Masoprocol decreases rat lipolytic activity by decreasing the phosphorylation of HSL

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Masoprocol decreases rat lipolytic activity by decreasing the phosphorylation of HSL. Am J Physiol Endocrinol Metab 279: E593–E600, 2000.—Masoprocol (nordihydroguaiaretic acid), a lipoxygenase inhibitor isolated from the creosote bush, has been shown to decrease adipose tissue lipolytic activity both in vivo and in vitro. The present study was initiated to test the hypothesis that the decrease in lipolytic activity by masoprocol resulted from modulation of adipose tissue hormone-sensitive lipase (HSL) activity. The results indicate that oral administration of masoprocol to rats with fructose-induced hypertriglyceridemia significantly decreased their serum free fatty acid (FFA; P < 0.05), triglyceride (TG; P < 0.001), and insulin (P < 0.05) concentrations. In addition, isoproterenol-induced lipolytic rate and HSL activity were significantly lower (P < 0.001) in adipocytes isolated from masoprocol compared with vehicle-treated rats and was associated with a decrease in HSL protein. Incubation of masoprocol with adipocytes from chow-fed rats significantly inhibited isoproterenol-induced lipolytic activity and HSL activity, associated with a decrease in the ability of isoproterenol to phosphorylate HSL. Masoprocol had no apparent effect on adipose tissue phosphatidylinositol 3-kinase activity, but okadaic acid, a serine/threonine phosphatase inhibitor, blocked the antilipolytic effect of masoprocol. The results of these in vitro and in vivo experiments suggest that the antilipolytic activity of masoprocol is secondary to its ability to inhibit HSL phosphorylation, possibly by increasing phosphatase activity. As a consequence, masoprocol administration results in lower serum FFA and TG concentrations in hypertriglyceridemic rodents.

Adipocyte; free fatty acid; triglyceride; hormone-sensitive lipase

HISTORICALLY, EXTRACTS OF the creosote bush (Larrea tridentata) have been extensively used by native healers throughout the Southwest region of North America for the treatment of type 2 diabetes. We have recently used an in vivo guided fractionation approach to identify masoprocol as the major, if not the only, compound in L. tridentata responsible for its antihyperglycemic effect. Masoprocol, also known as nordihydroguaiaretic acid, is a known lipoxygenase inhibitor (21, 34) and has been shown to lower plasma glucose concentrations and increase insulin-mediated glucose uptake in genetic models (db/db and ob/ob) of type 2 diabetes in mice (14).

In addition, rats were made insulin resistant and hyperinsulinemic by feeding a fat-enriched diet, and they were rendered hyperglycemic by injecting them with a modest amount of streptozotocin. In this situation, the insulin concentrations decline to levels similar to those in chow-fed rats and were no longer able to compensate for the insulin resistance, and hyperglycemia ensued (26). Masoprocol has been shown to effectively lower glucose concentrations in this nongenetic model of type 2 diabetes without any change in plasma insulin concentration but with decreases in free fatty acid (FFA) and triglyceride (TG) concentrations (26). In addition, masoprocol treatment has been shown to prevent hypertriglyceridemia in nondiabetic rats fed a fructose-enriched diet by significantly reducing hepatic TG secretion and increasing clearance of TG from the plasma (28).

Hormone-sensitive lipase (HSL) is a cytosolic neutral lipase that catalyzes the hydrolysis of intracellular TG (lipolysis) in adipose tissue (12, 32), skeletal muscle (20), and heart (31). HSL activity is regulated by multisite phosphorylation-dephosphorylation reactions in response to hormones (1, 29). Hormones (e.g., catecholamines) and other agonists that elevate cAMP levels stimulate HSL enzymatic activity through enhanced phosphorylation catalyzed by protein kinase A (2, 5). Insulin is thought to inhibit lipolysis by inactivating HSL due to the net dephosphorylation of the enzyme protein (5, 7). Okadaic acid, a polyether fatty acid, is a very potent inhibitor of protein phosphate 1 and protein phosphatase 2A (6), two of the four major protein phosphatases in cytosol of mammalian cells that catalyze hydrolysis of phosphoserine and phosphothreonine residues (4, 30). It is cell permeable and, when added to adipocytes, mimics the action of insulin in stimulating glucose transport (6, 9) and protein kinase activity (9).

In an effort to define the mechanism responsible for the metabolic effects of masoprocol, we have recently...
demonstrated that isoproterenol-induced lipolytic rate is significantly decreased when masoprocol is incubated with isolated adipocytes obtained from normal rats (8). The present studies were initiated to extend these observations and to specifically test the hypothesis that the ability of masoprocol to inhibit lipolytic activity is secondary to a direct effect on HSL.

**MATERIALS AND METHODS**

**Animals and treatments.** Male Sprague-Dawley rats obtained from Harlan Sprague-Dawley (Indianapolis, IN) were used in these studies. For in vitro studies, rats weighing ~220 g were fed Purina Rat Chow (no. 5012; St. Louis, MO) and water ad libitum and were maintained on a 12:12-h light-dark cycle. The animals were fasted for 4 h and decapitated by cervical dislocation, and epididymal fat pads were removed for preparation of adipocytes. For these studies, adipocytes were pooled from three to four animals. All experiments were performed in duplicate and were repeated at least three to six times (n = 3 or 6).

For in vivo studies, rats weighing 175–200 g were used. Rats were first maintained on a Chow diet for ~1 wk and then were switched to a high-fructose diet (TD 78463; Harlan Teklad, Madison, WI) that provided 60% of total calories as fructose. After 10 days on the high-fructose diet, the degree of hypertriglyceridermia was evaluated by determining the total plasma TG levels. The hypertriglyceridermic animals (TG > 250 mg/dl) were then divided into two groups (12 animals in each group, with comparable plasma TG concentrations). On day 0 of treatment, the rats were fasted for 4 h, and tail vein blood was collected for baseline measurements of serum TG, glucose, insulin, and FFA. The two groups of rats were then treated with either vehicle (Gelucire 44/14) or masoprocol at a dose of 80 mg/kg body wt twice a day for 7 days. After 4 days of treatment, blood was collected from the tail vein, 3 h after the last dose of vehicle or masoprocol. Serum samples were used to measure TG and glucose concentrations by enzymatic calorimetric methods (15, 33) using Sigma Diagnostic kits (St. Louis, MO). Serum insulin concentrations were measured by RIA using a Linco Rat Insulin RIA kit (St. Charles, MO). FFA concentrations were measured using the nonesterified fatty acid (NEFA) C kit by the ACS-ACOD method following the instructions of the manufacturer. Rats were killed by decapitation, and epididymal fat pads were removed quickly and used for adipocyte isolation (to measure lipolytic activity) and quantitation of HSL activity.

**Preparation of adipocytes.** Adipocytes were prepared from the epididymal fat pads by a slight modification of the procedure of Rodbell (27) as previously described (24). In brief, fat pads were minced with scissors, placed in plastic flasks in Krebs bicarbonate buffer containing 3.5% BSA, 3 mM glucose, and 1 mg collagenase/ml, and incubated for 1 h at 37°C in a gyratory water-bath shaker. The released cells were washed three times in fresh Krebs buffer with 2% albumin and allowed to separate from the infranatant by flotation. Suitable aliquots of diluted cells were taken for measurement of rates of lipolytic activity. A 100-μl aliquot of diluted cells was also fixed in a solution of 2% osmium tetroxide in collidine buffer and was counted in a coulter counter (Hialeah, FL) for determination of cell numbers.

**Measurement of rates of lipolytic activity.** Rate of adipocyte lipolysis was determined using an established procedure of this laboratory (24). Aliquots of diluted cells (~1 × 10⁶ cells/ml) were placed in plastic vials and preincubated in the presence or absence of 50 μM masoprocol in 1 ml of Krebs buffer containing 2% albumin at 37°C for 60 min, with continuous shaking at 40 counts/min. Subsequently, cells were incubated with or without (~)-isoproterenol (3 nM) or 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP, 0.5 mM) for 60 min at 37°C. In some instances, cells were also incubated with insulin (400 μM) for 60 min at 37°C with isoproterenol. At the end of the incubation, the cells were centrifuged, and the infranatant was collected for the quantification of glycerol and FFA, as described below. To further establish the specificity of masoprocol, the effect of esculetin, another specific inhibitor of lipoxigenase (18), was evaluated. Aliquots of adipocytes were preincubated with 4 or 40 μM esculetin for 60 min at 37°C and then were incubated with 5 nM isoproterenol for 60 min at 37°C to measure rates of lipolytic activity. For some other studies, adipocytes were also preincubated with 500 nM okadaic acid, a serine/threonine protein phosphatase inhibitor (6), or 1 μM wortmannin, a phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor (19), for 15 min at 37°C before additional incubation with masoprocol and/or isoproterenol, as described above. Glycerol concentration in the infranatant was measured by an enzymatic method (23) using the TG kit. FFA concentration in the infranatant was measured using the NEFA C kit by the ACS-ACOD method.

**HSL activity.** To assess the HSL protein and activity levels, adipose tissues from animals treated with masoprocol or vehicle or isolated adipocytes were homogenized using a Polytron in 50 mM Tris·HCl buffer (pH 7), 250 mM sucrose, and 5 μM EDTA. The homogenates were sequentially centrifuged at 1,500 (10 min) and 43,000 × g (15 min) at 4°C (17). The clear supernatants (43,000 g) were used for measurement of HSL activity and HSL protein content by Western blotting. Protein content of supernatants was determined by a modified (22) of the technique of Lowry et al.

HSL activity was assayed as neutral cholesteryl esterase by following the release of [1-14C]oleate acid from cholesteryl [1-14C]oleate as described by Nakamura et al. (17) with minor modifications. The incubation medium in a final volume of 200 μl contained 100 nM potassium phosphate buffer, pH 7.4, 0.025% BSA, 1.25 nmol cholesteryl [1-14C]oleate (~3 × 10⁶ dpm) added in 4 μl acetone, and 10 μg supernatant. After incubation (10 min), the reaction was terminated (10) by addition of 1 ml of borate/carbonate buffer (0.1 M, pH 10.5) followed by 3 ml of chloroform-methanol-heptane (1:3.9:1.28:1). The reaction tubes were vortexed vigorously for 1 min and centrifuged (1,500 g for 20 min at 10°C), and the released [1-14C]oleate in the aqueous phase was determined by a scintillation counter. The results are expressed as picomoles [14C]oleate released per minute per milligram protein.

**Western blotting of HSL protein.** An aliquot of adipose supernatant (25 μg protein) was mixed with equal volumes of 2× sample-loading buffer [4.6% (wt/vol) SDS, 16% (wt/vol) sucrose, 10% (vol/vol) β-mercaptoethanol, and 0.1 M...
Table 1. Effect of masprocol treatment on serum glucose, insulin, triglyceride, and FFA concentrations in fructose-fed rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle Baseline</th>
<th>Vehicle Treatment</th>
<th>Masprocol Baseline</th>
<th>Masprocol Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>259 ± 2</td>
<td>258 ± 4</td>
<td>256 ± 3</td>
<td>250 ± 13</td>
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<tr>
<td>Liver weight, g</td>
<td>N/A</td>
<td>9.99 ± 0.3</td>
<td>N/A</td>
<td>9.79 ± 0.4</td>
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<tr>
<td>Serum insulin, µU/ml</td>
<td>43 ± 6</td>
<td>44 ± 4</td>
<td>40 ± 5</td>
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<tr>
<td>Serum glucose, mg/dl</td>
<td>137 ± 6</td>
<td>135 ± 6</td>
<td>139 ± 7</td>
<td>140 ± 7</td>
</tr>
<tr>
<td>Serum triglycerides, mg/dl</td>
<td>224 ± 25</td>
<td>219 ± 32</td>
<td>242 ± 15</td>
<td>79 ± 5†‡</td>
</tr>
<tr>
<td>Serum FFA, mg/l</td>
<td>0.64 ± 0.03</td>
<td>0.69 ± 0.02</td>
<td>0.61 ± 0.02</td>
<td>0.55 ± 0.02*</td>
</tr>
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</table>

Data are means ± SE. The two groups of rats (n = 12) were treated with either vehicle or masprocol at a dose of 80 mg/kg twice a day for 4 days, delivered by oral gavage at a volume of 2.5 ml/kg body wt. FFA, free fatty acid. *P < 0.05 vs. vehicle. †P < 0.01 vs. vehicle. ‡P < 0.001 vs. masprocol baseline. NA, not applicable.

Tris·HCl, pH 6.8], heated at 95°C for 5 min, and cooled to room temperature. The sample was subjected to electrophoresis on a 7% SDS-polyacrylamide gel (11). After electrophoretic separation, proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) using standard techniques. The membranes were blocked in PBS-T buffer (pH 7.4; 10 mM sodium phosphate, 0.15 M NaCl, 2.5 mM MgCl₂, 0.1% Tween 20, and 5% (wt/vol) nonfat dried milk) for 1 h at 37°C. After being washed, the membranes were incubated with rabbit anti-HSL IgG (10) in blocking solution with agitation at 4°C overnight. Subsequently, the membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit IgG. The signals corresponding to HSL were detected using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL) according to the manufacturer’s instructions. The Western blots were scanned by a densitometer.

Immunoprecipitation and detection of HSL phosphorylation. Anti-phosphoserine antibody was used to detect HSL phosphorylation. Aliquots of adipocyte extracts (200 µg protein) were first incubated with 5 µg of polyclonal anti-HSL in 2× immunoprecipitation buffer (2% Triton X-100, 300 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, 2 mM EGTA, 4 mM sodium vanadate, 4.0 mM phenylmethylsulfonyl fluoride, and 1.0% Nonidet P-40) in a final volume of 1 ml for 2–3 h at 4°C. After incubation, protein A/G plus-agarose conjugate (50 µl) was added to each sample, and the tubes were vortexed and incubated with agitation overnight at 4°C. Immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 min at 4°C. The pellets were carefully washed four times with 1× immunoprecipitation buffer as before. The pellets were resuspended in 25 µl of 2× electrophoresis buffer, boiled for 5 min, and centrifuged for 5 min at 2,500 rpm. The released supernatants were subjected to SDS-PAGE followed by Western blotting, as described above with some minor modifications. The primary antibody used for detecting HSL phosphorylation was rabbit anti-phosphoserine IgG at a dilution of 1:100.

RNA preparation. Approximately 1–2 g of adipose tissue, cut into small pieces, was homogenized in 5 ml of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M β-mercaptoethanol (added before use)) at room temperature using a Polytron (setting 7 for 30 s). The solution was then extracted two times with an equal volume of chloroform to de lipidate the sample (13). The aqueous phase was transferred to a polypropylene tube, and 0.1 ml of 2 M sodium acetate, pH 4.1, 0.1 ml of water-saturated phenol, and 0.2 ml of chloroform-isoamyl (49:1) mixture were sequentially added per 1 ml of aqueous phase. The RNA was then purified according to the procedure described by Chomczynski and Sacchi (3), dissolved in diethyl pyrocarbonate (DEPC)-treated water, and stored at −70°C.

Construction of HSL and 18S rRNA cDNA probes. A 470-bp Ava I-Pst I (position 687–1156) fragment from rat HSL cDNA was subcloned into the Ava I-Pst I sites of pBlue-script KSII+ (pBS KSII+, Stratagene, La Jolla, CA). A 274-bp Dra II-Tha I (position 134–408) fragment of 18S ribosomal RNA cDNA (25) was subcloned into the Dra II-Eco R V sites of pBS KSII+. The plasmids were linearized with appropriate restriction endonucleases (Apa I for HSL and Bam H I for 18S ribosomal RNA), and 3′-ends of the linearized HSL plasmids were filled using Klenow fragment, extracted two times with phenol and two times with chloroform, precipitated in ethanol, and redissolved in DEPC-treated water. The linearized plasmids were used for riboprobe synthesis.

Preparation of riboprobes. The antisense [32P]cRNA probes were synthesized using [α-32P]CTP and the appropriate T3 or T7 RNA polymerase according to instructions supplied by

Fig. 1. Isoproterenol-induced glycerol (A) and free fatty acid (FFA; B) release by adipocytes isolated from fructose-fed rats treated with either vehicle or masprocol. Isolated adipocytes were incubated with or without isoproterenol (100 nM) for 60 min at 37°C. After incubation, the media were collected and assayed for released glycerol and FFA. The basal rate of lipolysis in adipocytes from fructose-fed rats in the absence of added isoproterenol was 715 ± 8 nmol·10⁻⁵ cells⁻¹·h⁻¹. In vivo administration of masprocol did not alter basal lipolysis (787 ± 20 nmol·10⁻⁵ cells⁻¹·h⁻¹). The results represent means ± SE of 9 individual measurements in each group.
the Strategene’s in vitro transcription kit. Because of their high liability, the riboprobes were always freshly prepared before hybridization.

**mRNA quantitation by RNase protection assay.** The HSL mRNA levels were determined using a sensitive RNase protection assay. Aliquots of adipose tissue RNA or control tRNA (10 μg) were dried under vacuum and redissolved in 30 μl of hybridization buffer containing 10^4 cpm of probe [i.e., the radiolabeled HSL riboprobe or the 18S rRNA riboprobe that was used as an internal standard for quantification]. The mixture was incubated for 5 min at 85°C to denature RNA and was then rapidly transferred to a hybridization temperature of 42°C for incubation overnight (16–18 h). The unprotected probe was hydrolyzed by digestion with 40 μg/ml RNase A and 2 μg/ml RNase T1 for 1 h at 30°C. The RNase digestion was terminated by the addition of proteinase K (50 μg) and SDS (2 mg) and incubation for 15 min at 37°C followed by phenol-chloroform extraction. The protected RNA-RNA hybrids were ethanol precipitated in the presence of yeast tRNA, and the pellets were dissolved in 15 μl of RNA loading buffer and heated for 5 min at 85°C. The protected fragments were separated on 6% acrylamide-urea denatur-
The prolipolytic effects of isoproterenol (100 nM) on adipocytes isolated from fructose-fed rats treated with vehicle or masoprocol are shown in Fig. 1. These results demonstrate that release of both glycerol (P < 0.002) and FFA (P < 0.02) by adipocytes from masoprocol-treated rat is decreased compared with adipocytes from rats receiving vehicle alone. HSL activity in adipocytes isolated from rats treated with vehicle or masoprocol is shown in Fig. 2A. The significant decrease (P < 0.001) in HSL activity in adipocytes from masoprocol-treated rats was associated with a concomitant fall in HSL protein, as determined by Western blot analysis (Fig. 2B).

Figure 3A shows the results obtained using a highly sensitive RNase protection assay to assess the levels of HSL mRNA in the adipose tissues of rats treated with vehicle or masoprocol. As a control, the expression of the stable marker 18S rRNA was also examined. The results presented in Fig. 3B (expressed as a ratio of HSL to 18S rRNA) demonstrate that expression of HSL mRNA was not significantly altered after exposure of rats to masoprocol and suggest the possibility that masoprocol modulates HSL activity posttranscriptionally.

In vitro studies. The next series of experiments were performed on adipocytes obtained from chow-fed rats, with masoprocol being added in vitro. The inhibitory effect of masoprocol and insulin on glycerol and FFA released during isoproterenol-induced lipolysis is shown in Fig. 4. It is clear from Fig. 4, A and B, that adipocytes pretreated with 50 μM masoprocol had a significant reduction in both glycerol (83 ± 3 vs. 165 ± 14 nmol·10⁻⁶ cells·h⁻¹; P < 0.01) and FFA (180 ± 30 vs. 440 ± 50 neq·10⁻⁵ cells·h⁻¹; P < 0.02) release compared with adipocytes incubated with isoproterenol alone. It can also be seen that this effect was comparable to the inhibition produced by 400 pM insulin. Masoprocol also significantly (P < 0.05) reduced 8 CPT-cAMP-induced lipolysis as shown in Fig. 5.
The effects of masoprocol and esculetin on isoproterenol-induced lipolytic activity in isolated adipocytes were also examined. As expected, pretreatment with 50 μM masoprocol significantly (P < 0.01) inhibited lipolytic activity, whereas esculetin (4 and 40 μM) did not (data not shown). The lipolytic effect of okadaic acid, a serine/threonine phosphatase inhibitor, in the presence or absence of masoprocol is shown in Fig. 6. Okadaic acid (500 nM) significantly enhanced isoproterenol-induced lipolytic activity (P < 0.05) by isolated adipocytes. Moreover, okadaic acid decreased the antilipolytic effect of masoprocol. Masoprocol (50 μM) inhibited isoproterenol-induced lipolytic activity by ~40%, but, in the presence of okadaic acid, masoprocol inhibited isoproterenol-induced lipolytic activity by only 5%. Wortmannin, a specific inhibitor of PI 3-kinase, did not affect the ability of masoprocol to inhibit isoproterenol-induced lipolytic activity (data not shown).

Figure 7 shows the in vitro inhibitory actions of masoprocol on isoproterenol-induced HSL activity in isolated adipocytes. In the presence of 3 nM isoproterenol, HSL activity was reduced by ~40% when adipocytes were preincubated with 50 μM masoprocol. Evidence that masoprocol decreases HSL activity by changing the phosphorylated state of HSL is presented in Fig. 8. In this experiment, an anti-phosphoserine antibody was used to detect the phosphorylation of HSL. The results clearly show that HSL protein immunoprecipitated from adipocytes treated with masoprocol had a decreased phosphoserine band.

**DISCUSSION**

This study was initiated to test the hypothesis that masoprocol inhibits the activity of HSL and that this
action accounts for its ability to decrease the rate of adipocyte lipolysis and lower plasma FFA, TG, and insulin concentrations. The results presented not only provide strong support for this view, but they define a molecular mechanism to explain how masoprocol inhibits lipolytic activity.

At the simplest level, the studies of fructose-induced hypertriglyceridemia demonstrate that administration of masoprocol lowers plasma FFA and TG concentrations in nondiabetic rats, similar to earlier results demonstrating the same phenomenon in rats with an experimental form of type 2 diabetes (26). However, the two situations differ with regard to the effect of masoprocol administration on insulin and glucose concentrations. In both instances, there is evidence based on the serum insulin measurements that insulin sensitivity was enhanced after masoprocol administration. In the fructose-fed rat, the physiological response of the pancreatic β-cell to enhanced insulin sensitivity would be to secrete less insulin, leading to lower insulin but unchanged glucose concentrations, as shown in Table 1. In contrast, the masoprocol-induced increase in insulin sensitivity in the fat-fed/streptozotocin rat model of type 2 diabetes would lead to increased glucose disposal and a fall in glucose concentration. However, because these animals remain hyperglycemic, the pancreatic β-cell continues to secrete as much insulin as before. In addition to its effect on enhancing insulin sensitivity, the results presented indicated that isoproterenol-induced stimulation of adipocytes isolated from masoprocol-treated rats was associated with significantly less FFA and glycerol release. Finally, HSL activity was significantly lower in adipose tissue from masoprocol-treated rats, associated with a decrease in HSL protein.

The results of the in vivo administration of masoprocol raised the possibility that the antilipolytic effect of masoprocol was mediated via its ability to inhibit phosphorylation of HSL, and this conclusion received strong support from the in vitro effects of masoprocol on isolated adipocytes. More specifically, the results of the in vivo administration of masoprocol on isolated adipocytes isoproterenol and govern activation properties in vitro. J Biol Chem 273: 215–221, 1998.


