Contribution of quinolinic acid in the development of anemia in renal insufficiency

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Quinolinic acid (QA) is a potential endogenous excitotoxin; elevation of its concentration in an organism has been implicated in the pathogenesis of various disorders. The purpose of this study was the assessment of QA impact on the process of erythropoiesis. Marked increase of QA concentration was observed in plasma and peripheral tissues of uremic rats. These changes were proportional to the amount of the removed renal tissue and positively correlated with the concentration of creatinine but negatively correlated with hematological parameters, i.e., hematocrit and Hb red blood cells count. The changes were accompanied by a slight decrease in the concentration of endogenic erythropoietin (EPO) in the plasma of animals with uremia. Chronic treatment with QA diminished the increase in EPO concentration after introduction of cobalt in rats. These changes were associated with the decrease in all hematological parameters after QA administration. The in vitro study in the conditions of hypoxia showed that QA inhibited the EPO release from HepG2 cells to the culture base. Additionally, in HepG2 cells QA had a dose-dependent inhibitory effect on hypoxia- and cobalt-induced EPO gene expression without any cell toxicity. In conclusion, the erythrinhibitory effect on hypoxia- and cobalt-induced EPO gene expression has been shown to occur at the EPO mRNA level. Thus we propose that QA can be a uremic toxin responsible for anemia in animals or patients with renal failure.

ERYPHTROPOIESIS DEPENDS ON the proliferative capacity of erythroid progenitor cells in the bone marrow and their stimulation, mainly by erythropoietin (EPO) (2). In chronic renal failure (CRF), the most important trigger of anemia is disturbances in erythropoiesis caused by reduced renal production of EPO as well as resistance of bone marrow cells to this hormone (3).

The literature data and our observations have indicated that in CRF patients, an increased degradation of tryptophan occurs, accompanied by a significant increase in the concentration of its plasma metabolites (18, 19). Quinolinic acid (QA) is the product of tryptophan oxidation that increases after enzymatic changes in the kynurenine pathway (Fig. 1) (21). QA is an endogenous, specific N-methyl-d-aspartate (NMDA) receptor agonist, which on activation may direct disturbances in cellular metabolic processes promoting apoptosis (22). In in vitro experiments, it has been demonstrated that QA possesses a suppressive effect on erythroid colony and lymphocyte blast formation (12). QA is excreted in the urine of healthy subjects, and it could be accumulated in the blood of uremic patients (17). Therefore, the increased blood concentration in CRF may account for uremic symptoms, such as anemia.

The present study was undertaken to investigate plasma QA concentrations in rats with chronic renal insufficiency and its influence on EPO production. We provide experimental evidence supporting the hypothesis that the inadequate EPO production in uremic patients might be at least partially attributed to the inhibitory effect of QA on EPO production. We also used the human hepatoma HepG2 cell line, which is a well-characterized in vitro system, to study the mechanisms of EPO production (8). We have demonstrated that these cells release EPO in the culture medium in response to hypoxia or transition metals (cobalt) and that this regulation has been shown to occur at the EPO mRNA level. In addition, we examined the relationship between the QA plasma concentration and the essential hematological and biochemical parameters of healthy rats and animals with experimental renal failure.

MATERIALS AND METHODS

Chemicals. The chemicals, which were of analytic reagent grade, were potassium dihydrogen phosphate, phosphoric acid, methanol, hydrochloric acid, and potassium chloride (Merck, Darmstadt, Germany); cobalt chloride hexahydrate, sodium citrate, potassium phosphate, Dulbecco’s modified Eagle’s medium, penicillin, streptomycin, heat-inactivated fetal bovine serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), isopropanol, Tris(hydroxymethyl)- aminomethane hydrochloride, and magnesium chloride.

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Fig. 1. Scheme of kynurenine pathway.

(Sigma, St. Louis, MO); QA (ICN); and pentobarbital sodium (Biomed, Pulawy, Poland).

Animals. Inbred adult (2 mo old) male albino rats (Wistar strain) of initial ~180–200 g body wt were used in the experiment. The animals were housed in conventional conditions, at 22 ± 1°C, with a relative humidity of 50 ± 10% and a 12:12-h light-dark cycle. They were allowed free access to drinking water (redistilled water) and rat chow (LSM, total protein 15.9%, dry diet, Fodder Manufactures, Motycz, Poland).

Ethics. Procedures involving the animals and their care conformed to the institutional guidelines, in compliance with national and international laws and Guidelines for the Use of Animals in Biomedical Research (7).

Surgical induction of experimental chronic renal insufficiency in rats. Rats were anesthetized with pentobarbital sodium (40 mg/kg ip). The resection of renal tissue was carried out by using the method described by Ormrod and Miller (16). In sham-operated rats, surgical extraction of the renal capsule was performed. The other experimental groups were as follows. “Moderate” renal insufficiency (CRF1) was induced by the removal of the left kidney, while the right kidney was decorticated in 60%. The rats with “severe” renal failure (CRF2) were subjected to the same surgical procedure as was CRF1, and after 1 wk the additional 20% of the right kidney cortex was removed. The “severe” group of animals was divided into two subgroups, CRF2 and CRF3. The blood and tissues for the biochemical analyses were taken a month after the surgical procedure on CRF2, with the exception of CRF3, in which the biological samples were collected 2 mo after the last surgical intervention.

Effects of QA on erythropoiesis in rats. The erythropoietic effects of sustained intraperitoneal administration of cobalt chloride in a dose of 10 mg/kg daily for 20 days in rats were compared with that in the animals that received the QA in doses of 10 or 100 mg/kg ip for 20 days 6 h before each cobalt injection. Blood was removed for determination of hematological and biochemical parameters at days 0–5, 10, 15, and 20 after treatment.

Blood and tissues sampling. The animals were anesthetized with pentobarbital sodium (40 mg/kg ip), and the blood was drawn by heart puncture and put into a tube containing 3.13% sodium citrate (citrate/blood = 1:9). The plasma was obtained by blood centrifugation at 3,000 rpm for 15 min (4°C). After bleeding, rat tissues (kidney, liver, lung, intestine, heart, spleen, and muscle) were prepared and slices (500 mg) were homogenized in ice-cold water. Homogenates were additionally sonificated and centrifuged at 14,000 g for 30 min at 4°C. Samples were stored at −80°C until assayed.

Determination of QA. QA was measured by using the HPLC technique as described by Werner-Felmayer et al. (26). The chromatographic system (Hewlett-Packard) was composed of an HP 1050 series pump with a Rheodyne injection valve fitted with a sample loop (20 µl). Partisil 10 SAX 250 × 4.6 mm (Phase Separations) column was eluted with 50 mM potassium phosphate (pH 2.0) containing 12% methanol at a flow rate of 2 ml/min. The amount of 2 ml plasma or supernatant of tissue homogenates was concentrated on Sep-Pack cartridges (Waters Accell Plus QMA), washed in 2 ml of water, and eluted with 0.2 ml 4 M H3PO4 (92% recovery of spiked QA). Using an HP 1050 series UV detector, the column effluent was monitored (272 nm). The output of the detector was connected to a single instrument LC-2D ChemStation. Chromatography was carried out at 24°C.

Determination of other biochemical parameters. The following parameters were measured with commercially available kits: creatinine (CRT; Creatinine 30, Cormay), urea (UR; Urea 30, Cormay), and EPO (EPO-Trac 1T 25β RIA Kit, Diasorin). The biochemical and hematological parameters were measured by the standard methods using an automatic Konelab 4.0.5 and Technikon H1 analyzers. Reticulocytes were stained with methylene blue, and their values [corrected reticulocyte count (CRC)] were adjusted to the degree of anemia (13).

Cell cultures. The HepG2 cell line was obtained from the American Type Culture Collection (HB 8065; tissue: hepatoblastoma, liver; sex: male; age stage: 15 yr; and ethnicity: caucasian). These cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% heat-inactivated fetal bovine serum in a humidified atmosphere (5% CO2-95% air) at 37°C (Heraeus incubator). Starting from the day before the experiment, 5 × 10⁵ cells/cm² of confluent cultures were fed with a serum-free medium (24-well polystyrene dishes). At the beginning of the 24-h experimental period, HepG2 cells received fresh medium containing QA (1, 10, 100, and 1,000 µM). In preliminary experiments, we observed that the addition of QA to cultures had no effect on the EPO levels (normoxic conditions) compared with a control group. EPO production by HepG2 was induced by incubation of the cultures with a low (1%) oxygen tension atmosphere or 100 µM of cobalt chloride for 24 h. At the end of the incubation period, supernatants were harvested, clarified by centrifugation, and stored frozen at 80°C until assayed.

Cytotoxicity of QA toward HepG2 cells. QA cytotoxicity (1, 10, 100, and 1,000 µM) was carried out according to Moosmann (15). Exposition time to QA was 24 h. MTT was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. At the times indicated below, stock MTT solution (100 µM/ml medium) was added to all wells of the assay and plates were incubated at 37°C for 4 h. Acid-isopropanol (1 ml of 0.04 M HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, the plates were read on a Multiskan EX Labsystems micro-ELISA reader to ensure that all crystals were dissolved, using a test wavelength of 570 nm.
570 nm and a reference wavelength of 630 nm. Plates were normally read within 1 h of adding the isopropanol.

Quantitation of EPO mRNA. We have examined the relative levels of EPO mRNA by using a semiquantitative RT-PCR procedure. The total RNA was extracted from HepG2 cells by using TRZol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. RNA was quantified spectrophotometrically at 260 nm. RNA was then stored in RNase-free water at −80°C. cDNA synthesis was performed in 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 1 mM 2-deoxynucleotide 5′-triphosphate mix, 2.5 μM oligo(dT)₁₅, 20 U RNasin ribonuclease inhibitor, and 200 U M-LV RT (Promega, Madison, WI) with 1 μg of total RNA in a final volume of 20 μl. The mixture was incubated at 42°C for 15 min and then heated to 95°C for 5 min. PCR was performed in 10 mM Tris·HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 50 μM 2-deoxynucleotide 5′-triphosphate mix, 200 nM of each primer, 1 unit of DyNAzyme II DNA polymerase (Finnzymes), and 5 μl of cDNA mixture in a final volume of 25 μl. The expression of the housekeeping gene, β-actin, was considered as a semiquantitative control. Sequences of specific primers for PCR were used: sense 5′-ATCACGAGGGGTGCTGCT-GAACAC-3′, positions 335–358, GenBank accession no. X02157, and antisense 5′-GGGAGATGGCTTCCTTCTGG-GGTC-3′, positions 623–630, GenBank accession no. X02157 for EPO (25); and sense 5′-CCAGAATCATTTATGAGACCT-3′, positions 913–932, GenBank accession no. BC009275, and antisense 5′-GCACAGCTTCCCTTAATGT-3′, positions 1204–1185, GenBank accession no. BC009275 for β-actin. PCR was carried out under the following conditions: 30 s of denaturation at 94°C, 30 s of annealing at 58°C, and 30 s of extension at 72°C for 30 cycles, with an additional 5 min of extension for the last cycle on an MJ Research Thermocycler (model PTC-200, Watertown, MA). Amplification products were run on a 2% agarose gel. Ethidium bromide-stained gels were visualized under UV illumination and photographed, and for each sample the intensity of the signal was measured with One Dscan/Zero Dscan v2.02 and v1.0 software (Scana-lytics). Ratios of the corresponding peak areas, EPO/β-actin, were calculated for each sample and used for quantitative calculations and comparisons.

Table 1. Effect of varying degrees of experimental renal insufficiency on biochemical and hematological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control-Sham (n = 6)</th>
<th>Moderate (n = 8)</th>
<th>Severe 1 (n = 8)</th>
<th>Severe 2 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine, μM</td>
<td>23.8 ± 3.1</td>
<td>63.8 ± 4.9†</td>
<td>91.3 ± 6.1‡</td>
<td>288.5 ± 16.3‡</td>
</tr>
<tr>
<td>Urea, mM</td>
<td>8.3 ± 0.1</td>
<td>12.2 ± 1.1*</td>
<td>21.4 ± 3.0†</td>
<td>85.6 ± 9.3†</td>
</tr>
<tr>
<td>RBC, 10⁶/l</td>
<td>6.64 ± 0.22</td>
<td>6.01 ± 0.53</td>
<td>5.25 ± 0.21*</td>
<td>3.02 ± 0.10‡</td>
</tr>
<tr>
<td>Hb, g/l</td>
<td>108.3 ± 3.5</td>
<td>93.4 ± 6.8</td>
<td>70.1 ± 5.8‡</td>
<td>45.2 ± 3.3‡</td>
</tr>
<tr>
<td>Hct, %</td>
<td>36.7 ± 0.5</td>
<td>30.6 ± 1.2*</td>
<td>25.9 ± 1.4‡</td>
<td>17.3 ± 1.8‡</td>
</tr>
<tr>
<td>MCV, 10⁻¹⁵/l</td>
<td>52.1 ± 1.9</td>
<td>49.3 ± 2.2</td>
<td>48.7 ± 1.7</td>
<td>50.6 ± 2.4</td>
</tr>
<tr>
<td>MCH, pg</td>
<td>18.4 ± 0.8</td>
<td>16.9 ± 1.0</td>
<td>19.2 ± 0.4</td>
<td>17.7 ± 1.1</td>
</tr>
<tr>
<td>MCHC, g/l</td>
<td>34.9 ± 1.4</td>
<td>30.3 ± 2.7</td>
<td>36.5 ± 3.1</td>
<td>319 ± 1.8</td>
</tr>
<tr>
<td>CRC, 10⁵/l</td>
<td>35.2 ± 4.0</td>
<td>28.6 ± 3.5</td>
<td>22.4 ± 2.2*</td>
<td>20.5 ± 2.8†</td>
</tr>
<tr>
<td>Iron, μg/l</td>
<td>681.3 ± 19.8</td>
<td>729.6 ± 24.3</td>
<td>701.8 ± 20.8</td>
<td>697.1 ± 26.1</td>
</tr>
<tr>
<td>Transferrin, g/l</td>
<td>0.53 ± 0.11</td>
<td>0.45 ± 0.07</td>
<td>0.58 ± 0.05</td>
<td>0.50 ± 0.12</td>
</tr>
<tr>
<td>TIBC, μg/l</td>
<td>69.7 ± 5.8</td>
<td>68.2 ± 2.1</td>
<td>73.1 ± 6.3</td>
<td>76.5 ± 4.2</td>
</tr>
<tr>
<td>EPO, U/l</td>
<td>28.1 ± 4.1</td>
<td>36.7 ± 3.7</td>
<td>34.2 ± 5.2</td>
<td>24.1 ± 6.1</td>
</tr>
<tr>
<td>‡EPO, U/l</td>
<td>76.3 ± 8.2</td>
<td>63.1 ± 6.3</td>
<td>49.2 ± 5.4‡</td>
<td>37.7 ± 7.6†</td>
</tr>
</tbody>
</table>

Values are means ± SE. RBC, red blood cells count; Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, MCH concentration; CRC, corrected reticulocyte count; TIBC, total iron-binding capacity; EPO, erythropoietin; ‡EPO, plasma EPO concentration in rats 24 h after administration of cobalt chloride (10 mg/kg ip). Multiple group comparisons were performed by one-way ANOVA; for homogeneity of variances, a Bartlett’s test was used. Significant differences in comparison with the control group (†P < 0.05, ‡P < 0.01, §§P < 0.001) were assessed by a Tukey-Kramer test.

Statistical analysis. The values are expressed as means ± SE; n represents the number of experiments. Multiple group comparisons were performed by one-way analysis of variance, and significant intergroup differences were assessed by a Tukey-Kramer test. Values of P < 0.05 were regarded as significant. Correlations between plasma concentrations of studied QA and other biochemical or hematological parameters were analyzed by using a Spearman test.

RESULTS

Effect of experimental renal insufficiency in rats on biochemical and hematological parameters. The parameters of renal insufficiency are summarized in Table 1. Rats with moderate, severe 1, and severe 2 renal failure had significantly increased blood CRT and UR compared with the control group. At the same time, these changes were associated with the decrease in hematological parameters, i.e., red blood cells count (RBC), Hb, Hct, and CRC. However, mean corpuscular volume, mean corpuscular Hb, mean corpuscular Hb concentration, and parameters of iron metabolism, such as serum iron concentration, transferrin, and total iron-binding capacity, were not changed.

In rats with CRF compared with control animals, the more extensive range of EPO plasma concentrations was demonstrated. However, the mean values of CRF of studied groups and the control group were not significantly different (P > 0.05). The administration of cobalt resulted in a significant increase in EPO plasma concentration, this effect was noticeably weaker in animals with partial ablation of the kidney (severe 1 and 2).

Effect of renal insufficiency on QA plasma and tissue concentration in rats. The essential increase in plasma QA concentrations in moderate (CRF1) and severe 1 and 2 groups (CRF1 and CRF2, respectively) of renal insufficiency was observed (Fig. 2). At the same time, increases in QA concentrations in the tissues (kidney,
liver, lung, intestine, and spleen) were observed mainly in CRF2 rats. Additionally, the relationship between the increase in the plasma QA concentration and the stage of renal insufficiency was demonstrated. The multiple regression analysis (Fig. 3) showed that there was a linear correlation between plasma concentration of CRT and QA (r = 0.848, P < 0.05) or UR and QA (r = 0.808, P < 0.05). There was a significant negative correlation between QA plasma concentrations and hematological parameters such as RBC (r = -0.841, P < 0.001), Hb (r = -0.704, P < 0.001), Hct (r = -0.843, P < 0.001), and CRC (r = -0.427, P < 0.023) (Fig. 4).

Effect of chronic QA administration in cobalt chloride-stimulated erythropoiesis in rats. After 1 h, administration of QA in 10 and 100 mg/kg ip doses produced plasma concentrations of 11.2 ± 5.3 and 67.9 ± 16.4 

μM, respectively (Fig. 5). The level of QA in rats then decreased and at 4 h was 0.5 ± 0.1 and 6.3 ± 4.5 μM, respectively. During the next 2 h, the plasma concentration of QA raised to 0.9 ± 0.1 and 2.2 ± 0.8 μM, respectively.

The exposure of rats to cobalt chloride on day 1 resulted in a significant increase in plasma EPO titers, reached the highest level after day 2, and then gradually declined (Fig. 6A). However, significant increases in RBC (Fig. 6B), Hb (Fig. 6C), and Hct (Fig. 6D) were observed after 15 days of intraperitoneal administration of cobalt chloride. Chronic treatment with QA in a dose of 100 mg/kg ip inhibited the increase in EPO concentration after cobalt chloride administration. Additionally, these changes were associated with the decrease in all hematological parameters, i.e., RBC, Hb, and Hct (P < 0.001).

Effect of QA on cell viability. As shown in Fig. 7A, the viability of HepG2 cells was decreased only at a higher concentration of QA (1,000 μM). QA in concentrations of 1, 10, and 100 μM had no effect on this parameter.

Effect of QA on medium concentration of EPO. HepG2 cells, when grown in 1% O2 for 24 h, produced approximately five- to sixfold more EPO compared with the cells grown in 21% O2 (Fig. 7B). QA (100 and 1,000 μM) inhibited hypoxia-induced EPO production by 32.1 ± 2.9 and 49.6 ± 2.2% in a dose-dependent man-

ner. However, 24-h exposure to 10 μM of QA had no effect on the EPO production, but 48-h HepG2 exposition significantly decreased the EPO medium concentration by 30.6 ± 4.1%. In normoxic HepG2 cultures, cobalt chloride significantly stimulated EPO production during a 24-h incubation period, but this effect was weaker than under hypoxic conditions. The addition of QA in concentrations of 100 or 1,000 μM inhibited EPO production by 24.9 ± 1.6 and 37.7 ± 2.4%, respectively.

Effect of QA on EPO mRNA induction. In HepG2 cells, hypoxia or cobalt chloride induced EPO gene expression (Fig. 7C). We observed a strong, dose-dependent reduction in EPO gene expression by QA in hypoxic conditions at concentrations ranging from 1 to 100 μM, without any cell toxicity. We observed 35.2 ± 5.4% inhibition of mRNA synthesis with 1 μM of QA, and this concentration is achievable in the plasma of uremic rats. However, this dose of QA had no effect on the induction of EPO mRNA by cobalt chloride. The inhibition of EPO gene expression in these conditions was observed in higher concentrations of QA (100 and 1,000 μM).

DISCUSSION

The present study was undertaken to investigate QA concentrations in rats with chronic renal insufficiency
and their influence on the EPO production in both in vivo and in vitro experimental conditions. We provide experimental evidence supporting the hypothesis that the inadequate EPO production in uremic patients might be partially attributable to the inhibitory effect of QA on the EPO production.

We used a well-established model of CRF in rats to define mechanisms that may be involved in anemia development, which is observed in kidney failure (16). The course of experimental renal failure was monitored by means of the plasma concentration of CRT and UR, and both of them increased proportionally to the extent of renal tissue resection. In addition, we evaluated basal hematological parameters (Hb, Hct, RBC, and CRC) as the markers of the renal insufficiency progression. We found that they were significantly decreased depending on the stage of the experimental renal insufficiency.

In CRF, a great number of the endogenous metabolites that are normally excreted in urine accumulate in the blood (24). Among these are the products of kynurenate sodium (KYN) degradation (18, 19). We observed a significant increase in the QA concentrations in plasma and peripheral tissues of uremic animals. These changes were proportional to the amount of the removed renal tissue and correlated with the concentration of CRT, a marker of decreased glomerular filtration rate and impaired kidney excretory function. In the CRF2 group, QA plasma concentration was 10-fold higher than in the control animals. The changes in QA concentration are in accordance with other reports that demonstrated similar patterns in the plasma QA of human patients with CRF. As reported by Saito et al. (20), the increase in serum QA concentrations during uremia is due to the decline in the activity of aminocarboxymuconate-semialdehyde decarboxylase (the enzyme responsible for the degradation of QA to the glutarate pathway). Although serum concentration of QA in CRF was elevated, its renal clearance values were slightly decreased by 20% compared with control subjects. Because urinary excretion of QA also increased, the authors presume that increased solute concentration is not related to a decrease in renal excretion but to an increase in production and decrease in degradation (20).

Fig. 4. Statistical correlations between the plasma concentration of QA and hematological parameters of rats with chronic renal failure. Depicted are Hb, Hct (B); RBC, red blood cells count (C); and CRC, corrected reticulocytes count (D).

Fig. 5. Kinetics of QA in plasma after intraperitoneal administration in doses of 10 and 100 mg/kg.
Our laboratory's earlier study demonstrated that hemodialysis is one of the therapeutic approaches that significantly reduces QA plasma concentration; despite this, QA was still elevated in uremic patients compared with the healthy volunteers (17). Saito et al. (20) found that serum KYN and QA concentrations after hemodialysis were significantly decreased by 30 and 75%, respectively, compared with prehemodialysis values. The rise in dialysis frequency decreases QA concentration (17) and simultaneously increases erythropoiesis (11).

In our study, the plasma level of QA negatively correlated with hematological parameters. The predominant reason for insufficient erythropoiesis in renal diseases is the impossibility of increasing EPO production in response to the initial anemia (3). The mean values of EPO in CRF and control rats were not significantly different. The cobalt administration in rats resulted in a significant increase in EPO plasma concentration in the control group, and this effect was significantly weaker in animals with partial ablation of the kidney. Although the rate of EPO production is clearly related to the degree of anemia and in turn to the supply of oxygen to the tissues, this relationship is quite broad, suggesting that a number of other factors play a role. Among potential agonists are cobalt (4), androgens, and insulin-derived growth factors (1). It is known that cobalt may lead to the depression of respiration, oxidative phosphorylation, and reduced oxygen uptake in kidneys. This metal has been found to mimic the hypoxia-induced expression of the EPO gene (4). Antagonists include inflammatory cytokines, such as tumor necrosis factor, IL-1, and transforming growth factor-β (6). It would seem likely that toxic metabolites retained in CRF may also impair activation of the EPO gene expression, but relevant observations are not available.

In the next step of our study, we used an animal model to define mechanisms that may be involved in the observed changes (16). We investigated several parameters of erythropoiesis in healthy rats in response to 20 days of daily exposure to cobalt chloride. The plasma concentration of EPO was significantly increased, and peak concentration was seen after 1–4 days from the start of the experiment. The increase in EPO production results in enhanced red cells formation in bone marrow, which causes the elevation in RBC, Hct, and Hb. This effect was observed after 15 days of cobalt chloride exposure. The chronic administration of QA clearly inhibited the EPO level after cobalt chloride treatment. Additionally, these changes were associated with the decrease in all hematological parameters, i.e., RBC, Hb, and Hct. The concentrations of QA obtained after the lower dose (10 mg/kg) reached the level observed in patients with chronic renal insufficiency (17). QA in a dose of 100 mg/kg caused an increase in the plasma concentration that persisted for 3 h and was much higher that those observed in uremic patients. QA in a dose of 10 mg/kg caused a decrease in the EPO plasma concentration and the inhibition of erythropoiesis, both induced by cobalt chloride. As mentioned above, the resulting concentration is typical...
for patients with chronic renal insufficiency. Thus we assume that also in vivo QA inhibits erythropoiesis.

We also used the human hepatoma HepG2 cell line, which is a well-characterized in vitro system, to study mechanisms regulating EPO production (8). In this experiment, HepG2 cells were grown in 1% O2 or in the presence of cobalt chloride. The increased EPO production during 24 h in cells grown in 1% O2 was much stronger compared with cells grown in 21% O2. These results demonstrated that QA dose dependently inhibited the production of EPO stimulated by hypoxia or transition metal (cobalt), without any cell toxicity. The lack of changes in β-actin mRNA and α-fetoprotein levels (data not shown) indicated that observed activity of QA on EPO production is specific. The specific effects of QA on EPO synthesis in HepG2 cells suggest that QA may also be an important regulator of EPO production in vivo. Kynurenic acid (NMDA receptor antagonist), which acts as an antagonist of QA (21), did not eliminate the QA effect on EPO production by HepG2 cells (data not shown), thus the inhibitory activity of QA on EPO synthesis seems to be not connected with the NMDA receptor. In addition, the changes observed in EPO synthesis resulted from the inhibition of the EPO mRNA level. The fact that in these studies the concentrations of QA used to inhibit hypoxia-induced expression of the EPO gene in HepG2 cells were within the range of QA in the plasma of humans (17) or rats (19) with chronic renal insufficiency suggests that QA could play a role in the anemia observed in uremia. In our in vitro HepG2 study, we observed that QA in the concentrations of 1 and 10 μM inhibits EPO concentration in the medium. The first cell reaction after stimulus (in our case QA) is the change in the EPO gene expression, which is followed by the activation of intracellular processes and modulation of EPO synthesis and release. The decrease in the EPO mRNA synthesis does not always reflect the EPO concentration (in the same interval time). Twenty-four-hour HepG2 exposition to 10 μM of QA (the QA concentrations observed in patients with chronic renal insufficiency) inhibits the EPO gene expression but without the decrease in EPO concentration in the medium. In contrast, 48-h HepG2 exposition to 10 μM of QA significantly decreased the EPO concentration in the medium.

Tissue hypoxia, whether due to altered O2 tension, O2-carrying capacity, or O2 affinity of the blood, is the primary stimulus for EPO production (14). The oxygen sensor is likely to be a heme protein, perhaps a cytochrome b-like flavo-heme NADPH oxidase that signals by activated oxygen compounds. H2O2 generated by NADPH oxidase in a O2-dependent manner is a possible candidate for an intracellular messenger (5). It is a freely diffusible signaling molecule between the sensor and the transcriptional activator-hypoxia inducible factor (HIF). Hypoxia- or cobalt-induced expression of the EPO gene depends on the activation of an enhancer element by HIF. HIF is a heterodimeric nonheme iron protein composed of α- and β-subunits (25). HIF1-α is continually synthesized but rapidly degraded in normoxia. H2O2 can react with iron in HIF1-α to generate OH radicals. Hypoxia induces stabilization of the HIF-1α subunit, allowing the formation of the complex HIF1-α-aryl hydrocarbon nuclear translocator protein. The dimerization induces a conformational change that allows it to bind DNA (9). Furthermore, there is evidence for a possible role of the nitric oxide-cGMP system in hypoxic regulation of EPO production (10). QA may lead to the formation of radical species (including nitric oxide) that can induce degradation of HIF1-α and, finally, negatively regulate EPO gene expression. Moreover, decarboxylation and conversion of QA to nicotinate mononucleotide by phosphoribosyltransferase is a step in biosynthesis of NAD7. It is also possible that the concentration of NAD can influence the redox situation.

In conclusion, this study provides the evidence for the accumulation of QA in the plasma and peripheral tissues in the course of CRF. The erythropoiesis in CRF could be attributed to the influence of QA on EPO

Fig. 7. Effect of QA on the HepG2 cells viability (A), EPO medium concentration (B), and EPO mRNA induction by hypoxia or cobalt chloride (C). *P < 0.05, **P < 0.01, ***P < 0.001, significant differences vs. control group.
synthesis. Thus we proposed that QA could be an uremic toxin responsible for anemia in CRF.

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REFERENCES


