutamine, trypsin-ethylene diamine tetraacetate, penicillin, streptomycin and Leibovitz L-15 medium were purchased from Life Technologies (Grand Island, NY). Fetal bovine serum was purchased either from Life Technologies or Novagen (Madison, WI). Wortmannin and LY294002 were purchased from Calbiochem (La Jolla, CA). Protein A/G plus Sepharose was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). General chemical and buffer components were purchased from either Sigma or Fisher Scientific Co. (Pittsburgh, PA).

Cell Culture

3T3-L1 preadipocytes were obtained from the American Type Culture Collection and were propagated at 37°C in a humidified atmosphere of 5% CO_2 in DMEM containing 10% donor calf serum, sodium pyruvate, glutamine, penicillin and streptomycin. Cells were serial passed one to two days postconfluence using mild trypsinization. Preadipocytes were maintained below 12 passes from original stock culture. Cells were plated into either 6-well or 24-well plates for differentiation. Induction of differentiation was done one to two days postconfluence. Cells were maintained in differentiation medium (DMEM, 10% fetal bovine serum, 0.25 mM dexamethasone, 0.5 mM 1-isobutyl-3-methylxanthine (IBMX), and 1 μg/mL insulin) for four days, medium changed every two days. At day

4, the dexamethasone and IBMX were removed with insulin remaining on the cells for an additional two days. Differentiation was allowed to continue in DMEM supplemented with 10% fetal bovine serum and the adipocytes were used between day 9 and 14 post-induction. Cells typically were 85% to 95% differentiated at day 6 post-induction. Prior to the cellular assays, cells were serum starved in DMEM for three hours or in Leibovitz L-15 medium with 0.2% bovine serum albumin, washed two times with KRPH buffer (5 mM Na_2HPO_4, 20 mM HEPES, pH 7.4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, 4.7 mM KCl) and equilibrated 15 minutes in KRPH. Treatment with insulin (100 nM) and/or MHCP (0.1 mg/mL) was allowed to proceed for 30 minutes.

Glucose Uptake Assay

Glucose uptake assays were performed based upon previously described methods [17,18]. The 3T3-L1 adipocytes were differentiated in 24-well plates. Glucose uptake proceeded in radiolabeled sugar (200 μM 2-deoxy-D-[1,2-^{3}H]-glucose, 0.1 μCi/mL) for the times indicated in the results. Cells were washed and lysed with 0.5N NaOH/0.1%SDS. Samples from each lysate were counted using Scintiverse BD (Fisher Scientific). Uptake was routinely measured in triplicate with each experiment repeated a minimum of twice.

Glycogen Synthesis Assay

Glycogen synthesis assays were performed as described [17]. 3T3-L1 cells were differentiated in 24-well plates. Glycogen synthesis proceeded for one hour in 5 mM glucose (2.0 μCi/mL [^{14}C]D-glucose). Cells were washed and lysed in 0.5 N NaOH. The radioactivity incorporated into the glycogen was measured by precipitating glycogen on E-31 paper (Whatman, Inc, Hillsboro, OR) in 66% ethanol. The papers were washed with 66% ethanol three times over one hour, dried and counted by scintillation. Uptake was routinely measured in triplicate.

Glycogen synthesis assays were also performed in the presence of inhibitors, wortmannin or LY294002. For experiments with wortmannin, inhibition was evaluated either with 1) the cells exposed to wortmannin for 30 minutes prior to treatment with insulin or MHCP, 2) the cells exposed during the treatment and/or 3) exposure during both the treatment and the production of glycogen, as indicated in the text of the results. Wortmannin concentrations ranged from 25 nM to 500 nM. Inhibition with LY294002 was done similarly, except that cells were not tested with a pretreatment. Concentrations of LY294002 ranged from 25 to 100 μM.

Glycogen Synthase Assay

The glycogen synthase assay method was based upon previously published methods [15,16,19,20]. 3T3-L1 adipocytes were cultured in 6-well plates. After treatment, cells were lysed in glycogen synthase extraction buffer (100 mM NaF, 10 mM EDTA, 1 mM benzamidine and 50 mM Tris-HCl, pH 7.8). For some experiments, cells were allowed to incubate for one hour in KRPH + 5 mM glucose prior to lysis. Lysates were homogenized and the supernate collected after centrifugation for 20 minutes at 10,000 $\times$ g. 50 μL of the supernate was added to an equal volume of glycogen synthase assay buffer (50 mM Tris-HCl, pH 7.8, 20 mM EDTA, 25 mM KF, 7 mg/mL glycogen, 200 μM uridine diphosphate glucose, 0.5 μCi/mL [^{14}C]-UDP D-glucose). Samples were incubated for 15 minutes at either 30°C or 37°C. 75 μL aliquots were removed and spotted on paper as described above for glycogen synthesis. Glycogen synthase activity is the amount of glycogen produced in the presence of 0.1 mM glucose 6-phosphate divided by the glycogen produced in 10 mM glucose 6 phosphate. Samples were performed in duplicate.

GSK-3β Assay

GSK-3β assays were performed as described for PC12 cells [21]. 3T3-L1 cells were differentiated in 6-well plates. Cell lysates were homogenized in extraction buffer (100 mM Tris, pH 7.4, 100 mM KCl, 2 mM EDTA, 0.1% Triton X-100, 1 mM benzamidine, 0.1 mM Na_3VO_4, 1mg/mL glycogen, 10 μg/mL pepstatin, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 100 nM okadaic acid) using a glass homogenizer and precipitated at 10,000 $\times$ g for 20 minutes prior to use. Protein concentrations were determined and 10 μg of protein was diluted to 150 μL in immunoprecipitation buffer (50 mM sodium glyceroposphate, pH 7.3, 1 mM EGTA, 1 mM benzamidine, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, 1 μg/mL pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 100 nM okadaic acid). Antibodies against GSK-3β (Transduction Laboratories) (0.375 μg) were added and the mixture was rotated for two hours at 4°C. 5 μg of rabbit anti-mouse IgG (Upstate Biotechnology, Inc.) was added for 30 minutes. Protein A/G Plus Sepharose (60 μL of a 30% solution) was added, and the incubation was rotated for one hour at 4°C. Immune complexes were recovered by centrifugation and washed once with extraction buffer and twice with immunoprecipitation buffer. Kinase activity was assayed by adding 25 μL of kinase buffer (250 mM sodium glycerophosphate, pH 7.4, 10 mM NaCl, 100 mM $MgCl_2$, 5 mM EGTA, 5 mM benzamidine, 5 mM dithiothreitol, 0.5 mM Na_3VO_4, 100 nM Okadaic acid) containing 20 μM phosphoglycogen synthase peptide-2 (Upstate Biotechnology) and 50 μM [γ^{32}P]ATP (1 μCi). After 10 minutes of incubation at 30°C, 20 μL of the reaction mixture was spotted onto Whatman P-81 phosphocellulose paper. Filters were washed in four changes of 175 mM phosphoric acid, rinsed in acetone, dried and counted. For some experiments, an additional incubation period of one hour was incorporated into the assay after treatments.

Immunoprecipitation and Detection of the Insulin Receptor

Adipocytes were differentiated in 6-well plates. After treatment, cells were lysed in an immunoprecipitation buffer (10 mM phosphate, pH 7.2, 0.14 mM NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM phenylmethylsulfonylfluoride, 50 mM NaF, 1.25 mM Na_2MoO_4, 1.25 mM Na_3VO_4, 12.5 mM $Na_4P_2O_7$, 10 μg/mL pepstatin, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). Cell lysates were clarified by centrifugation. Precipitating antibody was added (1–4 μg per reaction) and incubated with rocking overnight at 4°C. 30 μL of Protein A/G Plus Sepharose was added and incubated for one hour with rocking. Beads were washed three to four times with immunoprecipitation buffer. 25 μL of sodium dodecyl sulfate (SDS)-sample buffer was added, and the beads were denatured for five minutes at 100°C. Proteins were separated on a 7.5% SDS-polyacrylamide gel electrophoresis gel and transferred to polyvinyldifluoride membrane. Blots were blocked in TST (10 mM Tris, pH 7.4, 0.9% NaCl, 0.1% Tween 20) containing either 5% milk or 10% horse serum. Primary antibody was incubated on the blots at a 1:1000 dilution in TST overnight at 4°C. Blots were washed several times with TST, and the secondary antibody was added (1:3000–1:5000 dilution) for 1½ hours at room temperature. Blots were again washed several times. Immunoreactive proteins were detected with chemiluminescense using either ECL Plus (Amersham, Arlington Heights, IL) or SuperSignal (Pierce Chemical Co., Rockford, IL).

RESULTS

Phosphorylation of the Insulin Receptor

Treatment of a purified truncated version of the insulin receptor with MHCP in vitro established that the MHCP was capable of activating the kinase activity, resulting in autophosphorylation of the receptor [12]. To determine whether MHCP treatment resulted in the phosphorylation of the receptor in intact cells, immunoprecipitation experiments were performed using antibodies against the insulin receptor β domain. The proteins were separated, blotted and probed with antiphosphotyrosine antibodies and the results are shown in Fig. 1. MHCP stimulation led to the phosphorylation of the insulin receptor in a dose-dependent manner. The control treatment demonstrated very little if any phosphorylation, whereas the insulin control was also phosphorylated in a dose-dependent manner.

Glucose Uptake

Adipocytes were treated with either insulin, MHCP or a combination of MHCP and insulin. In initial experiments, the cells were treated for 10 minutes and allowed to take up radiolabeled deoxyglucose for 5 or 10 minutes. The use of deoxyglucose allows the assay to depend upon adequate ATP and functional hexokinase levels to trap the deoxyglucose taken up by cells as deoxyglucose 6-phosphate [22] without the incorporation into glycogen. Insulin treated adipocytes showed a two-and-a-half-fold increase in glucose transport over basal levels, just slightly lower than values reported by other investigators [19,22]. However, cells treated with MHCP did not incorporate glucose above controls within the first 10 minutes of incubation. Upregulation of glucose uptake upon MHCP stimulation had been expected based upon the glucose oxidation observed in rat fat pads [10]. The MHCP was then tested further by increasing the treatment time to 30 minutes and extending the glucose exposure time. As shown in Fig. 2, insulin-stimulated glucose uptake and the glucose incorporation rose gradually over the one-hour testing period. With

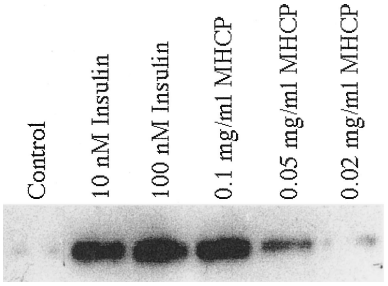

Fig. 1. Tyrosine phosphorylation of the insulin receptor in 3T3-L1 adipocyte cells. 3T3-L1 adipocytes were stimulated with various concentrations of insulin or methylhydroxychalcone polymer (MHCP) for 30 minutes, washed and lysed. The insulin receptor β subunit was immunoprecipitated, separated by SDS-PAGE and transferred to PVDF membrane. The receptor was probed with antiphosphotyrosine antibodies and detection was with chemiluminescence.

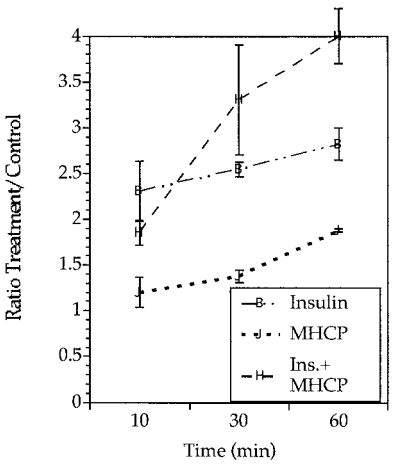

Fig. 2. Effect of MHCP or insulin on glucose uptake stimulation. 3T3-L1 adipocytes were treated with insulin (100 nM), MHCP (0.1 mg/mL) or insulin + MHCP for 30 minutes. The uptake of 2-deoxy-D-[1,2-^{3}H]glucose was measured in cell lysates at 10, 30 and 60 minutes. Data are expressed as the counts obtained in the treatment divided by the counts from the control. The uptake curves represent the mean ± standard deviation of triplicate samples from three experiments.

MHCP stimulation, both the 10-minute and 30-minute time points were just above control levels. However, after 60 minutes, there was a significant increase in the amount of glucose uptake. These results demonstrate that MHCP was able to induce glucose uptake in the adipocytes and that there may be a lag phase in the cellular response to MHCP. Also observed was that the stimulation with either insulin or MHCP did not diminish within the first 60 minutes after treatment.

In addition to treating the cells with insulin or MHCP alone, cells were treated with the compounds together. At the 10-minute time point, it appeared that the MHCP inhibited the insulin response. This result was in contrast to the results obtained at 30 minutes and 60 minutes where a potentiated response was observed. With the control set at 1, an additive value for insulin + MHCP would be approximately 2.8 at 30 minutes and 3.5 at 60 minutes. Experimentally, the values are 3.3 at 30 minutes and 4.0 at 60 minutes. These results suggest that MHCP and insulin may act synergistically.

Glycogen Synthesis

Adipocytes were evaluated for glycogen production after treatment with insulin and/or MHCP, as was done with glucose uptake. For glycogen production, cells were treated for 30 minutes, washed and exposed to radiolabeled glucose for one hour. In order to ensure that just the radioactive sugar incorporated into the glycogen stores was measured, a portion of the lysate was precipitated with ethanol as reported for glycogen [23]. This method removes 98% of the UDP-glucose from the filters [23]. Experimentally, lower standard deviations were observed with this assay, so it was used to evaluate the effective range of concentrations for MHCP stimulation and to study the synergistic effects further. These results are given in Table 1. MHCP and insulin stimulated glycogen production in a dose-related manner. Because the MHCP is a mixture of polymers, the effective molar concentrations cannot be compared between insulin and MHCP. When used in combination, potentiation of the response was again observed. For example, the additive value for 10 nM insulin + 0.1 mg/mL MHCP would be 3.0 and the experimentally derived value is 4.7. Again, 50 nM insulin + 0.2 mg/mL MHCP would be 4.8 and the experimental number is 10.9. These results clearly demonstrate that there is synergism when insulin and MHCP are used in combination.

Effect of PI-3-kinase Inhibitors on Glycogen Synthesis

After determining that MHCP induced both glucose uptake and glycogen synthesis, it was of interest to evaluate the role of PI-3-K in this process. PI-3-K is involved in both the upregulation of glucose uptake and glycogen production upon insulin stimulation (for review see [14]). To study this enzyme, inhibitory studies were performed using wortmannin and LY294002. Wortmannin inhibits a number of PI-3-K isoforms with nanomolar efficiency [24]. However, there is conflicting information regarding the range of targeted PI-3-K forms and reversibility. Recently, both an irreversible and a reversible type of inhibition of the insulin stimulation of glucose uptake, indicating two different sites of inactivation, was observed in 3T3-L1 adipocytes [24]. To compare MHCP with insulin, wortmannin inhibition was tested in several ways. First, the cells were pretreated with wortmannin for 15 minutes. Pretreatment with wortmannin followed by insulin stimulation is a commonly used for testing wortmannin inhibition. In these experiments, insulin stimulation was inhibited (data not shown). However, at no time was the response to MHCP inhibited by pretreatment with wortmannin. In the second set of experiments, no pretreatment was done and the wortmannin was incorporated into the treatments. As shown in Fig. 3, the cells treated with MHCP appeared to be activated by the wortmannin treatment rather than inhibited. As this result was unexpected, these results were verified by repetition. The potentiation response using both insulin and MHCP was also inhibited by wortmannin, but only partially. Together, these results suggest that the insulin signal contributing to the potentiation effect was inhibited, whereas the MHCP signal was not. The third means of testing wortmannin was to incorporate the wortmannin into both the treatment and the glycogen synthesis. Finally, this means of wortmannin exposure resulted in effective inhibition in all of the treatments: insulin, MHCP and insulin with MHCP, as shown in Fig. 4. The effective inhibitory concentration observed here was similar to the range given for the irreversible inhibition of glucose transport and GLUT4 translocation [24].

The results with wortmannin described above suggest, albeit not strongly, that a PI-3-K is triggered by the MHCP stimulation and plays a role in the synergistic response caused by the dual treatments. To establish clearly the importance of a class

Table 1. Effect of MHCP or insulin on glycogen synthesis stimulation

Insulin (nM)	0	2	10	50	200
Control	1.0	1.2 ± 0.2	1.6 ± 0.3	2.6 ± 0.2	3.5 ± 0.4
0.05 mg/mL MHCP	1.5 ± 0.2	1.5 ± 0.1	2.2 ± 0.7	5.0 ± 0.9	6.3 ± 1.8
0.1 mg/mL MHCP	2.4 ± 0.7	2.7 ± 0.8	4.7 ± 1.7	7.6 ± 2.3	7.7 ± 1.6
0.2 mg/mL MHCP	3.2 ± 0.3	4.4 ± 1.5	8.0 ± 3.8	10.9 ± 1.2	ND

3T3-L1 adipocytes were treated with varying concentrations of insulin, MHCP or insulin + MHCP for 30 minutes.
The incorporation of D-[^{14}C]glucose into the glycogen pools was allowed for 60 minutes. The radioactivity incorporated into the glycogen was measured in the glycogen precipitate. The data represent the mean ± standard deviation of triplicate samples from at least three experiments.

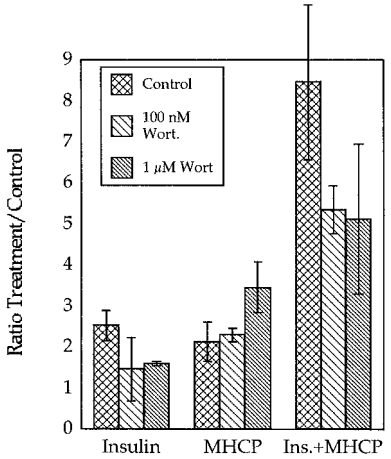

Fig. 3. Effect of wortmannin treatment on the stimulation of glycogen production in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with insulin (100 nM), MHCP (0.1 mg/mL) or insulin + MHCP in combination with various concentrations of wortmannin for 30 minutes and washed. The incorporation of D-[^{14}C]glucose into the glycogen pools (without wortmannin present) was allowed for 60 minutes. The radioactivity incorporated into the glycogen was measured in the glycogen precipitate. The data represent the mean ± standard deviation of triplicate samples from three experiments.

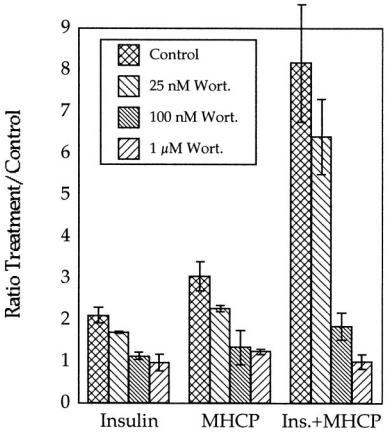

Fig. 4. Effect of wortmannin on the stimulation of glycogen production in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with various concentrations of wortmannin in the presence of insulin (100 nM), MHCP (0.1 mg/mL) or insulin + MHCP for 30 minutes. The incorporation of D-[^{14}C]glucose into the glycogen pools in the presence of wortmannin was allowed for 60 minutes. The radioactivity incorporated into the glycogen was measured in the glycogen precipitate. The data represent the mean ± standard deviation of triplicate samples from two experiments.

I PI-3-K in the MHCP stimulation of adipocytes, the inhibitor LY294002 was tested. This agent is an specific inhibitor of the class I PI-3-kinases and has been shown to inhibit insulin-stimulated glucose uptake and GLUT4 translocation. When adipocytes were pretreated with LY294002 and subsequently stimulated with insulin or MHCP, only the insulin response was inhibited (data not shown). Because of the results observed with wortmannin, follow-up experiments used LY294002 in both the treatment and in the glycogen production. As shown in Fig. 5, LY294002 effectively inhibited the insulin response, the MHCP response and the potentiation response. Cell viability would not be an issue in these experiments as LY294002 is delivered in ethanol and has not been shown to be toxic to cells. These results conclusively show that a class I PI-3-K is triggered upon MHCP treatment and that this PI-3-K is involved in the strong synergistic response observed when both MHCP and insulin were used.

Glycogen Synthase

The results with the glucose uptake and the glycogen production assay both demonstrated that there is a synergistic effect when insulin and MHCP are used in combination. It was of interest to not only determine whether GS was upregulated by MHCP alone, but also to determine the level of stimulation using the combination treatment. The enzymatic activity of GS is measured using radiolabeled UDP-glucose as the tag into glycogen. Experimentally, the activity was measured in the presence of either low glucose-6-phosphate (G6P) (0.1 mM) or in high G6P (10mM). The low G6P/high G6P ratio has been reported to be very sensitive to changes in the enzymic properties of GS caused by phosphorylation and provides a more proper definition of the *in vivo* activity state of the enzyme [20]. The concentration of G6P is high enough to be practically saturating for all samples, regardless of their degree of phosphorylation. The activity measured should be almost constant for any sample phosphorylated to different degrees. The low concentration of G6P in the assay is almost saturating for the

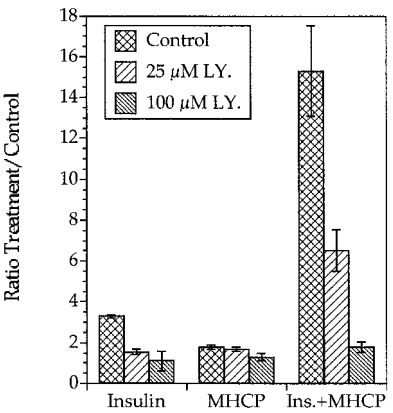

Fig. 5. Effect of LY294002 on the stimulation of glycogen production in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with various concentrations of LY294002 in the presence of insulin (100 nM), MHCP (0.1 mg/mL) or insulin + MHCP for 30 minutes. The incorporation of D-[^{14}C]glucose into the glycogen pools in the presence of LY294002 was allowed for 60 minutes. The radioactivity incorporated into the glycogen was measured in the glycogen precipitate. The data represent the mean ± standard deviation of triplicate samples from three experiments.

non-phosphorylated forms, and maximum sensitivity is obtained [20]. As shown in Fig. 6, MHCP stimulated glycogen synthase activity. Noticeably, in these experiments, the 0.1 mg/mL MHCP stimulation of GS activity equaled the 100 nM insulin stimulation. By comparison, in the glucose uptake experiments, treatment of cells with the 0.1 mg/mL MHCP resulted in less than a two-fold activation of glucose uptake compared with the two-and-a-half-fold activation of glucose uptake in the cells treated with 100 nM insulin (Fig. 1). Thus, MHCP stimulated GS activity to a greater degree than stimulating glucose uptake. Surprisingly, there was no potentiation effect seen when both cinnamon and insulin were used, shown in Fig. 6. The 200 nM insulin treatment established that the 100 nM insulin treatment did not stimulate GS to its maximal level. Thus, GS could have been stimulated to a higher level by the dual treatments if synergism was to occur.

In comparison with the other assays in which synergism was observed, one noticeable difference involves the timing. In all assays, cells were treated for 30 minutes. For glucose uptake, cells were tested at various time points over one hour, and the level of glycogen was measured at one hour. GS activity, by comparison, is tested 10 minutes after lysis of the cells. This timing can be compared with the 10-minute glucose uptake incubation in which only a low level of stimulation by MHCP was observed (Fig. 2). Likewise, the 10-minute timepoint was too early to see any potentiation effects in the glucose uptake. Potentiation was initially observed at 30 minutes and most prominently seen at 60 minutes. The synergism observed in the glycogen production was when the glycogen was measured at 60 minutes. To ensure that the absence of potentiation in the GS was not due to premature measurement in the assay, the cells were allowed to incubate for 60 minutes prior to testing for GS activity. These experiments also did not indicate a potentiation effect with the combined treatments (data not shown). At 60 minutes, glycogen synthase activity was still upregulated as compared with the controls, but their level was decreased from the 10-minute timepoint.

GSK-3β

In the enzymatic pathway stimulating glycogen production, GS activity is downregulated by phosphorylation by GSK-3β. It was of interest to determine whether GSK-3β was affected by MHCP treatment and whether an effect on this enzyme might account for the potentiation effects observed in glycogen production. As shown in Fig. 7, treatment of adipocytes with MHCP resulted in an inhibition of GSK-3β activity at a level greater than the inhibition by insulin treatment. Although insulin stimulation was only slightly inhibitory in these trials, insulin has been shown to inhibit GSK-3β activity in transfected cells which express a higher level of GSK-3β [19]. The dual treatment resulted in only a slightly lower level of inhibition than seen with MHCP alone. These data indicate that there is an additive effect when MHCP and insulin are used together, but there does not appear to be strong synergism. This may reflect the weak ability of insulin to inhibit GSK-3β.

As was done with the GS assay, the GSK-3β assay was modified to assess potentiation after a 60-minute incubation. When the enzyme was tested at this timepoint, all of the treatments inhibited the activity (data not shown). MHCP inhibited at 54%, insulin inhibited at 25% and the combined

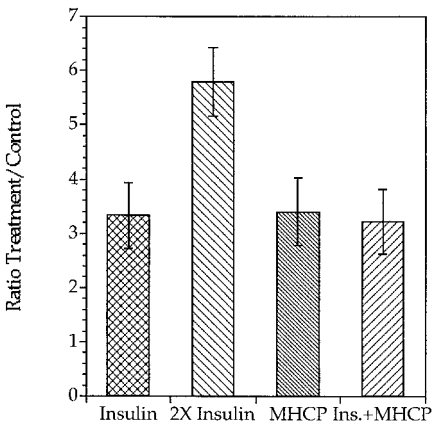

Fig. 6. Effect of MHCP or insulin on glycogen synthase activity. 3T3-L1 adipocytes were treated with insulin (100 nM), MHCP (0.1 mg/mL) or insulin + MHCP for 30 minutes, washed and the cell supernate obtained. Glycogen synthase assays were performed using uridine diphosphate D-[^{14}C]glucose in the presence of either 0.1 mM glucose-6-phosphate (G6P) or 10 mM G6P and the radioactivity incorporated into the glycogen precipitant was measured. The low G6P/high G6P ratio was determined and the ratio from the treatment was divided by the ratio obtained from control cells. The data represent the mean ± standard deviation of triplicate samples from three experiments.

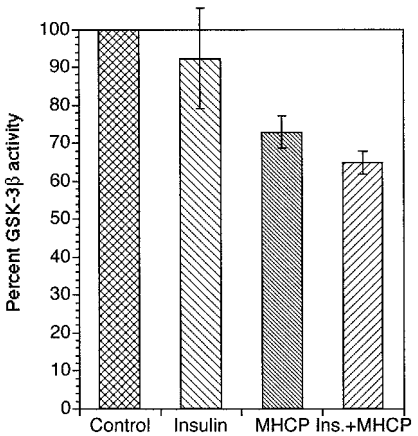

Fig. 7. Effect of MHCP or insulin on glycogen synthase kinase3β activity. 3T3-L1 adipocytes were treated with insulin (100 nM), MHCP (0.1 mg/mL) or insulin + MHCP for 30 minutes, washed and the cell supernate obtained. GSK-3β assays were performed to measure the incorporation of ^{32}P into the phosphoglycogen synthase peptide 2 (Upstate Biotechnologies). The level of incorporation of the control cells was set at 100% and the other values were set in comparison. The data represent the mean ± standard deviation of triplicate samples from three experiments.

treatment of MHCP and insulin inhibited 56% of the activity. This demonstrates that inhibition of GSK-3β continues on for at least 60 minutes after treatment. In this trial, there was no additive effect between MHCP and insulin. The long term effect of GSK-3β inhibition would be to keep GS in the more active non-phosphorylated form with glycogen production at a higher level. Thus, these results may indicate that GSK-3β plays a role in the potentiation response seen in glycogen production, as MHCP alone was able to inhibit this enzyme to a much greater degree than insulin at the 10-minute timepoint. However, there was no synergistic inhibition of this enzyme using the combined treatment.

DISCUSSION

These results establish that MHCP stimulated the autophosphorylation of the insulin receptor, upregulated glucose uptake, glycogen synthesis and GS activity in 3T3-L1 adipocytes and also downregulated GSK-3β activity. Glycogen synthesis stimulation is through a class I PI-3-K dependent pathway. These events are all characteristic of a 3T3-L1 adipocytes response to insulin treatment. MHCP stimulated glucose uptake by the cells at a lower level than did insulin. However, when MHCP is compared to insulin in the other assays, similar levels of glycogen production and the glycogen synthase activity were observed. Thus, the MHCP acts an insulin mimetic, most likely by triggering the same cascade as the insulin signaling pathway. Results presented here also establish that the MHCP acts as an insulin potentiating agent. In both the glucose uptake assay and the glycogen production analysis, treatment with insulin and MHCP resulted in a synergistic effect. This response in glycogen production was over a two-fold increase of the corresponding additive values (Table 1) and was often a four-to-five-fold increase over what insulin alone stimulated.

Even though the MHCP triggered similar responses as insulin, there were noticeable differences. First, the response of the cells to MHCP is slower than seen with insulin. Cells were incubated for 30 minutes with MHCP. Insulin stimulation usually uses a 5-to-10-minute treatment window. In addition, the glucose uptake results (Fig. 2) indicated that there may be a lag phase in the response to MHCP. Stimulation of cells with insulin gave a linear slope over the 60-minute treatment window, whereas the MHCP stimulation yielded only a slight increase at 10 minutes, with a dramatic rise in uptake observed between 30 and 60 minutes. A second difference between the MHCP and insulin treatment was observed in the inhibition trials with wortmannin and LY294002. The insulin signal was effectively inhibited when the cells were pretreated with either inhibitor. However, for MHCP, inhibition was only observed when these inhibitors remained on the cells during the entire trial period. Finally, the third difference is that MHCP was able to inhibit endogenously expressed GSK-3β to a much greater degree than insulin. This increase in inhibition was observed both within the first 10 minutes of treatment and again one hour later.

One potential hypothesis to account for these differences is that the MHCP may have to enter the cells prior to interacting with one or more of the enzymes involved in the signaling cascade. The data discussed above, previous data and a comparison to another insulin mimetic supports this hypothesis. The need to treat the cells for 30 minutes, an absence of glucose uptake at the 5-to-10-minute timepoint and the observed lag phase in the glucose uptake trials suggest a period of time before the cell is capable of a response. Secondly, previous results with MHCP indicate that the MHCP is able to alter intracellular target proteins. MHCP stimulated the autophosphorylation of a purified insulin receptor kinase domain and inhibited protein tyrosine phosphatase I (PTP-1) using *in vitro* assays [12]. The phosphorylation of the insulin receptor by the adipocytes upon MHCP stimulation, as shown in Fig. 1, supports the interaction with the receptor *in vivo*. Entrance of MHCP into the cells would be the only mechanism *in vivo* to stimulate both the kinase domain of the insulin receptor and to inhibit PTP-1. Finally, several insulin mimetic compounds have been described with distinctly different properties. An insulin mimetic, described by Zhang, was shown to stimulate the glucose uptake after only a five-minute incubation [25]. This fungal extract was discovered using a screening assay which selected for compounds which interacted with the extracellular region of the insulin receptor. Likewise, the insulin mimetic phosphoinositol-glycan-peptide stimulated a rapid onset and reversibility of glucose transport with a half-time of six to eight minutes [26,27]. Research indicated that this compound stimulated cells through an unknown receptor and the pathway proceeded through IRS-1 and PI-3-K [26,27]. The possibility of the MHCP crossing the cell membrane also provides a potential explanation for the inhibitor results with MHCP. Once inside, the MHCP may remain active in the cell for a period of time and may hypothetically retrigger the pathways. In both the wortmannin trials and with LY294002, the cell had to be exposed to the inhibitors for the duration of the experiment for inhibition to be observed. A second argument for the MHCP remaining active can be seen in the glucose uptake results. The response at one hour is significantly higher than at 30 minutes post treatment as compared to the insulin response, which remained fairly steady in the level of glucose taken into the cell.

The second major finding of these results was the synergistic effect observed in both the glucose uptake and glycogen production when insulin and MHCP were combined. There are several possible explanations for this observation. First, MHCP and insulin may stimulate the insulin receptor-IRS proteins-PI-3-K pathway through separate mechanisms, and when used in combination a greater response is generated. Inhibition by LY294002 established that both compounds act through a class I PI-3-K. The wortmannin trials give suggestive evidence for the signals being separate. When wortmannin was used as a

pretreatment, the insulin signal was inhibited, and the MHCP signal was not (Fig. 3). In the combined treatment, there was only partial inhibition of the synergistic effect. A second possible explanation for the synergism is that the MHCP may cause multiple effects inside the cell. One effect would be to trigger an insulin-like response through the receptor, the other to act upon regulating enzymes. MHCP has now been shown to inhibit both PTP-1 [12] *in vitro* and GSK-3β in vivo, both regulatory enzymes. PTP-1 is involved in downregulating the phosphorylation from the kinase activity of the insulin receptor. Inhibition of this enzyme by MHCP would maintain the receptor in an activated state, keep the level of tyrosine phosphorylation on the IR constant and might contribute to the potentiation observed in the glucose uptake and glycogen synethesis assays, which occurred 60 minutes after treatment. Inhibition of another phosphatase, the one responsible for activating GSK-3B, would result in the inhibition of GSK-3B. This may be reflected in the significantly greater inhibition of this enzyme after MHCP treatment as compared to insulin treatment. The kinase activity of GSK-3β regulates the activity of GS. The net result would be to keep GS activated and glycogen synthesis at full levels, results observed after MHCP treatment. In addition, previous studies have suggested that GSK-3β inhibition is not the only mechanism whereby insulin stimulates glycogen production. An alternative pathway, involving protein phosphatase activation, for stimulating glycogen production has been suggested [28]. Inhibition of GSK-3β, PTP-1 and action upon other potential phosphatases would interfere with the downregulation of the insulin signal and may contribute to the continued stimulation of glycogen production.

CONCLUSION

In summary, this study analyzed the ability of MHCP to upregulate many of the cellular responses seen with insulin. Although there are noticeable differences between the responses, the MHCP is fully capable of mimicking insulin. Thus, the MHCP can be used as another tool in studying the pathways leading to glucose uptake and glycogen synthesis. Because MHCP can work alone, it may function as a therapeutic agent for use with insulin resistant cells. A significant observation from this research is the synergism between insulin and the MHCP. The actual events leading to this heightened response are unknown. A study of this synergistic effect may lead to a fuller understanding of the pathways involved in the insulin signaling pathways and of alternative pathways leading to upregulation of glucose uptake by the cells.

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