Glitazones regulate glutamine metabolism by inducing a cellular acidosis in MDCK cells

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Coates, Greg, Itzhak Nissim, Harold Battarbee, and Tomas Welbourne. Glitazones regulate glutamine metabolism by inducing a cellular acidosis in MDCK cells. Am J Physiol Endocrinol Metab 283: E729–E737, 2002. First published April 2, 2002; 10.1152/ajpendo.00485.2001.—We studied the effect of the antihyperglycemic glitazones, ciglitazone, troglitazone, and rosiglitazone, on glutamine metabolism in renal tubule-derived Madin-Darby canine kidney (MDCK) cells. Troglitazone (25 μM) enhanced glucose uptake and lactate production by 108 and 92% (both P < 0.001). Glutamine utilization was not inhibited, but alanine formation decreased and ammonium formation increased (both P < 0.005). The increase in net alanine formation occurred with a change in alanine aminotransferase (ALT) reactants, from close to equilibrium to away from equilibrium, consistent with inhibition of ALT activity. A shift of glutamine’s amino nitrogen from alanine into ammonium was confirmed by using L-[2-15N]glutamine and measuring the [15N]alanine and [15N]ammonium production. The glitazone-induced shift from alanine to ammonium in glutamate metabolism was dose dependent, with troglitazone being twofold more potent than rosiglitazone and ciglitazone. All three glitazones induced a spontaneous cellular acidosis, reflecting impaired acid extrusion in responding to both an exogenous (NH4 Cl) and an endogenous (lactic acid) load. Our findings are consistent with glitazones inducing a spontaneous cellular acidosis associated with a shift in glutamine amino nitrogen metabolism from predominantly anabolic into a catabolic pathway.

The glitazones troglitazone, ciglitazone, and rosiglitazone are proxisome proliferator-activated receptor-γ (PPARγ) agonists (9) that exhibit antihyperglycemic activity (11, 13, 18), halt mesangium expansion in experimental models of type 2 diabetes mellitus (20, 26), and act directly on mesangial cells to limit extracellular matrix formation (33). In cultured mesangial cells, troglitzone inhibits glutamine utilization (34) and, specifically, glutamate transamination and alanine formation (Fig. 1, R2). In addition to the decreased alanine formation, there is an increased ammonium formation without increased glutamine breakdown (Fig. 1, R1), consistent with a shift of the glutamate flux from the transamination (Fig. 1, R2) into the deamination pathway (Fig. 1, R3). As shown in Fig. 1, one way for troglitazone to affect increased deamination (R3) at the expense of transamination would be to shift glutamate from the transamination pathway (R2) into the mitochondrion, where the deamination pathway, glutamate dehydrogenase (GDH; R3) is located. If the transamination pathway is predominantly cytosolic, then this response would reflect a shift of glutamate from the cytosol into the mitochondrial compartment. Thus an important point to establish from the perspective of this model is the intracellular localization of the alanine aminotransferase.

Furthermore, if the functional glutaminase activity is indeed within the cytosolic compartment (23, 32), glutamate formed within the cytosol would be effectively disrupted from subserving functions localized to this compartment, including matrix synthesis, and be directed into the mitochondrial pathway. Noteworthy is that not all investigators view the mitochondrial glutaminase as being functional within the cytosolic compartment and instead assign it to the mitochondrial matrix (21) or matrix surface of the inner mitochondrial membrane (12). Kvamme et al. (23), on the other hand, found the matrix space glutaminase inactive and the inner membrane glutaminase population to be functionally active on the cytosolic surface.

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Fig. 1. Putative glutamine and glucose metabolism in relation to intracellular acidosis in Madin-Darby canine kidney (MDCK) cells. Glucose (Gluc) uptake is shown coupled to lactic acid production, with lactic acid (LAc) released to the medium and H⁺ pumped out by the Na⁺/H⁺ exchanger (NHE). Glutamine (Gln) is converted to ammonium (NH₄⁺) and glutamate (Glu⁻) by mitochondrial glutaminase (R1) shown present on the outer surface of inner membrane (22, 30). Note that glutaminase may also be functional within the matrix space (10, 19), in which case the glutamate flux would be reversed. Cytosolic conversion of glutamate to alanine (ALA) is catalyzed by alanine aminotransferase (R2) and may be inhibited by acidosis. Transport of glutamate into the mitochondrial glutamate dehydrogenase (GDH) pathway (R3) and ammonium formation are facilitated by acidosis. Glitazones may shift glutamate from alanine to ammonium by producing a cellular acidosis, either by stimulating acid production or inhibiting acid extrusion.

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Cytosolic glutamate represents a pivotal point in the metabolism of glutamate as depicted in Fig. 1. If glutamate undergoes transamination or conversion to proline, largely cytosolic reactions (2, 25), then the amino nitrogen as well as carbon skeleton may be distributed into amino acids, which serve as building blocks for protein synthesis and particularly collagen, as in the case of glutamate conversion to proline (2, 24). On the other hand, if glutamate is deaminated by GDH, a mitochondrial matrix space activity (35), then the amino nitrogen is released as ammonium, and the carbon skeleton, α-ketoglutarate, is oxidized and therefore would not contribute to biosynthetic processes. From this perspective, our purpose was to determine whether glitazones effect this putative shift in glutamine metabolism in a well-established epithelial cell line, Madin-Darby canine kidney (MDCK) cells, which actively produces basement membrane components, e.g., collagens (16). Our second goal was to determine whether a shift of glutamate from transamination into deamination was associated with a cellular acidosis, and, if so, whether this was attributable to impaired acid extrusion or increased acid production.

METHODS

MDCK cells obtained from American Type Culture Collection (ATCC catalog no. CCL-34; Manassas, VA) were grown to confluence in T150 flasks in DMEM plus 10% fetal calf serum containing (in mM) 28 sodium bicarbonate, 10 sodium pyruvate, 5 D-glucose, and 2 L-glutamine at 37°C and 5% CO₂ (pH 7.4). Confluent cells were subcultured by detachment with the use of trypsin-EDTA (GIBCO-BRL, Rockville, MD) and reseeded onto 6-well culture plates (Corning Cell Wells, Corning, NY) for metabolic studies or in specially designed 30-mm chambers (Bioptechs; Biological Optical Technologies, Butler, PA) equipped with a heating element and cap port for O₂-to-CO₂ aeration. The chambers were placed uncapped inside a 60-mm covered tissue culture dish and incubated at 37°C and 5% CO₂. The cells were allowed to gain confluence, usually 3–4 days for 6-well plates and 2 days for cells in chambers.

Metabolic Studies. Studies were performed on confluent MDCK cells grown in the 6-well plates over 16 h in DMEM containing DMSO (vehicle) or DMEM plus troglitazone (kindly supplied by Dr. Tagata, Sankyo, Tokyo, Japan) or ciglitazone (Cayman Chemical, Ann Arbor, MI). Media samples were promptly treated with an equal volume of ice-cold 5% trichloroacetic acid, left on ice for 10 min, and then centrifuged at 10,000 g for 10 min. Aliquots of the protein-cleared supernatant containing free amino acids were then treated with O-phthalaldehyde (OPA; FLUKA, Buchs, Switzerland) for precolumn derivatization and injected onto a C₁₈ 4.6 × 250-mm column (Microsorb; Varian, Walnut Creek, CA) for separation of the derivatized amino acids. The column effluent was passed through a fluorescence detector with peaks for the major amino acids at characteristic retention times for standards (in min): glutamate, 11.2; glutamine, 16.4; homoserine (internal standard), 17.8; and alanine, 20.5 (34). The medium concentration of the glutamate, glutamine, and alanine was obtained from the peak areas divided by the peak areas for their respective standards. Utilization or production rates for the respective amino acids were obtained from the concentration differences multiplied by the medium volume of 2 ml. Ammonium concentration was determined by the microdiffusion method (27), and formation rate was determined as above, by subtracting the medium blank, and expressed on the basis of milligrams protein. Glucose and lactate medium concentrations were measured enzymatically (Sigma, St. Louis, MO). Utilization of glucose and production of lactate were calculated as for the amino acids and expressed on the basis of milligrams protein.
For analysis of alanine and ammonium formed from L-[2-\textsuperscript{15}N]glutamine, medium glutamine was replaced with L-[2-\textsuperscript{15}N]glutamine (99 atom % excess; Cambridge Isotope Laboratories, Andover, MA). After 16 h of incubation in the prescribed media above, medium samples were taken and treated with ice-cold 40% perchloric acid. The concentration of ammonium and alanine and their \textsuperscript{15}N enrichment were determined on the neutralized supernatants. Briefly, the amino acids underwent precolumn derivatization with OPA and separation by HPLC and fluorescence detection as described above. Analysis of \textsuperscript{15}N in the amino acids was done by GC-MS, as previously described (6, 29). Formation of \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} was determined after conversion of ammonium to norvaline (6). To calculate the conversion of \textsuperscript{15}N]glutamine to ammonium and alanine, the isotopic enrichment (atom % excess) of \textsuperscript{15}N in the particular metabolite was multiplied with the amount present and expressed as nanomoles per milligram of protein. Ammonium concentration was measured by the previously described microdiffusion method (27).

The distribution of alanine aminotransferase between the cytosolic and mitochondrial compartments was estimated as previously described for liver homogenates (14) in homogenates obtained from confluent monolayers of control cells. Briefly, the cells were scraped into 1 ml of a mannitol-sucrose buffer (0.225 M mannitol, 0.075 M sucrose, 0.01 M Tris, pH 7.8, and 0.05 M EDTA) and gently homogenized on a motor-driven Potter-Elvejhem homogenizer using 10 passes of a loose-fitting pestle. This homogenate was then centrifuged at 14,000

\[ g \] for 45 min at 10°C with the resulting supernatant and pellet fractions separated; the pellet was resuspended in 1 ml of a KHPO\textsubscript{4}-glycerol buffer (0.05 M KPO\textsubscript{4}, pH 7.8, 0.025 M alanine, 0.005 M cysteine, and 50% glycerol), and both fractions were assayed for ALT and LDH activity under optimal conditions. The distribution of ALT between the particulate and soluble fractions was corrected for by the fraction of the cytosolic lactate dehydrogenase (LDH) present in the particulate fraction. Monolayer contents of pyruvate and \( \alpha \)-ketoglutarate were determined on TCA extracts after neutralization to pH 7.4 using 5 M K\textsubscript{2}CO\textsubscript{3}. Determinations were performed by modifications of the enzymatic analyses, using LDH or GDH, and coupled to the oxidation of NAD\textsuperscript{+} + H\textsuperscript{+} monitored at 340 nm as otherwise described (3, 7). Recoveries for pyruvate and \( \alpha \)-ketoglutarate (25 nmol) standards were 91 ± 8 (mean ± SD, n = 4) and 101 ± 13% (mean ± SD, n = 6), respectively.

**Cell pH measurements.** After confluence, chambers were transferred to the stage of an Olympus IMT-2 microscope equipped with a heated stage insert (model no. TIS04201501; Kent Scientific). Fluorescent measurements were made at 37°C through an inverted epifluorescence microscope with a UV-F\times40 objective with use of a Photon Technology International (Brunswick, NJ) RM-D microspectrophotometer outfitted for photometric ratio fluorescence studies. After an autofluorescence measurement made in Krebs-Henseleit medium containing 10 mM d-glucose, the cells were loaded with the pH-sensitive fluorescent dye, the acetoxymethyl ester of 2\',7\'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM, 5 mM in DMSO stock; Molecular Probes, Eugene, OR), dissolved in Krebs-Henseleit medium to 5 \( \mu \)M and added to the chambers for 25 min at 37°C. The chamber was then washed three times with Krebs-Henseleit medium, and fluorescence measurements were obtained after equilibration of the cells in Krebs-Henseleit medium with HEPES substituted for bicarbonate (KHHEPES). Light emitted from a 75-W xenon arc lamp alternately exposed cells to wavelength of 490 and 440 nm. Excitation wavelengths were chopped (10 Hz) at 490 and 440 nm, and emissions from a minimum of three to four aggregated cells were monitored at 535 ± 25 nm by use of a low-pass optical filter. Instrument components and data acquisition and analysis were computer controlled using fluorescence software (FeLiX; Photon Technology International, Brunswick, NJ). Changes in the emission ratio (490/440 nm) were taken as an index of changes in the intracellular pH. The recording periods at the various data acquisition intervals were minimized to avoid BCECF photobleaching. Only preparations with 20-fold greater fluorescence intensity than that of the autofluorescence were used. The high K\textsuperscript{+}-nigericin technique (38) was used to clamp the intracellular pH to medium standards of known pH (confirmed on a Corning 240 pH meter at 37°C after withdrawal of the sample from the chamber), obtaining a pH calibration of the 490-to-440 signal ratio \((r^2 = 0.99\) for 490/440 ratio vs. pH; \(n = 28\)).

To monitor the spontaneous pH\textsubscript{i} response to glitazones, the medium was replaced with fresh Krebs-Henseleit medium with 24 mM HEPES buffer replacing bicarbonate, and the 490-to-440 signal ratio was followed continuously for 8 min. The medium was exchanged a second time with the above KHHEPES containing 25 \( \mu \)M troglitazone, followed by another 8 min of continuous recording. The control and glitazone media were collected and analyzed for lactate concentration as above. Comparisons were then made between control and troglitazone-treatment differences in pH\textsubscript{i} and acid production with the use of the Student \( t \)-test. To test for acid extrusion capability, the cells were incubated in HEPES-buffered Krebs-Henseleit medium (pH 7.40) and then acid loaded with a 4-min exposure to Krebs-Henseleit medium in which 20 mM NaCl had been replaced with 20 mM NH\textsubscript{4}Cl. After returning the Krebs-Henseleit medium, we continued to monitor the spontaneous pH\textsubscript{i} response for 4 min. The recovery response was taken as the change in pH\textsubscript{i} per time interval (d pH/min). Time control experiments for a repetitive acid load were also performed, establishing that the recovery rates were not different for the loading periods. Differences within the 4-min recovery period between control and glitazone ΔpH/min responses were determined by use of the paired Student’s \( t \)-test. For repeated measurements, differences between control and test groups were assessed with the use of ANOVA and a corrected \( t \)-test (Dunnett’s).

**RESULTS**

Table 1 shows the effect of 25 \( \mu \)M troglitazone on glutamine and glucose metabolism. A hallmark of the therapeutic effectiveness of glitazones is their glucose-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gln Uptake</th>
<th>Ala Formed</th>
<th>NH\textsubscript{4} Formed</th>
<th>Glucose Uptake</th>
<th>Lac\textsuperscript{−} Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1,091 ± 199</td>
<td>977 ± 125</td>
<td>1,130 ± 49</td>
<td>3,219 ± 213</td>
<td>6,395 ± 447</td>
</tr>
<tr>
<td>Troglitazone (25 ( \mu )M)</td>
<td>811 ± 196</td>
<td>577 ± 90</td>
<td>1,614 ± 60</td>
<td>6,700 ± 543</td>
<td>12,253 ± 1,549</td>
</tr>
<tr>
<td>( P )</td>
<td>NS</td>
<td>&lt;0.0003</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>

Results are means ± SE in nmol/mg protein from 6 plates/group. Gln, glutamine; Ala, alanine; Lac\textsuperscript{−}, lactate; NS, not significant.
lowering action (11, 13), largely the result of increased utilization (13, 18, 20). In MDCK cells, 25 μM troglitazone increased glucose uptake by 108% ($P < 0.0001$) and lactate formation by 92% ($P < 0.0005$). Despite this large increase in glucose uptake, there was not a reciprocal decline in glutamine utilization ($1,091 \pm 170$ vs. $811 \pm 160$ nmol/mg protein for control and troglitazone treatment, respectively), as might be expected if these substrates were competing as oxidative fuel (31). Despite the maintained glutamine uptake, the products formed from subsequent glutamate metabolism were markedly altered. In the presence of troglitazone, ammonium formation increased by 43% ($1,614 \pm 60$ vs. $1,130 \pm 49$ nmol/mg protein; $P < 0.0001$); this large increase occurs without an increase in glutamine utilization (Fig. 1, R1), consistent with an increased glutamate flux via the deamination pathway (Fig. 1, R3). Note that alanine production decreased also by 43% ($557 \pm 90$ vs. $977 \pm 125$ nmol/mg protein; $P < 0.02$).

The ratio of total ammonium to alanine produced rises from $1.27 \pm 0.20$ to $3.40 \pm 0.68$ ($P < 0.01$) as a result of both the increase in ammonium formed and the decrease in alanine produced.

Because the ALT catalyzes a near-equilibrium reaction (14, 36), we measured the cellular reactants after 4 h in control and troglitazone-treated monolayers to assess whether this reaction remains close to equilibrium. As shown in Table 2, the ALT reaction was close to equilibrium in the control monolayers, given the equilibrium constant of 1.6 (36) and a measured mass action ratio of 1.85 ± 0.36. In monolayers exposed to 25 μM troglitazone for 4 h, the mass action ratio fell 59% to $0.77 \pm 0.06$ ($P < 0.06$), indicating that the reaction has moved away from equilibrium, consistent with reduced ALT activity. This movement away from the near-equilibrium condition of the controls reflects a 66% decrease in cellular alanine content (from $45 \pm 9$ to $15 \pm 2$ nmol/mg protein; $P < 0.02$). In line with the decrease in cellular alanine content, the net alanine flux measured for 4 h slowed by 76% (from $397 \pm 108$ to $96 \pm 12$ nmol·h⁻¹·mg protein⁻¹; $P < 0.03$). The α-ketoglutarate content did not change, but there was a small increase ($P < 0.05$) in pyruvate content (from $13 \pm 1$ to $16 \pm 1$ nmol/mg protein). The other substrate, glutamate, decreased, whereas ammonium production increased 1.8-fold (from $235 \pm 12$ to $412 \pm 22$ nmol·h⁻¹·mg protein⁻¹; $P < 0.002$), consistent with a shift of glutamate metabolism from the ALT into the GDH pathway (Fig. 1). We also measured the assayable ALT activity and the soluble vs. particulate distribution within these cells. Although troglitazone exerts a dose-dependent inhibition of both assayable ALT activity and net alanine flux in the proximal tubule-like cell line LLC-PK₁·F⁺ (39), there was no reduction in assayable ALT in this cell line ($21 \pm 3$ vs. $22 \pm 2$ U/mg protein for control and troglitazone treatment, respectively; $n = 7$ pairs). The subcellular distribution of ALT was predominantly within the soluble fraction (59%; Table 3), and when corrected for the activity trapped within the particular fraction, amounts to almost 76% associated with the cytosolic compartment. This distribution is similar to that observed within enterocytes (25). Although the decrease in alanine production indicates a decreased flux through the ALT, the increase in ammonium production could represent the contribution of both the amide and the amino nitrogen of glutamine and, therefore, by itself would not necessarily indicate an increased deamination of glutamate.

To confirm that the rise in the ammonium-to-alanine production ratio does indeed reflect the putative shift in the utilization of the amino nitrogen of glutamine from the transamination into the deamination pathway as shown in Fig. 1, we measured the formation of [¹⁵N]ammonium and [¹⁵N]alanine formed from L-[2-¹⁵N]glutamate. With this tracer approach, the formation of labeled ammonium quantitates the deamination flux and also localizes it to the mitochondrial matrix space. As shown in Fig. 2, 25 μM troglitazone increases the ammonium formation from the amino nitrogen of glutamine by 2.74-fold (from $179 \pm 3$ to $491 \pm 18$ nmol/mg protein; $P < 0.0001$), confirming that the increase in ammonium formation shown in the balance study above is largely ($312/484 \times 100 = 65\%$) derived from the deamination of labeled glutamate. Alanine formation from the amino nitrogen of glutamine decreased ($P < 0.001$) from $491 \pm 21$ to $118 \pm 12$ nmol/mg, accounting for 93% of the fall in alanine formed. As a consequence, there is a rise in the ratio of ammonium to alanine formed from the amino nitrogen of glutamine from $0.36 \pm 0.03$ to $4.26 \pm 0.48$ ($P < 0.001$).

This shift in glutamate into the deamination pathway at the expense of the transamination pathway is dose dependent, as shown in Fig. 3A for troglitazone, Fig. 3B for ciglitazone, and Fig. 3C for rosiglitazone. Both troglitazone and ciglitazone produced a decrease

### Table 2. Mass action ratio and reactants for alanine aminotransferase

<table>
<thead>
<tr>
<th></th>
<th>MAR</th>
<th>Ala</th>
<th>AKG²⁺</th>
<th>Glu⁻</th>
<th>Py⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.85 ± 0.36</td>
<td>45 ± 7</td>
<td>28 ± 2</td>
<td>54 ± 2</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>Troglitazone</td>
<td>0.77 ± 0.06</td>
<td>15 ± 1</td>
<td>34 ± 4</td>
<td>43 ± 2</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>$P$</td>
<td>=0.01</td>
<td>=0.004</td>
<td>NS</td>
<td>=0.01</td>
<td>=0.02</td>
</tr>
</tbody>
</table>

Results are means ± SE in nmol/mg protein from 4 monolayers/treatment. Measurements were made after 4 h of exposure to troglitazone (25 μM) or vehicle DMSO. MAR, mass action ratio; AKG²⁺, α-ketoglutarate; Glu⁻, glutamate; Py⁻, pyruvate.

### Table 3. Distribution of ALT and LDH between particulate and soluble fractions of MDCK cells

<table>
<thead>
<tr>
<th></th>
<th>ALT Activity</th>
<th>Corrected</th>
<th>LDH Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Particulate</td>
<td>Soluble</td>
<td>Particulate</td>
</tr>
<tr>
<td>1</td>
<td>42.6</td>
<td>57.4</td>
<td>65</td>
</tr>
<tr>
<td>2</td>
<td>41.0</td>
<td>59.0</td>
<td>88</td>
</tr>
<tr>
<td>3</td>
<td>40.0</td>
<td>60.0</td>
<td>75</td>
</tr>
<tr>
<td>Mean</td>
<td>41.2</td>
<td>58.8</td>
<td>76</td>
</tr>
<tr>
<td>SE</td>
<td>±1.3</td>
<td>±1.3</td>
<td>±6.6</td>
</tr>
</tbody>
</table>

LDH, lactate dehydrogenase; MDCK cells, Madin-Darby canine kidney cells; ALT, alanine aminotransferase. Nos. at left are individual plates.
(P < 0.05) in alanine production at 25, 50, and 100 μM (Fig. 3, A and B), whereas rosiglitazone (Fig. 3C) lowered alanine only at 100 μM. In contrast, all three glitazones increased ammonium production over the concentration range of 10–100 μM. The ratio of ammonium produced to alanine produced increases in the ciglitazone-treated monolayers from 0.56 ± 0.08 to 0.96 ± 0.28, 0.72 ± 0.10, 0.83 ± 0.17, 1.36 ± 0.34, and 2.23 ± 0.18 at 5, 10, 25, 50, and 100 μM, respectively (P < 0.05 at 10, 25, 50, and 100 μM). For rosiglitazone, the ratios increased from 0.70 ± 0.05 to 0.84 ± 0.05, 0.99 ± 0.07, 1.20 ± 0.10, 2.16 ± 0.31, and 4.66 ± 0.6, respectively (P < 0.05 at 10, 25, 50, and 100 μM). In the troglitazone-treated monolayers, this ratio increases from 0.53 ± 0.02 to 0.72 ± 0.11, 0.98 ± 0.13, 2.31 ± 0.71, 2.80 ± 0.62, and 4.02 ± 0.17 at 5, 10, 25, 50, and 100 μM, respectively (P < 0.05 at 10, 25, 50, and 100 μM). Although all three glitazones increase this ratio, troglitazone appears more potent than ciglitazone, whereas rosiglitazone appears almost as potent as troglitazone at 25 μM and above.

One testable mechanism by which glitazones might shift glutamate from the transamination into the deamination pathway would be to lower the cytosolic pH and thereby accelerate glutamate transport across the mitochondrial inner membrane and into the matrix space (Fig. 1). To determine whether troglitazone decreases cytosolic pH, MDCK cells were loaded with BCECF, and the spontaneous pHi was monitored over 8 min after addition of KHHEPES or KHHEPES containing 25 μM troglitazone. A representative recording is shown in Fig. 4A, with results from 20 experiments presented in Table 4. The steady-state pHi for MDCK cells did not change with the addition of the KHHEPES (ΔpH/min = 0.0006 ± 0.001, measured over the first 4 min) and averages 7.13 ± 0.10 (measured at 8 min). In marked contrast, addition of KHHEPES plus 25 μM troglitazone produced a prompt decline in the sponta-

![Figure 2](http://ajpendo.physiology.org/)

![Figure 3](http://ajpendo.physiology.org/)

**Fig. 2.** Proof that troglitazone shifts the amino nitrogen of glutamine from transamination to deamination pathway. Troglitazone (30 μM) increases [15N]ammonia (NH4+) formation and decreases [15N]alanine (ALA) formation from L-[2-15N]glutamine. Results are means ± SE (in nmol/mg protein) from 3 monolayers/group incubated for 16 h.

**Fig. 3.** A: dose response for ammonium and alanine production to troglitazone. B: dose response of ammonium and alanine production to ciglitazone. C: dose response of ammonium and alanine to rosiglitazone. A–C: results are means ± SE from 4 wells/dose. *Different from control (P < 0.05) by ANOVA and Dunnett’s corrected t-test.
neous pH\(_i\) (−0.08 ± 0.01; \(P < 0.0001\) vs. KHHEPES alone over first 4 min). The resting pH\(_i\) after 8 min of exposure to troglitazone drops to 6.51 ± 0.05 (\(P < 0.0001\) vs. control). These results show that troglitazone induces a spontaneous cellular acidosis. Note that lactic acid production did not acutely increase (21 ± 3 and 23 ± 3 nmol/min; Table 4), indicating that this large rise in intracellular hydrogen ion concentration was not the result of an increased endogenous acid production. To determine whether ciglitazone also induces a spontaneous cellular acidosis, MDCK cells were exposed to KHHEPES and to KHHEPES plus 25 μM ciglitazone, and the spontaneous pH\(_i\) was continuously monitored over the following 8 min. As shown in the representative experiment in Fig. 4B, pH\(_i\) decreased with 25 μM ciglitazone in contrast to a steady or slightly rising pH\(_i\) for the KHHEPES alone; results from four additional experiments show that ciglitazone acidifies the cell compared with control (6.85 ± 0.05 vs. 7.13 ± 0.10; \(P < 0.05\)) at a rate of pH\(_i\) decline that is less than that exerted by troglitazone (−0.04 ± 0.02 vs. −0.08 ± 0.01 ΔpH/min). Figure 4C shows that rosiglitazone at 25 μM also produces an acute cellular acidosis, with the steady-state pH\(_i\) falling from 7.29 ± 0.07 to 6.76 ± 0.06 (\(P < 0.01\), \(n = 4\)). Therefore, all three glitazones induce a spontaneous cellular acidosis in the MDCK cell line, but with the drop in pH\(_i\) being about twice as great for troglitazone (0.62 ± 0.06) and rosiglitazone (0.53 ± 0.02) as for ciglitazone (0.28 ± 0.04).

To assess the duration of the intracellular acidosis elicited by troglitazone, monolayers were incubated in either KHHEPES (Fig. 5A) or DMEM (Fig. 5B) and aerated with 5% CO\(_2\) in the presence of vehicle or 25 μM troglitazone. As shown for the KHHEPES medium (Fig. 5A), the acidosis induced by troglitazone was maintained for at least 45 min. Because the metabolic studies were performed in HCO\(_3\)-CO\(_2\)-buffered DMEM, we wished to determine whether an acidosis would occur in this medium as well. As shown in Fig. 5B, troglitazone elicits an intracellular acidosis that is maintained for at least 20 min. Taken together, these findings demonstrate that troglitazone elicits a spontaneous cellular acidosis that is maintained for at least 20–45 min and is not dependent on increased acid production.

To assess whether troglitazone impairs cellular acid extrusion, cells were exposed to a 20 mM NH\(_4\)\(^+\) acid load for 4 min and then allowed to recover in KHHEPES or KHHEPES plus 25 μM troglitazone (Fig. 6). Exposure to KHHEPES containing 20 mM NH\(_4\)\(^+\) results in an initial alkalinization (NH\(_4\)\(^+\) in Fig. 6 re-
corded discontinuously) followed by a sharp fall in the pHi on removal of the NH₄⁺-containing KHHEPES and replacement with standard KHHEPES; this sharp fall off in pHi reflects the dissociation of intracellular NH₄⁺ and diffusion of NH₃ leaving H⁺ to acidify the cell interior. Note that because of the strict dependency of the major acid extruder, the NHE, on the prevailing pHi, it is important that the recovery response begin with the same degree of acidosis. In the representative example shown in Fig. 6, cell pHi indeed shows a similar drop in both the control (pHi = 6.55) and the troglitazone (pHi = 6.50) trials. Despite the same degree of initial acidosis, the recovery response is slowed in the presence of 25 μM troglitazone (0.12 ± 0.03 vs. 0.04 ± 0.02 ΔpH/min; P < 0.01) and is incomplete (pHi = 6.66 ± 0.08 vs. 6.98 ± 0.08; P < 0.05). Figure 7 shows that 25 μM rosiglitazone also inhibits the acid extrusion after the standard acid load (0.18 ± 0.07 vs. 0.08 ± 0.04 ΔpH/min; P < 0.01). These findings are therefore consistent with glitazone inhibition of acid extrusion as the mechanism for the spontaneous drop in cell pHi.

Table 4. Troglitazone induces a spontaneous acidosis without increasing lactic acid production

<table>
<thead>
<tr>
<th>Condition</th>
<th>pHᵢ</th>
<th>ΔpH/min</th>
<th>Lactate, nmol/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.13 ± 0.10</td>
<td>0.0006 ± 0.001</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Troglitazone (25 μM)</td>
<td>6.51 ± 0.05</td>
<td>-0.077 ± 0.014</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>NS</td>
</tr>
</tbody>
</table>

Results are means ± SE from 20 chambers. pHᵢ, intracellular pH; ΔpH/min, change in pHᵢ per time interval.
DISCUSSION

The expressed purpose of these studies was to determine 1) whether glitazones would alter glutamine metabolism, and 2) if so, whether this effect would be associated with a cellular acidosis. We chose to perform the study on a well-established renal tubule epithelial cell line, MDCK, because these cells readily elaborate extracellular matrix proteins (16) and therefore offer an available cellular model for the thickening of the tubular basement membrane that occurs in diabetes mellitus (16, 20, 26). It is noteworthy that in an animal model of type 2 diabetes mellitus, troglitazone inhibits this thickening (26). We know that production of extracellular matrix proteins is glutamine dependent (2, 10, 24, 34, 37), and, therefore, troglitazone might act to halt matrix expansion at least in part through glutamine metabolism.

The three glitazones studied clearly exert a major and previously unrecognized effect on glutamine metabolism in this well-established renal epithelial cell line. Specifically, troglitazone shifts glutamate from the transamination pathway(s) and into the deamination pathway (Fig. 1, from R2 into R3). The evidence for this is an increased ammonium production and decreased alanine formation (Table 1) without increased glutamine utilization. The proof for this effect on the fate of the amino nitrogen of glutamine is the rise in $[^{15}\text{N}]$ ammonium from [2,15$\text{N}$]glutamine and the decline in $[^{15}\text{N}]$ alanine formation (Fig. 2). The observation that the rise in ammonium and fall in alanine are related (Table 1 and Fig. 3, A and B, at 25 $\mu$M for troglitazone and at 25 $\mu$M and above for ciglitazone) is consistent with a coupled action on both the transamination and deamination pathways. The observation that the former is predominantly, but not exclusively, a cytoplasmic pathway (Table 3) may explain the apparent lower sensitivity for inhibition of alanine formation if the mitochondrial ALT reaction compensates for a falling contribution from the cytosolic ALT. The increased ammonium formation from [2,15$\text{N}$]glutamine is consistent with a shift of glutamate from the predominant cytosolic transamination pathway into the mitochondria, since GDH is exclusively an intramitochondrial activity (35). This shift in pathways could reflect inhibition of the dominant cytosolic ALT reaction, activation of the mitochondrial glutamate transporter, or both. The observed apparent near-equilibrium state for the ALT activity in control cells and the shift away from this near equilibrium (Table 2) are consistent with inhibition of the ALT activity; the finding of unchanged assayable ALT activity indicates that some factor external to the ALT enzyme is inhibitory. If both the cytosolic ALT activity and the putative mitochondrial glutamate uptake (35) have similar pH sensitivity, then much of what is observed can be explained by a developing cytosolic acidosis.

The pH-sensitive fluorescent probe BCECF is distributed predominantly within the cytosol (28), providing a measure of the acid-base balance in this compartment. Using this approach, we observed a pHi (7.13 ± 0.10) for MDCK cells in KHHEPES buffer that approximated the values found by others in MDCK cells (pHi = 7.20 ± 0.01; Ref. 13). The addition of each glitazone induced a prompt decrease in the pHi (Fig. 4, A–C), occurring within 1 min and then declining at least 0.28 units within 4 min (representing almost a doubling of the cytosolic hydrogen ion concentration). The potency of these three glitazones in inducing the cellular acidosis (see Fig. 4, A–C) roughly parallels their effect on glutamine metabolism (see Fig. 3, A–C). Whether the spontaneous acidosis is maintained or in turn involves activation of a pH-responsive signaling system (40), or both, remains to be determined.

The mechanism responsible for the spontaneous decline in the pHi was shown to be a reduction in the acid extrusion rather than an increase in acid production. Of course, acid produced from lactate formation did increase (Table 1), but this was not observed in the acute study (Table 4) nor would an increase in acid production alone be sufficient to produce the degree of acidosis observed in the present study; this is because the NHEs are activated at their internal pH-sensitive site to accelerate the rate of acid extrusion as pHi falls (1). Therefore, a spontaneous cellular acidosis of the severity observed should not develop as the result of increased acid production alone.

Troglitazone clearly impaired the ability of these cells to respond to an exogenous NH$_4^+$ acid load, a response that is attributed to direct inactivation of the NHE activity by troglitazone in an endothelial cell line (15); it is noteworthy that these investigators did not find an inhibition of the NHE activity with rosiglitazone, suggesting that either different cell lines or unique NHE isoforms may account for this apparent difference. Our findings are more consistent with an indirect action of glitazones on potential membrane signaling systems (20) capable of modulating NHE activity (22). There is another potential acid extruder in these cells, the sodium-independent proton secretion (17), but this extruder is far less active than the NHEs under NH$_4^+$ loading conditions (17). Further studies are required to better characterize the responses of other cell lines, particularly cell lines that have a single, well-characterized NHE; the intracellular acid-base balance and cell signaling events with long-term exposure to glitazones; and the resulting consequences for matrix protein turnover.

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REFERENCES

GLITAZONES AND CELLULAR ACID-BASE METABOLISM