INHIBITION OF METOPROLOL METABOLISM BY AMINO ACIDS IN PERFUSED RAT LIVERS

Insights into the Food Effect?

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ABSTRACT:

A mixture of amino acids inhibits propranolol metabolism in perfused rat livers. To obtain mechanistic information about the interaction, a related but less tissue-bound drug, metoprolol, was used to determine V_max and K_M for parent drug and two metabolites in the presence and absence of amino acids. Six groups of 4 livers from 24 male Sprague-Dawley rats were perfused in the single-pass mode at 3 ml/min/g liver for 130 min with oxygenated buffer containing 3.74, 4.49, 5.61, 7.48, 18.7, or 44.9 µM metoprolol. From 50 to 90 min, a balanced amino acid mixture was included in the buffer. Samples of liver effluent taken every 5 min were analyzed by HPLC for metoprolol and two metabolites, α-hydroxymetoprolol and O-demethylmetoprolol. Steady-state concentrations of drug determined before, during, and after amino acids were used to determine V_max and apparent K_M values by nonlinear curve-fitting under each condition. Amino acids reversibly reduced the V_max values of metoprolol and both metabolites by 50% without significantly affecting apparent K_M values. As a result, large increases in availability occurred, especially at low metoprolol inlet concentrations (>90%). Amino acids also increased oxygen consumption until the effluent buffer was almost depleted. Possible mechanisms influencing V_max include direct inhibition of metabolic enzymes by amino acids or cosubstrate (NADPH or oxygen) limitation. Amino acid-mediated pericentral oxygen depletion in the hepatic sinusoids could result in inhibition of drug-metabolizing enzymes, and is consistent with a reduction of V_max and oxygen depletion in the effluent buffer during amino acid coinfusion. We postulate that one or more of these mechanisms could contribute to the interaction between food and high first-pass drugs observed in humans.

1 Abbreviations used are: AUC, area under the plasma concentration-time curve; O_2, oxygen; OH, hydroxy; V_max and K_M, velocity of metabolism; CL, organ clearance;Css, concentration at steady-state; f_in, time to steady-state; C_in, inlet concentration; C_out, outlet concentration; Q, buffer flow rate; E, organ extraction ratio; CL, organ clearance; F, organ availability; r, velocity of metabolism; C_out, outlet concentration of metabolite; [S], substrate concentration; MW, molecular weight; CYP, cytochrome P450.

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Rac-metoprolol is one of several drugs known to exhibit increased oral availability when coadministered with a high-protein meal, including propranolol, propafenone, labetolol, zuclopenthixol, and dixyrazine (1). Although this food interaction was first observed two decades ago, its mechanism remains to be fully elucidated, despite intensive study (1). The mechanism is likely to be complex, with contributions from more than one of the physiological responses to food combining to cause a net increase in AUCoral (1). Because drugs that show the “food effect” are almost completely absorbed from the gastrointestinal tract, it is generally agreed that the interaction is due to a reduction in first-pass metabolism (1). Studies to date on propranolol have concentrated on the liver, although recent studies in rabbits suggest the possible contribution of intestinal metabolism (2). A reduction in hepatic first-pass metabolism could occur through changes to hepatic blood flow, plasma protein binding, or metabolic activity. The hypothesis that the increase in AUCoral of propranolol could be caused by a transient increase in hepatic blood flow (3) was questioned, because flow changes could not account for the magnitude of the increase (4, 5). It is also unlikely that food causes an increase in the unbound fraction of propranolol in plasma (6). Experiments in humans to test for transient changes in metabolic activity have shown that food causes inhibition of presystemic primary conjugation of propranolol (7), although data relating to the more important phase I pathways are inconclusive (8, 9). Nevertheless, simulations have indicated that propranolol availability would be most sensitive to changes in apparent V_max and somewhat sensitive to changes in apparent K_M, both of which contribute to intrinsic clearance (10).

To explore further whether nutrients could inhibit propranolol metabolism, amino acids were coinfused into rat livers perfused with buffer containing propranolol (11). Global inhibition of metabolism was observed, indicating that at concentrations achieved after a high protein meal, amino acids could inhibit all of the pathways of propranolol metabolism and that the degree of metabolic inhibition was related to the concentration of amino acids in the buffer. Hepatic tissue binding prevented further exploration of the mechanism using propranolol as a model drug. Pilot studies showed, however, that metoprolol is much less extensively tissue bound and its metabolism was inhibited by amino acids, making it an appropriate model drug for mechanistic studies involving the measurement of Michaelis-Menten parameters. The metabolism of metoprolol is depicted in fig. 1. We describe herein experiments that confirm our pilot observations in perfused rat livers and that focus on amino acid effects on the apparent V_max and K_M of metoprolol metabolism. As part of the liver viability assessment, effluent buffer O_2 content was measured.
Materials and Methods

Chemicals. (+)-Metoprolol tartrate and nadolol (internal standard) were purchased from Sigma Chemical Co. (St. Louis, MO). H119/66 p-OH benzoate (α-hydroxymetoprolol), H105/22 p-OH benzoate (O-demethylmetoprolol), and H117/04 HCl (metoprolol acid) were gifts from Astra Hässle (Mölndal, Sweden). Solvents were HPLC grade (BDH, Toronto, Canada). All other chemicals used were of analytical grade.

Animals. Livers used in the study were from 24 male Sprague-Dawley rats (198–250 g, Charles River, St. Constant, Quebec, Canada) maintained on standard laboratory chow and water ad libitum in accordance with the guidelines of the Canadian Council on Animal Care. Animals were randomly assigned to six groups of four in a parallel design, one group for each of six concentrations of metoprolol in the perfusion medium (3.74, 4.49, 5.61, 7.48, 13.46, and 44.9 μM). Perfusions were performed sequentially according to the random assignments.

Surgical Procedure. After anesthetizing the rat with methoxyflurane (Pitman-Moore, Mississauga, Ontario, Canada), the liver was isolated and perfused in the single-pass mode with oxygenated Krebs bicarbonate buffer in a temperature- and humidity-controlled cabinet as previously described (12, 13). During the surgical procedure, there was no prolonged period of anoxia because perfusion was started immediately after cannulation of the portal vein. The entire surgical procedure was conducted rapidly (<20 min) to minimize the chance of an unsuccessful preparation. Liver viability was assessed by: 1) measurement of the O2 tension in the liver effluent using a biological O2 monitor (YSI model 5300; YSI, Inc., Yellow Springs, OH) equipped with a micro-O2 probe attached to a low-flow cell; 2) the physical appearance of the liver; and 3) the maintenance of metoprolol steady-state concentrations.

Liver Perfusions. The study was designed both to evaluate the apparent Michaelis-Menten parameters and to measure the effect of a balanced mixture of amino acids on apparent Vmax and Km values for metoprolol and its metabolites. Metoprolol in Krebs bicarbonate buffer was infused at 3 ml/min/g liver into different livers at various concentrations over the range of saturation of metabolism. Because the liver outlet steady-state concentration reflected the velocity of metabolism or formation of each metabolite, plots of velocity vs. concentration were constructed from which the apparent Vmax and Km values for metoprolol and each metabolic pathway were calculated. To assess the effect of amino acids, the metoprolol and/or metabolite steady-state concentrations in the effluent were perturbed by adding a balanced mixture of amino acids to the buffer. Based on the measurement of portal venous concentration of phenylalanine (250 μM) in dogs after a high-protein meal (14), a balanced mixture of amino acids in the perfusion buffer-medium was added as a dilution of Aminosyn® II 10% Amino Acids Injection (Abbott Laboratories, Montreal, Quebec, Canada). Final concentrations of constituent amino acids in the inlet buffer were as previously described (11). Velocity vs. concentration plots were again constructed, and the apparent Vmax and Km values in the presence of amino acids were calculated for comparison with the preamino acid values. Once steady-state liver outlet concentrations had been measured in the presence of amino acids, buffer without amino acids was perfused again to verify the reversibility of the amino acid interaction.

Time 0 was defined by the initiation of perfusion with Krebs bicarbonate buffer containing metoprolol at 1 of the 6 inlet concentrations. A blank perfusion buffer sample was collected before time 0; after time 0, perfusate samples were collected over 20 sec at 1, 2.5, 5, 7.5, 10, and then every 5 min until 130 min. After the initial 50-min control phase, during which metoprolol and metabolite concentrations in the effluent buffer had been at steady state for ~30 min, the perfusion medium was switched to Krebs buffer containing amino acids in addition to metoprolol. The metoprolol and amino acids perfusion was continued for 40 min, then the perfusion medium was returned to the original Krebs buffer containing only metoprolol for 40 min. During the perfusion, the temperature and O2 tension in the effluent were recorded when samples were taken. Samples of liver effluent were analyzed for metoprolol and metabolites immediately following each perfusion experiment.

Analytical Method. After addition of the internal standard (50 μl of a 6.46 μM nadolol solution in water) to 1.0 ml of the perfusate sample, 50 μl of the mixed sample was directly injected onto the HPLC system consisting of a Waters model 510 Pump, a Shimadzu model SIL-9A Autoinjector, a NovaPak C18 8 × 100 mm, 4-μm particle size column, an ABI Spectroflow 980 Programmable Fluorescence detector (excitation wavelength 224 nm; no emission filter), and a Shimadzu model C-R3A Chromatopac Integrator. The mobile phase consisted of water:acetonitrile:triethylamine (91:9:0.3, v/v/v), adjusted to pH 3.0 with orthophosphoric acid and pumped at 3.0 ml/min. Run time was 22 min. The assay was validated according to generally accepted criteria (15).

Data Analysis. When the single-pass perfusion system was at steady-state, and the distribution/binding processes of the drug in the liver preparation were essentially complete, the rate of drug loss across the liver equaled the rate of metoprolol elimination (i.e. the rate of metabolism). Cn and tn were defined as previously described (12). The hepatic tissue binding of metoprolol was calculated from the effluent steady-state metoprolol data as previously described (12). Hepatic disposition of drug was described by standard steady-state mass balance equations for drug elimination by an organ (16). Parameters calculated from Cn, Cn, and Q included E, CL, (ml/min) and F. The (nmol/min/g liver)
was calculated from $Q$ (ml/min/g liver), $C_{in}$ and $C_{out}$ (μM), and the formation rates of α-hydroxymetoprolol and O-demethylmetoprolol from metoprolol (nmol/min/g liver) were calculated from $Q$ and the corresponding $C_{out,a}$ as previously described (17). The apparent $V_{max}$ and $K_{M}$ values for metoprolol metabolism and each metabolic pathway were estimated by nonlinear curve-fitting (PCNONLIN V4.0; SCI Software, Lexington, KY) to the Michaelis-Menten equation.

A repeated-measures ANOVA was used to determine whether coinfusion of amino acids significantly affected steady-state effluent concentrations of metoprolol and its metabolites. Comparison of steady-state concentrations in the effluent among the initial control phase (from 0 to 50 min), amino acid coinfusion phase (from 50 to 90 min) and after the removal of amino acid phase (from 90 to 130 min), and comparisons of the pharmacokinetic parameters between the initial control and amino acid coinfusion conditions were made by paired $t$ test ($p \leq 0.05$).

**O$_2$ Uptake.** Although it was originally intended that O$_2$ concentration values in the effluent perfusate be used only for liver viability assessment, the measurements were appropriate to determine O$_2$ uptake by the liver over time. Given that the buffer was equilibrated with 95% O$_2$/5% CO$_2$ and the solubility of O$_2$ in 37°C Ringers is 1.0755 μmol/ml (19), the influent O$_2$ concentration was ~1.02 μmol/ml. The O$_2$ monitor readout was in percentage saturation; the influent buffer was calibrated to 100%. O$_2$ content in the effluent buffer was thus calculated as the readout percentage saturation × 1.02 μmol/ml. For each liver, the mean O$_2$ content of the effluent buffer during each phase of the experiment was calculated (i.e. 5–50 min, 55–90 min, and 95–130 min). O$_2$ uptake during each phase was calculated as

$$O_{2\text{in}} - O_{2\text{out}} \times Q,$$

where $O_{2\text{in}}$ is the influent concentration of O$_2$, $O_{2\text{out}}$ is the mean effluent concentration of O$_2$ during a particular phase of the experiment, and $Q$ is the buffer flow rate for a particular liver expressed as ml/min/g liver.

We have observed that the O$_2$ uptake rate varies considerably between livers. Although the addition of metoprolol increases O$_2$ uptake slightly compared with blank buffer, the effects of even high concentrations of drug were deemed to be negligible compared with the interindividual variability of O$_2$ uptake. Therefore, for the purpose of comparing O$_2$ uptakes between the phases of the experiments, data from all the livers were pooled. Data were compared using paired $t$ tests ($p < 0.05$).

**Results**

**Assay Validation.** Separation of metoprolol (MW free base, 267.4) and its three metabolites α-hydroxymetoprolol (free base MW, 283.4), O-demethylmetoprolol (free base MW, 253.4), and metoprolol acid (zwitterion MW, 267.4) was achieved with no interferences from endogenous compounds (fig. 2). Retention times for α-hydroxymetoprolol, O-demethylmetoprolol, metoprolol acid, nadolol (internal standard), free base MW 309.4, and metoprolol were 2.7, 3.2, 3.85, 5.55, and 15.5 min, respectively.

The calibration curve for metoprolol was based on the unweighted least squares regression of the peak area ratios of metoprolol to nadolol (internal standard), free base MW 309.4, and metoprolol acid (0.75–18.7 μM) were plotted as the peak height ratios of each compound to nadolol against the corresponding concentrations of analytes. Each calibration curve was linear over the concentration ranges examined, and the correlation coefficients were ≥0.999. Blank samples of effluent liver buffer showed no interfering peaks with the analytes of interest. Variability was assessed by preparing replicate samples of each analyte at three concentrations covering the range from the limit of quantitation. The validated limits of quantitation was assessed by preparing replicate samples of each analyte at three concentrations covering the range from the limit of quantitation. The validated limits of quantitation were the lower concentrations of the ranges reported herein. For intraday variability, six replicates were prepared on 1 day; for interday variability, two replicates were prepared on six separate days. Accuracy for the analytical method was within 5% of the nominal concentration, except for the lowest concentration of metoprolol acid (16%). In most cases, intraday and interday coefficients of variation were <5%, except for metoprolol intraday variation (15%) and metoprolol interday variation (11%) at their lowest concentrations.

**Steady-State.** The time for the effluent concentrations of metoprolol, α-hydroxymetoprolol, and O-demethylmetoprolol to reach steady-
state were calculated for each of the six different inlet concentrations of metoprolol (table 1). Except in one case, the average time for metoprolol to reach steady-state was <20 min, regardless of the metoprolol inlet concentrations; the effluent concentrations of α-hydroxymetoprolol and O-demethylmetoprolol also quickly reached steady-state. The concentration of the metabolite metoprolol acid continued to rise in the effluent and did not reach steady-state during the perfusion.

**Hepatic Tissue Binding of Metoprolol.** Metoprolol hepatic tissue binding (nmol/g liver), calculated from the metoprolol concentrations in the effluent over the time 0–tss, increased as the metoprolol inlet concentration was increased (table 1). But, when the metoprolol inlet concentration was below 7.48 μM, the tissue binding remained relatively constant.

**Metoprolol and Its Metabolites in the Effluent.** Table 2 lists the steady-state concentrations of metoprolol, α-hydroxymetoprolol and O-demethylmetoprolol in the effluent during the three phases of liver perfusion, including “Pre-Amino Acids” (from 0 to 50 min as the control), “Amino Acids” (from 50 to 90 min), and “Post-Amino Acids” (from 90 to 130 min).

At all six metoprolol inlet concentrations, the metoprolol effluent concentrations during amino acid coinfusion were significantly higher (range: 23–97%) than during the control phase (0–50 min). The increases were relatively larger at low than at high inlet concentrations (table 2). Post-amino acid effluent concentrations returned toward pre-amino acid levels, but did not seem to recover completely, although only in the cases of 5.61 and 7.48 μM inlet concentrations were there significant differences between the post- and pre-amino acid acid periods.

For α-hydroxymetoprolol, the effluent concentrations during amino acid coinfusion were significantly lower (range: 26–54%) than during the control period at all six metoprolol inlet concentrations (table 2). The decreases were relatively larger at high metoprolol inlet concentrations than at lower concentrations. Except for when the metoprolol inlet concentrations were 4.49 and 5.61 μM, α-hydroxymetoprolol concentrations rose significantly when amino acid coinfusion was terminated. The post-amino acid concentrations returned to within 8% to 19% of pre-amino acid level, remaining significantly lower than the pre-amino acid effluent levels, except when the metoprolol inlet concentrations were 3.74 and 4.49 μM. This failure to return completely to the pre-amino acid levels is consistent with the observation of slowly declining effluent concentrations of α-hydroxymetoprolol in pilot studies with livers not confused with amino acids.

O-demethylmetoprolol effluent concentrations decreased significantly (15–48%) when metoprolol was coinfused with the amino acids, for all metoprolol inlet concentrations except 3.74 and 5.61 μM. The decreases were relatively larger at higher metoprolol inlet concentration (table 2). After removal of amino acids, the O-demethylmetoprolol concentrations increased toward control levels.

### Effect of Amino Acids on Pharmacokinetic Parameters.

Considering only the 50-min control period in all the livers, metoprolol E and CL decreased by >60% as the inlet concentrations of metoprolol were increased from 3.74 to 44.9 μM (table 3). Similarly, over the range of inlet concentrations, the formation CL of α-hydroxymetoprolol and O-demethylmetoprolol decreased by 62 and 40%, respectively. F increased by 167% as the inlet concentration of metoprolol was increased from 3.74 to 44.9 μM. When metoprolol inlet concentrations were between 3.74 and 5.61 μM, the parameters were similar. But, when the metoprolol inlet concentrations were 7.48 μM or more, large decreases in E and CL, as well as marked increases in F, occurred.

During the amino acid coinfusion period, E and CL of metoprolol decreased by 72%, as the inlet concentration of metoprolol was increased from 3.74 to 44.9 μM (table 3). Similarly, the formation CL of α-hydroxymetoprolol and O-demethylmetoprolol decreased by 73% and 68%, respectively. Metoprolol F in the presence of the amino acids increased by 67% as the inlet concentration was increased from 3.74 to 44.9 μM. As with the control period, changes in these parameters were greater when metoprolol inlet concentrations exceeded 7.48 μM.

Amino acid coinfusion decreased metoprolol E and CL by 35–54%, compared with the control phase, over the range of inlet concentrations. Similarly, the formation CL of α-hydroxymetoprolol decreased significantly by 28–55%. O-demethylmetoprolol formation CL decreased by 17–50%, but only at metoprolol inlet concentrations exceeding 5.61 μM. Amino acid increased F by 21–93% over the six inlet concentrations. The increase in F was greatest at lower metoprolol inlet concentrations.

### Estimation of Apparent V<sub>max</sub> and K<sub>M</sub> and the Effect of Amino Acids.

Amino acids significantly decreased the apparent V<sub>max</sub> values for metoprolol, α-hydroxymetoprolol, and O-demethylmetoprolol (table 4). Metoprolol apparent V<sub>max</sub> decreased by 50% if substrate concentrations were calculated based on the well-stirred model and by 55% if based on the parallel tube model. Similarly, the apparent V<sub>max</sub> value for α-hydroxymetoprolol formation decreased by 53% (well-stirred) or 56% (parallel tube). The apparent V<sub>max</sub> of O-demethylmetoprolol decreased by 53% (well-stirred) or 61% (parallel tube).

There were no consistent changes in the apparent K<sub>M</sub> in the presence of the amino acids. In 4 of the 6 cases, the apparent K<sub>M</sub> values declined in the presence of the amino acids, but the decreases were not statistically significant. The standard errors of the K<sub>M</sub> estimates were high; this would have prevented detection of any real differences between the control and amino acid conditions. The assumptions of the parallel tube model resulted in slightly higher estimated V<sub>max</sub> and K<sub>M</sub> values than those of the well-stirred model.

A weighting of 1/ν was used in the curve-fitting because the velocity of metabolism varied more between livers at higher than at lower concentrations (figs. 3 and 4).
**TABLE 2**

<table>
<thead>
<tr>
<th>Metoprolol Inlet Concentration</th>
<th>Pre-Amino Acids</th>
<th>Amino Acids</th>
<th>Post-Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3.74 μM metoprolol inlet concentration</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metoprolol</td>
<td>0.97 ± 0.19</td>
<td>1.94 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.16 ± 0.30&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-Hydroxymetoprolol</td>
<td>1.45 ± 0.07</td>
<td>1.02 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.38 ± 0.18&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-Demethylmetoprolol</td>
<td>0.28 ± 0.04</td>
<td>0.32 ± 0.04</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td><strong>4.49 μM metoprolol inlet concentration</strong></td>
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<td></td>
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<tr>
<td>Metoprolol</td>
<td>1.23 ± 0.15</td>
<td>2.40 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.46 ± 0.19&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-Hydroxymetoprolol</td>
<td>1.73 ± 0.04</td>
<td>1.27 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.69 ± 0.21</td>
</tr>
<tr>
<td>O-Demethylmetoprolol</td>
<td>0.43 ± 0.04</td>
<td>0.36 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.47 ± 0.12</td>
</tr>
<tr>
<td><strong>5.61 μM metoprolol inlet concentration</strong></td>
<td></td>
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</tr>
<tr>
<td>Metoprolol</td>
<td>1.68 ± 0.56</td>
<td>3.00 ± 0.90&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.09 ± 0.56&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>α-Hydroxymetoprolol</td>
<td>2.18 ± 0.42</td>
<td>1.59 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.98 ± 0.42&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-Demethylmetoprolol</td>
<td>0.43 ± 0.08</td>
<td>0.40 ± 0.08</td>
<td>0.47 ± 0.12</td>
</tr>
<tr>
<td><strong>7.48 μM metoprolol inlet concentration</strong></td>
<td></td>
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<tr>
<td>Metoprolol</td>
<td>2.88 ± 0.52</td>
<td>4.71 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.14 ± 0.45&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<tr>
<td>α-Hydroxymetoprolol</td>
<td>2.82 ± 0.32</td>
<td>1.80 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.61 ± 0.28&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-Demethylmetoprolol</td>
<td>0.59 ± 0.08</td>
<td>0.43 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td><strong>18.7 μM metoprolol inlet concentration</strong></td>
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<tr>
<td>Metoprolol</td>
<td>11.2 ± 1.38</td>
<td>15.2 ± 0.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.0 ± 0.82&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>α-Hydroxymetoprolol</td>
<td>4.27 ± 0.46</td>
<td>1.98 ± 0.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.53 ± 0.42&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-Demethylmetoprolol</td>
<td>1.07 ± 0.12</td>
<td>0.63 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.07 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>44.9 μM metoprolol inlet concentration</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Metoprolol</td>
<td>32.2 ± 1.8</td>
<td>39.6 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.7 ± 1.9&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>α-Hydroxymetoprolol</td>
<td>6.81 ± 0.99</td>
<td>3.46 ± 0.67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.54 ± 1.3&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>O-Demethylmetoprolol</td>
<td>2.17 ± 0.20</td>
<td>1.11 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.09 ± 0.24&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.05 in the repeated-measures ANOVA.

<sup>b</sup> p < 0.05, Pre-amino acids vs. Amino Acids and Pre-Amino Acids vs. Post-Amino Acids by paired t test.

<sup>c</sup> p < 0.05, Amino Acids vs. Post-Amino Acids by paired t test.

**TABLE 3**

<table>
<thead>
<tr>
<th>Metoprolol Inlet Concentration, μM</th>
<th>Control</th>
<th>AA</th>
<th>Control</th>
<th>AA</th>
<th>Control</th>
<th>AA</th>
<th>Control</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.74 μM</td>
<td>0.73 ± 0.06</td>
<td>0.47 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.06</td>
<td>0.53 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.1 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.60 ± 0.71&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.93 ± 0.27</td>
<td>1.83 ± 0.22</td>
</tr>
<tr>
<td>4.49 μM</td>
<td>0.72 ± 0.03</td>
<td>0.47 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28 ± 0.03</td>
<td>0.53 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.1 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.85 ± 0.85&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.30 ± 0.33</td>
<td>1.90 ± 0.42&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5.61 μM</td>
<td>0.69 ± 0.10</td>
<td>0.45 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.31 ± 0.10</td>
<td>0.55 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.8 ± 3.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.87 ± 2.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.87 ± 0.35</td>
<td>1.68 ± 0.38</td>
</tr>
<tr>
<td>7.48 μM</td>
<td>0.62 ± 0.09</td>
<td>0.39 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.38 ± 0.08</td>
<td>0.61 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.23 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.54 ± 0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.82 ± 0.22</td>
<td>1.36 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>18.7 μM</td>
<td>0.39 ± 0.06</td>
<td>0.20 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.61 ± 0.05</td>
<td>0.80 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.48 ± 1.41</td>
<td>4.82 ± 0.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.41 ± 0.20</td>
<td>0.79 ± 0.11b</td>
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<tr>
<td>44.9 μM</td>
<td>0.28 ± 0.04</td>
<td>0.13 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.72 ± 0.04</td>
<td>0.87 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.71 ± 0.97</td>
<td>3.14 ± 0.48&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.15 ± 0.12</td>
<td>0.58 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> Data reported are means ± SD of four experiments.

<sup>b</sup> p < 0.05, control (0–50 min) vs. amino acid coinfusion (50–90 min) by paired t test.

**O₂ Uptake.** In every liver, O₂ uptake increased upon coinfusion of amino acids so that the effluent buffer was almost depleted. The mean O₂ content of the effluent buffer declined from 0.166 ± 0.089 μmol/ml during the initial phase of the experiments to 0.053 ± 0.058 μmol/ml during amino acid coinfusion (compared with the inlet concentration of 1.02 μmol/ml). After the amino acid coinfusion was stopped, the mean effluent buffer O₂ levels reached a peak of 0.37 μmol/ml at 110 min, then declined to 0.31 μmol/ml at the end of the experiment (130 min). The mean buffer flow rate was 3.47 ± 0.38 ml/min/g liver; the O₂ delivery rate to the livers thus averaged 3.54 ± 0.39 μmol/min/g liver. The mean O₂ uptake was 2.96 ± 0.44 μmol/min/g liver during the pre-amino acid phase, increasing to 3.36 ± 0.44 μmol/min/g liver during amino acid coinfusion. The mean O₂ uptake after amino acid coinfusion averaged 2.56 ± 0.73 μmol/min/g liver. Paired t tests indicated that the increase in O₂ uptake with amino acid coinfusion and the return toward control values after amino acid coinfusion were statistically significant (p < 0.05).
Discussion

Analytical Method. Direct-injection HPLC methods with fluorescence detection using C18 columns have been used for the measurement of metoprolol and metabolite concentrations in human urine (20, 21), but neither method detected O-demethylmetoprolol. A method that allowed separation and detection of all three major metabolites has been described with conditions suitable for urine and microsomal preparations (22). This method was used as the basis for development of the present method. The conditions were modified to allow good separation of the analytes from interfering peaks present in directly injected rat liver perfusate (fig. 2). The present method also provides the appropriate compromise in sensitivity to allow quantitation of metoprolol and its metabolites when metoprolol is present at up to 50-fold higher concentration than one of the metabolites.

The Model System. Our study with propranolol revealed that a balanced mixture of amino acids could globally inhibit its metabolism, but revealed little about the mechanism of this inhibition (11). To gain more mechanistic information, our approach was to determine whether the inhibition occurs as a result of changes to the affinity or capacity (or both) of the metabolic enzymes. This required determination of the apparent $K_M$ (affinity) and $V_{max}$ (capacity) values of the metabolic pathways. To be able to observe both direct and indirect mechanisms, we conducted this study using isolated, perfused rat livers.

A weakness of propranolol as a model drug is that tissue binding prevents steady-state from being reached, except at very high, saturating inlet concentrations. The hepatic tissue binding of metoprolol was at least 6-fold lower than the 2 mM mol/g liver reported for propranolol and varied with inlet concentration (table 1) (12, 23). Even at nonsaturating inlet concentrations as low as 3.74 mM, steady-state was reached within 20 min (table 1). Thus, studies at steady-state could be conducted at concentrations covering the range of saturation of metabolism. This is an important advantage of metoprolol as a model drug.

Metoprolol is extensively metabolized in the human liver with; 85% of the administered dose recovered in urine as unchanged metoprolol (10%), $\alpha$-hydroxymetoprolol (10%), and metoprolol acid (65%) (24) (fig. 1). In rats, $\approx$95% of a 100 mg/kg (374 mMol/kg) oral dose was recovered in urine as unchanged metoprolol (4.5%), $\alpha$-hy-
droxymetoprolol (25%), O-demethylmetoprolol (3.5%), and metoprolol acid (62%) (25). Thus, the metabolism of metoprolol in rats is similar to what is observed in humans. Metoprolol acid is a secondary metabolite, occurring as an oxidation product of O-demethylmetoprolol (26). Therefore, in addition to the parent drug, all three of these metabolites were measured in the liver effluent. During the initial 50-min control phase, metoprolol, α-hydroxymetoprolol, and O-demethylmetoprolol approached steady-state levels in the effluent within 20 min, regardless of the inlet concentration used (table 1). Metoprolol acid concentrations, however, continued to rise throughout the perfusion experiments for unknown reasons. Therefore, it was excluded from the calculations in this study. We speculate that because this metabolite is more polar than the others (it is a form of amino acid and therefore exists as a zwitterion), it may not readily cross membranes into the effluent. Alternatively, it may be secreted into the bile, which was not collected separately in these experiments.

Michaelis-Menten Kinetics of Metoprolol. E declined by 62% from 0.73 to 0.28 when the metoprolol inlet concentration was increased from 3.74 to 44.9 μM, thus indicating that the inlet concentrations spanned much of the range of saturation of metabolism (table 3). The experimental design, whereby different livers were used for each point of the \( v \) vs. \( [S] \) curve gives rise to a lack of precision in the measurements not seen with microsomal preparations. Differences between rat liver preparations resulted in scattered data, especially at lower metoprolol inlet concentrations (figs. 3 and 4); thus, the computer-processed curve-fitting did not converge on precise values for apparent \( K_M \) (table 4). The mean apparent \( K_M \) values 3.6 (well-stirred) and 8.8 (parallel tube) μM are lower than the apparent \( K_M \) of metoprolol metabolism by microsomes reported by Arfwidsson et al. (25), likely due to differences in the experimental conditions. The \( V_{\text{max}} \) values from microsomes and the perfused liver preparation cannot be directly compared, but if a microsomal protein content of 45 mg protein/g liver is assumed (27), then the reported \( V_{\text{max}} \) for microsomes of 1.28 nmol/mg protein/min may be converted to a value of ~58 nmol/min/g liver. The estimates from the perfused livers compared favorably at 38 (well-stirred) and 46 (parallel tube) nmol/min/g liver (table 4). Using the same conversion factor of 45 mg protein/g liver, the apparent \( K_M \) and \( V_{\text{max}} \) values for α-hydroxylation were 18 μM and 13 nmol/min/g liver and for O-demethylation were 18 μM and 7.7 nmol/min/g liver in rat liver microsomes (24). These values compare more favorably with this study than the parent drug data (table 4). The parallel tube model gave somewhat higher estimates of apparent \( K_M \) and \( V_{\text{max}} \) than did the well-stirred model; these higher estimates were in general in closer agreement with the microsomal data. It should be noted that the values for \( K_M \) and \( V_{\text{max}} \) may depend to some extent on the range of concentrations of drug used in the experiments, because in humans both high-affinity, low-capacity and low-affinity, high-capacity enzyme systems for each pathway have been identified (28). These may also exist in the rat, although they have not yet been reported.

Effect of Amino Acids on Metoprolol Metabolism. Meal protein is digested and transported into the portal circulation as amino acids (14), so they were used in the perfusion buffer to mimic a high-protein meal. In pilot studies, a mixture of amino acids inhibited metoprolol metabolism similarly to propranolol metabolism (11). Whereas the main objective of this study was to determine whether amino acids would affect the apparent \( K_M \) and/or \( V_{\text{max}} \) of metoprolol, a second objective was to determine the sensitivity of metoprolol availability to amino acids at nonsaturating vs. saturating concentrations of metoprolol.

The increase in effluent metoprolol concentrations, coupled with the corresponding reductions in α-hydroxymetoprolol and O-demethylmetoprolol levels indicated that metoprolol metabolism was inhibited (table 2); no apparent effect of the amino acids on hepatic tissue binding of metoprolol was observed, because the new steady-state was reached immediately upon coinfusion of amino acids. The recovery toward pre-amino acid effluent concentrations of metoprolol and its metabolites indicated that the amino acid effect was reversible. The α-hydroxymetoprolol levels failed to recover completely after removal of the amino acids; however, under control conditions, its concentration in the effluent slowly declined over the course of the perfusion. The effect of amino acids was temporary, so it was likely that no irreversible alteration or damage to the hepatic metabolic enzyme system occurred.

Nonlinear curve-fitting of metabolism/formation velocity vs. metoprolol concentration in the liver water revealed that amino acids reduced \( V_{\text{max}} \) by ~50%, but had no significant effect on the apparent \( K_M \) of metoprolol and its two metabolic pathways (table 4). Therefore, amino acids can reversibly reduce the capacity for oxidative metabolism, ruling out competitive inhibition as a mechanism. With competitive inhibition, changes to \( K_M \) only would have been observed. It was evident that the hepatic elimination model used in the curve-fitting did not influence the estimated effect of the amino acids on the apparent \( V_{\text{max}} \) and \( K_M \) (table 4, figs. 3 and 4).

In an earlier study, we observed only a small amino acid-mediated increase in \( F \) of propranolol when it was infused at saturating concentrations, but predicted a larger increase if we had been able to observe the system at low (nonsaturating) concentrations (11). A principal advantage of metoprolol as a model drug is its low tissue binding, because we were able to observe the interaction at low, more clinically relevant steady-state concentrations. Over the entire range of inlet concentrations, coinfused amino acids caused significant decreases in metoprolol \( E \) and \( CL \), as well as the formation clearances of α-hydroxymetoprolol and O-demethylmetoprolol. At the highest inlet concentration, the observed increase in \( F \) was only 21%, similar to the observations with propranolol, but at the lowest inlet concentration, \( F \) increased by 93% from 0.27 to 0.53 (3.74 μM, table 3). Across the group of drugs affected by food, the average increase in AUC may be <50%, but increases of >100% may occur in individual patients. None of the mechanisms proposed to date has been shown to have the capability to produce sufficiently large \( F \) changes to explain the entire effect (29). The high sensitivity of \( F \) at low inlet concentrations of metoprolol in the perfused rat liver shows that metabolic inhibition under conditions that have relevance for the human clinical situation can cause increases in bioavailability similar in magnitude to those observed in humans.

Possible Mechanisms for the Effect of Amino Acids. This study indicates that the inhibition of metoprolol metabolism in the perfused rat liver during coinfusion of amino acids occurs through a reduction in the capacities of the oxidative pathways, implying that the dominant component of the mechanism may be direct noncompetitive or uncompetitive inhibition of cytochrome P450, such as by allosteric effects, or it may be indirect, such as by limiting the supply of cosubstrates for oxidation, NADPH, or \( O_2 \).

No reports were found relating to direct interactions between amino acids and cytochrome P450 enzymes. These should be observable in microsomal systems, however. Direct interactions would be expected to be somewhat specific toward enzyme forms rather than global, however, and all of the pathways of both metoprolol and propranolol are similarly affected (11). Although more than one enzyme seems to be involved in each metabolic pathway of metoprolol metabolism, our observations on metoprolol could be attributable to a single high-affinity, low-capacity enzyme system. In human microsomal experi-
ments, CYP2D6 catalyzes both \( \alpha \)-hydroxylation and \( O \)-demethylation with similar apparent \( K_{\text{m}} \) values to those observed in the present rat liver perfusion study, whereas the low-affinity, high-capacity pathway(s) were linear in the concentration range used in the present study and could remain unaffected (30). Direct inhibition of a single CYP enzyme is not consistent with our previous observations on propranolol, however. Not only ring-hydroxylation, but \( N \)-dealkylation and deamination are inhibited by amino acids; these metabolic reactions seem to be catalyzed by different enzymes (31).

A continuous supply of NADPH is required as a cofactor for cytochrome P450-dependent drug oxidation. NADPH is generated by multienzyme systems that exist in several intracellular compartments. Indirect regulation of metabolism by limitation of the supply of NADPH may occur \( \textit{in vivo} \), and this may be an important control mechanism in drug oxidation since maintenance of the redox state of the NADP: the NADPH couple is a highly regulated process in intact cells (32). In fasted rats, competing reactions for substrate and cofactor play a major role in determining the availability of NADPH for mixed-function oxidation. In this study, however, the ample supply of glucose in the perfusion buffer (11 mM) should have provided sufficient NADPH \( \textit{via} \) the pentose phosphate shunt to support cytochrome P450 activity. It is uncertain whether the amino acid mixture used would affect the redox state of the hepatocytes, because metabolic reactions involving both production and utilization of reducing equivalents may occur. In addition, the production of reduced NADPH and NADH is independently dependent on intracellular \( O_2 \) tension (33). When \( O_2 \) tension is low, the reduced form may be favored. Although the NADPH supply could have been limiting, the conditions used seem unlikely to cause its depletion. Other mechanisms of metabolic inhibition are more plausible, but this mechanism cannot yet be ruled out.

Among the reports related to the food effect on high first-pass drugs, we have encountered none indicating that nutrients may cause sufficient depletion of hepatic \( O_2 \) that it could be a limiting cofactor for drug metabolism. Nevertheless, coadministration of amino acids caused both decreased \( V_{\text{max}} \) values for the parent drug and metabolites and an increase in hepatic \( O_2 \) consumption sufficient to deplete almost completely the buffer of \( O_2 \) in every liver. \( O_2 \) limitation could have a role within the hepatocyte if other reactions with higher affinities were to compete successfully with cytochrome P450 for \( O_2 \). A much larger effect could be expected, however, if \( O_2 \) were to be used before it reached the cells containing the drug-metabolizing enzymes. Thus, the distribution of enzymes within the zones of the hepatic acinus may be an important determinant of the amount of \( O_2 \) reaching the cells containing the drug-metabolizing enzymes. It is known that an \( O_2 \) gradient exists between the portal and central venous sides of the liver lobule (33). The influent \( O_2 \) concentration \( \textit{in vivo} \) is \( \sim 85 \) \( \mu \)M; the difference across the lobule is \( \sim 50 \) \( \mu \)M. Handling of amino acids, including transport into the cells and gluconeogenesis, is an \( O_2 \)-consuming process that is conducted predominantly by the hepatocytes in the periportal zone of the acinus (34). The cytochrome P450 enzymes that perform drug metabolism are distributed downstream, mainly in the pericentral region of the acinus (32, 35). Therefore, when both amino acids and drug are present in the portal vein, the location of the metabolic enzymes would dictate that amino acid metabolism has priority, and this process may deplete \( O_2 \) supplies before sinusoidal blood reaches the pericentral zone. Propranolol clearance is less during antegrade perfusion than during retrograde perfusion of rat livers under hypoxic conditions, indicating that propranolol metabolism is sensitive to the local \( O_2 \) concentration in the pericentral region where most of the oxidative enzymes are located (36). It is important to note that this observation was made under slightly hypoxic conditions, wherein no extraoxidative load other than by drug metabolism was placed on the liver. Therefore the reported data cannot indicate whether increasing \( O_2 \) utilization in the periportal region would increase the sensitivity of propranolol clearance to hypoxia. Although it seems likely that placing an oxidative load upstream from a sensitive system could inhibit metabolism through \( O_2 \) depletion, this remains to be demonstrated.

Although the conditions used in this study were not designed to illustrate the effect of \( O_2 \) depletion by amino acids, they are comparable with those used in other liver perfusion studies. The pre-amino acid \( O_2 \) consumption of \( \sim 3 \) \( \mu \)mol/min/g liver is somewhat higher than the 2.3 \( \mu \)mol/min/g liver reported to be usual in liver perfusion studies (37). This figure is also higher than the 1.5–2.0 \( \mu \)mol/min/g liver rate of \( O_2 \) consumption reported in an early study on the effect of hypoxia on propranolol elimination (38), but somewhat lower than the 3.5 \( \mu \)mol/min/g liver consumption reported by the same group in a more recent study on the sensitivity of propranolol elimination to hypoxia (39). In the latter study, authors indicate that a higher \( O_2 \) delivery rate (6.4 \( \mu \)mol/min/g liver) is more physiological than lower rates (we used 3.5 \( \mu \)mol/min/g liver). They observed an immediate, direct, and reversible relationship between \( O_2 \) delivery and propranolol clearance below a delivery rate of 6 \( \mu \)mol/min/g liver. In our study, amino acids caused almost complete \( O_2 \) extraction from the buffer, and the studies cited herein indicate that it is likely that most of the \( O_2 \) was extracted upstream from the enzymes responsible for metoprolol metabolism.

As yet, we cannot be sure how relevant our results may be to the \( \textit{in vivo} \) situation in humans, but studies in humans indicate that meal protein (in contrast to meal carbohydrate or fat) results in \( O_2 \) depletion from the hepatic effluent, despite a large increase in hepatic blood flow (40, 41). This is consistent with the general observation that the food effect on high first-pass drugs is associated particularly with high protein meals. Under these circumstances, the reduced hepatic arterial flow and enhanced oxidative metabolism of amino acids in the periportal cells almost certainly led to reduced \( O_2 \) availability in the perivenous zone, wherein most of the cytochrome P450 activity resides. Sensitivity of human hepatocytes and their cytochrome P450 activities to reduced \( O_2 \) levels is unknown. Nevertheless, data from rats indicate that propranolol clearance is very sensitive to \( O_2 \) levels (39), and the maximum effect of a protein meal on hepatic \( O_2 \) consumption occurred close to the time of peak drug levels in the liver (10).

An amino acid-mediated decrease in metabolic capacity of metoprolol and other affected drugs, such as propranolol, is consistent with many, but not all, of the observations related to the food effect. In particular, recent observations that small increases in the propranolol AUC were observed in both humans and dogs when they were shown food but not allowed to ingest it indicate that other mechanisms may contribute to the interaction (42). It has been stated on numerous occasions that the food effect seems to be complex. We propose that one or more of the mechanisms discussed herein could contribute to this phenomenon. Further experimentation will be required to establish the importance of each.

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References


