Parenteral iron formulations differentially affect MCP-1, HO-1, and NGAL gene expression and renal responses to injury

Ali CM Johnson, Kirsten Becker, and Richard A. Zager

Department of Medicine, University of Washington, and Clinical Division, Fred Hutchinson Cancer Research Center, Seattle, Washington

Submitted 4 May 2010; accepted in final form 25 May 2010

Johnson AC, Becker K, Zager RA. Parenteral iron formulations differentially affect MCP-1, HO-1, and NGAL gene expression and renal responses to injury. Am J Physiol Renal Physiol 299: F426–F435, 2010. First published May 26, 2010; doi:10.1152/ajprenal.00248.2010—Despite their prooxidant effects, ferric iron compounds are routinely administered to patients with renal disease to correct Fe deficiency. This study assessed relative degrees to which three clinically employed Fe formulations [Fe sucrose (FeS); Fe gluconate (FeG); ferumoxytol (FMX)] impact renal redox-sensitive signaling, cytotoxicity, and responses to superimposed stress [endotoxin; glycerol-induced acute renal failure (ARF)]. Cultured human proximal tubule (HK-2) cells, isolated proximal tubule segments (PTS), or mice were exposed to variable, but equal, amounts of FeS, FeG, or FMX. Oxidant-stimulated signaling was assessed by heme oxygenase-1 (HO-1) or monocyte chemoattractant protein (MCP)-1 mRNA induction. Cell injury was gauged by MTT assay (HK-2 cells), %LDH release (PTS), or renal cortical neutrophil gelatinase-associated lipoprotein (NGAL) protein/mRNA levels. Endotoxin sensitivity and ARF severity were assessed by TNF-α and blood urea nitrogen concentrations, respectively. FeS and FeG induced lethal cell injury (in HK-2 cells, PTS), increased HO-1 and MCP-1 mRNAs (HK-2 cells; in vivo), and markedly raised plasma (~10 times), and renal cortical (~3 times) NGAL protein levels. Both renal and extrarenal (e.g., hepatic) NGAL production likely contributed to these results, based on assessments of tissue and HK-2 cell NGAL mRNA. FeS pretreatment exacerbated endotoxemia. However, it conferred marked protection against the glycerol model of ARF (halving azotemia). FMX appeared to be “bioneutral,” as it exerted none of the above noted FeS/FeG effects. We conclude that 1) parenteral iron formulations that stimulate redox signaling can evoke cyto/nephrotoxicity; 2) secondary adaptive responses to this injury (e.g., HO-1/NGAL induction) can initiate a renal tubular cytoresistant state; this suggests a potential new clinical application for intravenous Fe therapy; and 3) FMX is bioneutral regarding these responses. The clinical implication(s) of the latter, vis a vis the treatment of Fe deficiency in renal disease patients, remains to be defined.

ferumoxytol; Fe gluconate; Fe sucrose; heme oxygenase-1

PATIENTS WITH END-STAGE RENAL disease are typically Fe deficient because of poor gut Fe absorption and ongoing blood losses (e.g., due to dialysis, frequent diagnostic phlebotomies; Refs. 11 and 15). Because of this, intravenous (iv) Fe therapy, combined with erythropoietin-stimulating agents (ESAs), has become a mainstay in the management of dialysis patients (9). Currently employed iv Fe formulations consist of a ferric iron core surrounded by various carbohydrate “shields.” The latter are designed to mitigate free Fe$^{3+}$ exposure, and hence, Fe-mediated oxidative stress. The degree to which these various carbohydrate shields accomplish this goal, and thus, mitigate potential adverse clinical reactions, remains a subject of debate (1, 44, 45). So-called “first-generation” Fe compounds, the Fe dextrins (FeD), were associated with a risk of anaphylaxis (3, 14). Despite the subsequent release of improved, “low-molecular-weight” FeD formulations (e.g., “InFed”), ongoing concerns of potential life-threatening allergic reactions continue to limit FeD use. Currently, so-called “second-generation” agents, most notably Fe sucrose (FeS) and Fe gluconate (FeG), are most widely employed (2, 9, 28, 34). While reported to have a low risk of allergic reactions, both FeS and FeG still possess potent prooxidant effects (44, 45). Thus there is no completely “nontoxic” iv iron formulation (4).

Ferumoxytol (FMX) is an Fe oxide nanoparticle which is coated with a polyglucose sorbitol carboxymethyl ether (6, 21, 30, 35, 36). It was initially designed for use as a magnetic resonance imaging (MRI)-angiographic contrast agent, and as such, it was formulated to permit rapid iv administration of large Fe dosages (10). It was subsequently demonstrated that when so administered, FMX can effectively replete Fe stores in hemodialysis patients. One potential reason for FMX’s “relative safety,” despite high dose bolus injection, is a reportedly low level of free (“catalytic”) Fe, compared with other agents (FeS, FeG) (18, 20). However, this assumption is based solely on the results of in vitro testing with the bleomycin assay (18, 20). This measures the ability of bleomycin to complex free Fe, and then generate thiobarbituric acid-reactive substances (TBARs) from exogenously added DNA. Of note, the Fe-bleomycin assay is fraught with difficulties regarding specificity, reproducibility, and extreme sensitivity, potentially clouding data interpretation. Hence, it remains unclear as to whether FMX is, indeed, less prooxidant than current mainstay iv Fe formulations and whether in vivo correlates of potential in vitro toxicity exist.

The purpose of the present study was to gain insights into this issue and to better define potential iv Fe effects on the kidney. To this end, studies were undertaken in 1) human-derived cultured HK-2 proximal tubular cells; 2) isolated mouse proximal tubule segments (PTS); and 3) CD-1 mice. Direct Fe toxicity [blood urea nitrogen (BUN), LDH release, MTT assay, NGAL formation], redox-sensitive cytokine induction [TNF-α, monocyte chemoattractant protein (MCP)-1; Refs. 19 and 27], Fe-sensitive heme oxygenase-1 (HO-1) expression (26), and the ability of different Fe formulations to alter superimposed renal injury responses (induced by either endotoxin or glycerol-induced acute renal failure) were assessed.

Address for reprint requests and other correspondence: R. A. Zager, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N; Rm. D2-190, Seattle, WA 98109 (e-mail: dzager@fhcrc.org).
METHODOLOGY

Cell Culture Experiments

Cell viability following Fe exposure. All cell culture experiments were performed with HK-2 cells, an immortalized proximal tubule cell line derived from normal human kidneys (31). They were cultured with keratinocyte serum-free medium supplemented with pituitary extract, glutamine, and penicillin/streptomycin, as previously described in detail (31). For experimentation, the cells were trypsinized and seeded into 24-well Costar plates. Six hours later, wells in any given culture plate were exposed to either 500 or 1,000 µg/ml of elemental iron (Fe3+) in the form of either FeS (Venofer; American Regent Pharmaceuticals, Shirley, NY), FeG (Watson Pharmaceuticals; Morristown, NJ), or FMX (AMAG Pharmaceuticals, Lexington, MA). Coincubated cells maintained under control culture conditions served as controls. After 18-h incubations, viable cell numbers were assessed by MTT assay, as previously described (31). Viable cells transport MTT into their mitochondria, the compound is then reduced to formazin (purple color), and the latter is quantified colorimetrically. HK-2 cells oxidant responses. MCP-1 and HO-1 are highly redox-sensitive genes, and with an oxidant challenge, increases in their respective mRNAs result (9, 26, 27). Hence, we tested the relative abilities of FeG, FeS, and FMX to alter MCP-1 and HO-1 mRNA levels in HK-2 cells. To this end, HK-2 cells were incubated overnight under control conditions or with 100 µg/ml FeS or FMX. After 18-h incubations, the cells underwent total RNA extraction and HO-1 mRNA, MCP-1 mRNA, and GAPDH mRNAs were determined by competitive RT-PCR as previously described (40, 43–45). MCP-1 and HO-1 mRNA levels were expressed as a ratio to simultaneously obtained GAPDH levels (serving as a housekeeping gene; n = 4 determinations for each mRNA).

Dose titration of FeS concentrations and HK-2 cell HO-1 mRNA responses. The following experiment was undertaken to ascertain whether HK-2 cell responses to the Fe compounds can be observed at different post-Fe or -vehicle injections. Six mice were used for each Fe treatment, and the results were contrasted to those observed in six control mice, respectively.

Mouse Isolated PTS Experiments

The following experiment assessed whether the cytotoxicity results observed in HK-2 cells with the MTT assay could be recapitulated in a second in vitro system. To this end, isolated mouse PTS were used. Four sets of mouse PTS were isolated from male CD-1 mice (8–12 g; Charles River Laboratories, Wiltmin, MA) as previously described (40, 45). Mouse protocols were approved by the Fred Hutchinson Cancer Research Center’s Institutional Animal Care and Use Committee. In brief, the mice were anesthetized with pentobarbital sodium (~2 mg ip), the kidneys were immediately removed through a midline abdominal incision, and the cortices were recovered by dissection with a razor blade on an iced plate. The cortical tissues were minced with a razor blade, digested with collagenase, passed through a stainless steel sieve, and then pelleted by centrifugation (4°C). Viable PTS were then recovered by centrifugation through 32% Percoll (Pharmacia, Piscataway, NJ). After multiple washings in iced buffer, the PTS were resuspended (~2–4 µg protein/ml) in

experimentation buffer (in mmol/l: 100 NaCl, 2.1 KCl; 25 NaHCO3; 2.4 KH2PO4; 1.2 MgSO4; 1.2 MgCl2; 1.2 CaCl2; 5 glucose; 1 alanine; 4 Na lactate; and 10 Na butyrate, as well as 36-kDa dextran, 0.6%) and gassed with 95% O2-5% CO2 (final pH 7.4). Finally, they were rewarmed to 37°C in a heated shaking water bath over 15 min and then used for experimentation. Four PTS preparations were each divided into four equal aliquots (1.25 ml of PTS suspension placed into 10-ml Erlenmeyer flasks) and incubated for 60 min at 37°C either under control conditions (buffer only) or with 2 mg/ml of Fe3+ in the form of FeS, FeG, or FMX. After completion of the 60-min incubations, cytotoxicity was assessed by determining the % tubular lactate dehydrogenase (LDH) release (44, 45).

In Vivo Assessments of MCP-1 and HO-1 Expression

3-h Post-iv Fe administration: MCP-1 and HO-1 assessments. CD-1 mice were placed into restraining tubes and subjected to a tail vein injection of either FeS, FeG, or FMX (2 mg elemental Fe). Control mice received 100-µl tail vein saline injections. Three hours later, the mice were anesthetized with pentobarbital sodium, the abdominal wall was opened, a plasma sample was obtained from the inferior vena cava, and the kidneys were resected. The plasma samples were used to measure MCP-1 by ELISA (BD Biosciences, San Jose, CA). The kidneys were cooled to 4°C, the cortices were dissected, and total RNA was extracted (RNeasy Kit; Qiagen). An index of renal cortical oxidative stress, the samples were analyzed for MCP-1 and HO-1 mRNAs by RT-PCR, as previously described (30, 39, 43–45). The results were expressed as ratios to simultaneously obtained GAPDH product (n = 5, 4, 6, and 10 for FeS, FeG, FMX, and control mice, respectively).

18-h Post-iv Fe administration: MCP-1 and HO-1 assessments. The above experiment was repeated, except that the plasma and renal cortical samples were obtained at a more delayed time point: 18 h post-Fe or -vehicle injections. Six mice were used for each Fe treatment, and the results were contrasted to those observed in six controls. Terminal BUN concentrations were also assessed.

NGAL Assessments

Renal cortex. NGAL is widely recognized as a marker of subclinical or overt renal tubular injury (8, 13, 23). To further assess whether the Fe preparations have an intrinsic cytotoxic potential, renal cortical NGAL levels and its mRNA were assessed at different time points post-Fe injections. Mice were injected with either FeS (n = 8), FeG (n = 8), FMX (n = 8), or saline (n = 14) via the tail vein as noted above. Either 3 or 18 h later (one-half of the mice in each group at each time point), they were anesthetized, the kidneys were removed, and the cortices were subjected to protein extraction. NGAL levels were assessed using a commercially available assay (R&D Systems). Values were expressed as micrograms per milligram tissue protein extract. In addition, the 18-h samples were also extracted for total RNA to assess whether observed NGAL protein changes (see RESULTS) were paralleled by changes in NGAL mRNA. The primers used for NGAL mRNA are presented in Table 1.

Plasma NGAL concentrations and extrarenal NGAL mRNA expression. The following experiment was undertaken to ascertain whether renal cortical NGAL concentrations may have been impacted by high circulating NGAL concentrations, and if so, whether extra-renal organs respond to iv Fe injection with an increase in NGAL mRNA. Mice were injected via the tail vein with 2 mg of Fe in the form of FeS, FeG, or FMX or vehicle (6 mice/group). After 18 h, they were anesthetized and plasma was obtained from the inferior vena cava. In addition, three mice per group had pieces of liver, spleen, and lung excised for assessment of NGAL mRNA.

Assessment of direct Fe effects on proximal tubule NGAL induction. HK-2 cells were exposed to 25 µg/ml of either FeS, FeG, or FMX for 3 days. Coincubated wells of cells served as controls (n =
Calculations and Statistics

Renal cortical samples were subjected to RT-PCR to quantify TNF-
plasma samples were assayed for TNF-
tized, and plasma and renal cortical samples were obtained. The
Louis, MO). Two hours post-LPS injection, the mice were anesthe-
coli LPS (stock solution, 4 mg/ml saline; 0111:B4; L-2630; Sigma, St.
were made, the Bonferroni correction was applied.

were performed by unpaired Student’s t-test. If multiple comparisons
were made, the Bonferroni correction was applied.

RESULTS

Cell Culture Experiments

Cell viability. As shown in Fig. 1, at a dose of 500 µg/ml, only FeS caused a significant decrease in MTT uptake (36% of
values observed in the control cells). At the 1,000 µg/ml
concentration, each of the Fe preparations decreased MTT
uptake, with the degree of suppression being FeS >> FeG >
FMX.

HO-1 and MCP-1 mRNA induction. As shown in Fig. 2, FeS
induced marked increases in HK-2 cell HO-1 and MCP-1
mRNAs. Conversely, FMX evoked no increase in either
mRNA, consistent with a lack of significant oxidative stress.

HO-1 mRNA dose-response relationship. As shown in
Fig. 3, a steep dose-response relationship between FeS dose
and HK-2 cell HO-1 mRNA induction was observed. Even at
a 1 µg/ml FeS dose × 3 days, a significant increase in HO-1
mRNA was observed. The 25 µg/ml “clinically relevant” FeG
dose also significantly increased HO-1 mRNA. Conversely, 25

Fe Effects on Superimposed In Vivo Renal Injury

Fe “preconditioning”: effect on glycerol-induced rhabdomyolysis
acute renal failure. It has previously been documented that Fe-
induced oxidative stress, e.g., as evoked by glycerol or cisplatin ARF models;
Refs. 26 and 33). Because FeS, but not FMX, evoked oxidant stress,
it was hypothesized that pretreatment with FeS, but not with FMX,
might protect against subsequent ARF. To test this hypothesis, mice
were injected with 2 mg of either FeS (n = 7) or FMX (n = 5) or
control tail vein injections (n = 11), as noted above. Approximately
18 h later, the mice were lightly anesthetized with isoflurane and
subjected to intramuscular hypertonic glycerol injection (8 ml/kg;
50% solution; administered in equally divided doses in upper hind-
limbs). At 18 h post-glycerol injection, the mice were deeply anes-
thetized with pentobarbital sodium, a plasma sample was drawn from
the inferior vena cava, and the kidneys were removed. The plasma
samples were assayed for BUN, and the renal cortices were assayed
for HO-1 and MCP-1 mRNAs.

Fe preconditioning: effect on Gram-negative bacterial endotoxin
(LPS)-mediated TNF-α generation. We previously demonstrated that
prior iv FeS or FeG treatment sets the stage for an exaggerated TNF-α
response to LPS injection (41, 45). The following experiment assessed
whether this phenomenon is also evoked by FMX-induced Fe loading.
Mice received tail vein injections of either FeS (n = 4), FeG (n = 4),
FMX (n = 4), or saline (n = 8). Approximately 18 h later, each of the
mice received an intraperitoneal injection of 10 mg/kg Escherichia
coli LPS (stock solution, 4 mg/ml saline; 0111:B4; L-2630; Sigma, St.
Louis, MO). Two hours post-LPS injection, the mice were anesthe-
thetized, and plasma and renal cortical samples were obtained. The
plasma samples were assayed for TNF-α (ELISA; R&D Systems).
Renal cortical samples were subjected to RT-PCR to quantify TNF-α
mRNA levels (41, 45).

Calculations and Statistics

All values are presented as means ± SE. Statistical comparisons
were performed by unpaired Student’s t-test. If multiple comparisons
were made, the Bonferroni correction was applied.
µg/ml of FMX did not impact HO-1 mRNA levels (NS vs. controls).

**Isolated PTS Experiments**

As shown in Fig. 4, FeS caused the greatest toxicity of the three tested Fe preparations (% LDH release, 41%). FeG evoked mild, but statistically significant, cytotoxicity. Conversely, FMX had no overt cytotoxic effect.

**In Vivo Mouse Experiments**

3-h Post-Fe injection: MCP-1 and HO-1 assessments. As shown in Fig. 5, left, within 3 h of FeS or FeG injection, only slight, and nonsignificant, increases in renal cortical MCP-1 mRNA were observed. However, about threefold increases in circulating MCP-1 protein were noted (Fig. 5, right), consistent with either increased renal or extrarenal MCP-1 production. Conversely, FMX evoked no increase in serum MCP-1 levels. If anything, FMX mildly suppressed renal cortical MCP-1 mRNA levels following FMX treatment were significantly lower than the values obtained with FeS and FeG treatment (P < 0.05).
mRNA values compared with the FeS and FeG treatment groups (P < 0.05). As shown in Fig. 6, FeS and FeG each induced dramatic increases in renal cortical HO-1 mRNA levels, whereas FMX did not impact this marker of Fe-mediated oxidative stress.

18-h Post-Fe injection: MCP-1 assessments. By 18 h post-FeS or -FeG injection, significant increases in renal cortical MCP-1 mRNA values were observed vs. control values (Fig. 7, left). These increases corresponded with striking increases in plasma MCP-1 levels (Fig. 7, right). Conversely, by 18 h post-FMX injection, no significant changes in either MCP-1 mRNA or in plasma MCP-1 protein levels were observed.

None of the Fe formulations significantly altered BUN concentrations (<28 mg/dl for Fe and control groups).

NGAL Assessments

Renal cortex. As shown in Fig. 8, FeG injection caused approximately two- and fivefold increases in renal cortical NGAL concentrations at 3 and 18 h, respectively (compared with control values). FeS caused a fourfold NGAL increase, but this change was only observed at the 18-h time point. In contrast, FMX failed to evoke any renal cortical NGAL increase at either the 3- or 18-h time points. Consistent with these results, both FeS and FeG significantly increased renal cortical NGAL mRNA, whereas FMX did not alter renal NGAL mRNA levels.

Plasma NGAL concentrations and extrarenal NGAL mRNA levels. As shown in Fig. 9, left, FeS injection and FeG induced 8- to 20-fold increases in plasma NGAL levels (vs. control plasma samples). Conversely, FMX did not alter plasma NGAL concentrations. The high circulating NGAL levels likely reflected, at least in part, extrarenal NGAL production, given that FeS and FeG, but not FMX, markedly increased hepatic NGAL mRNA levels (Fig. 9, right). In contrast, none of the Fe compounds increased splenic or pulmonary mRNA levels (data not shown).

Direct Fe effects on proximal tubule NGAL induction ± HO-1 inhibition. As shown in Fig. 10, FeS and FeG markedly suppressed HK-2 cell NGAL mRNA (to ~20% of control values). This was not due to a Fe-mediated increase in HO-1 activity because the HO-1 inhibitor SnPP did not prevent the Fe-mediated NGAL mRNA suppression. That SnPP had effectively inhibited HO-1 in these experiments was indicated by the fact that it caused an approximate eightfold increase in HO-1 mRNA (Fig. 10, right). In striking contrast to FeS and FeG, FMX did not alter HK-2 NGAL mRNA levels.
Fe Effects on Superimposed In Vivo Renal Injury

Fe preconditioning: effect on glycerol-induced rhabdomyolysis ARF. As shown in Fig. 11, left, FeS pretreatment significantly attenuated the severity of glycerol-induced ARF, as denoted by a 50–60% reduction in BUN concentrations. A correlate of this protection was a 50–60% reduction in glycerol-induced HO-1 mRNA elevations (Fig. 11, right). A significant correlation existed between the BUN and MCP-1 mRNA concentrations (glycerol alone, 1.1 ± 0.4; FeS 0.52 ± 0.15; r = 0.56; P < 0.05), also consistent with a FeS-induced diminution in glycerol-induced oxidative stress. In contrast, FMX pretreatment did not attenuate the severity of glycerol-induced ARF, as assessed by BUN concentrations or HO-1 mRNA (Fig. 11).

Impact of Fe Loading on LPS-mediated TNF-α induction. None of the Fe formulations, by themselves, increased renal cortical TNF-α mRNA levels (controls, 0.3 ± 0.1; FeS, 0.5 ± 0.2; FeG, 0.4 ± 0.1; FMX 0.4 ± 0.1) or plasma TNF-α (undetectable levels). However, FeS and FeG each sensitized to LPS-driven TNF-α production, as reflected by preferential increases in plasma TNF-α concentrations and renal cortical TNF-α mRNA levels (P < 0.001, < 0.005), compared with non-Fe-treated LPS-injected controls. Conversely, prior FMX
administration did not alter LPS responsiveness, as gauged by renal TNF-α mRNA or circulating TNF-α levels (Fig. 12).

**DISCUSSION**

It has been firmly established that iv Fe therapy effectively repletes Fe stores and enhances erythropoietin (Epo)-mediated red blood cell production in patients with chronic renal disease. However, concerns persist that, despite its overall beneficial effects, iv Fe administration might also exert adverse effects. For example, in three large clinical trials that involved predialysis or dialysis patients, aggressive iv Fe/Epo use, targeting hemoglobin concentrations of ≥13 g/dl, was associated with increased cardiovascular complications and/or mortality compared with less aggressive therapy (2, 28, 34). Because iv Fe and Epo are typically used together, it has not been possible to clearly dissect out whether these increased complications were directly caused by Epo alone, Fe alone, or the combination of these two agents. That currently used iv Fe formulations have potent prooxidant effects (e.g., Refs. 44 and 45), and that oxidant stress is well known to evoke cardiovascular injury and systemic inflammation (5, 12, 22, 29, 37), provide a theoretical basis for assuming that iv Fe may have played a role. However, it is notable that in the TREAT study, no direct relationship between iv Fe dose and cardiovascular complications appeared to exist (28).

Because of structural differences in currently employed Fe formulations (7), the degrees to which they induce oxidant stress may vary. Indeed, the results of our previous study (45), which compared FeS, FeG, and FeD, supports this view, given that Fe-mediated cytotoxicity greatly varied among three test agents (FeS > FeG > FeD). Since the time of that initial study, FMX has gained FDA approval and has entered into clinical use. Hence, we sought to determine FMX’s relative cytotoxic and prooxidant potentials compared with the two most widely used parenteral Fe formulations (FeS, FeG). To gain initial insights, the ability of these three compounds to induce lethal cell injury in cultured HK-2 cells was assessed. As shown in Fig. 1, the rank order of toxicity, as assessed by MTT uptake, was FeS > FeG > FMX. It is noteworthy that a reduction in MTT uptake indicates cytotoxicity, in general, and not Fe-mediated oxidative stress per se. Hence, to more directly assess this latter issue, the impact of FeS and FMX on HK-2 cell expression of two redox-sensitive genes, HO-1 and MCP-1, was examined. As shown in Fig. 2, FeS increased both HO-1 and MCP-1 mRNAs. Conversely, FMX was without effect. Thus these two sets of HK-2 cell results support the initial hypothesis: that differing Fe shields, as used in parenteral Fe formulations, can, indeed, have a striking impact on Fe’s cytotoxic and prooxidant effects.
Unlike in vivo proximal tubules, which are almost completely dependent on mitochondrial (aerobic) ATP production, cultured proximal tubule (including HK-2) cells have substantial glycolytic capacity (16). Because mitochondrial oxygen consumption is the dominant source of oxygen free radical generation in the renal cortex (38), results obtained with glycolytic cells may not be directly relevant to the in vivo state. Thus, to confirm differential toxicity within proximal tubule epithelium, this issue was addressed using almost strictly aerobic proximal tubules, isolated from normal mice. As depicted in Fig. 3, both FeS, and to a lesser degree, FeG, exerted direct cytotoxicity, as assessed by LDH release. Conversely, FMX had no overt cytotoxic effect. Thus these data were entirely consistent with the HK-2 cell results.

Given the above in vitro observations, we next sought evidence of Fe-mediated cytotoxicity in the in vivo state. Of note, none of the three test Fe formulations evoked overt renal injury, as denoted by normal postinfusion BUN concentrations (<28 mg/dl). Hence, a more sensitive marker of cytotoxicity was sought. Over the past 8 years, NGAL has been recognized as having utility in this regard (8, 13, 23, 33). It is upregulated in response to diverse forms of cellular stress, and like heat shock proteins, it may confer a survival benefit upon sublethally damaged cells (24, 32). As shown in Fig. 8, FeG administration evoked an early (3 h) and progressive increase in renal cortical NGAL levels, such that by 18 h postinjection, an eightfold increase in NGAL protein, and a fourfold increase in its mRNA, were observed. FeS largely recapitulated these FeG results. Conversely, FMX did not evoke any increase in renal cortical NGAL protein or mRNA. Thus these in vivo results were entirely consistent with the HK-2 cell results.

Given the above in vitro observations, we next sought evidence of Fe-mediated cytotoxicity in the in vivo state. Of note, none of the three test Fe formulations evoked overt renal injury, as denoted by normal postinfusion BUN concentrations (<28 mg/dl). Hence, a more sensitive marker of cytotoxicity was sought. Over the past 8 years, NGAL has been recognized as having utility in this regard (8, 13, 23, 33). It is upregulated in response to diverse forms of cellular stress, and like heat shock proteins, it may confer a survival benefit upon sublethally damaged cells (24, 32). As shown in Fig. 8, FeG administration evoked an early (3 h) and progressive increase in renal cortical NGAL levels, such that by 18 h postinjection, an eightfold increase in NGAL protein, and a fourfold increase in its mRNA, were observed. FeS largely recapitulated these FeG results. Conversely, FMX did not evoke any increase in renal cortical NGAL protein or mRNA. Thus these in vivo results were entirely consistent with the HK-2 cell and isolated tubule data: i.e., that FMX appears to have a low cytotoxic potential compared with the FeS and FeG formulations.

It is important to note that increases in renal cortical NGAL mRNA and protein are not necessarily mechanistically linked. For example, it has recently been demonstrated that injury-induced renal NGAL mRNA elevations occur predominantly within the loop of Henle and collecting duct (32). This is a seeming paradox, given that the proximal tubule is the dominant site of both nephrotoxic and ischemic renal damage. Thus it is now hypothesized that the bulk of injury- induced renal cortical NGAL protein accumulation arises from extrarenal NGAL production, followed by glomerular filtration, and megalin/24p3R receptor-mediated proximal tubule update (32). Given these considerations, we assessed the impact of the iv Fe formulations on plasma NGAL levels and tested whether the Fe preparations impact extrarenal, as well as proximal tubule-specific (HK-2 cell), NGAL gene induction. As shown in Fig. 9, FeS and FeG markedly increased plasma NGAL levels, and corresponding increases in extra renal NGAL mRNA (e.g., in the liver) were observed. Conversely, FeS and FeG induced profound suppressions of proximal tubule (HK-2) cell NGAL mRNA production. Thus, when these in vivo and in vitro results are viewed together, they strongly suggest that the FeS/FeG- mediated renal cortical NGAL protein increases were derived, at least in part, from the systemic circulation, and not from proximal tubule cells. The reason why FeG and FeS suppressed HK-2 cell NGAL mRNA remains unknown. Of note, it cannot simply be explained by an Fe-mediated concomitant increase in cytoprotective HO-1 expression, given that HO-1 inhibition (with SnPP) did not reverse Fe’s suppressive effect. NGAL is currently viewed as an intracellular Fe delivery system. Thus it seems possible that when proximal tubules become Fe overloaded (e.g., with iv Fe therapy), a compensatory downregulation of the NGAL-Fe delivery system results. In striking contrast to FeG and FeS, FMX neither increased plasma NGAL nor altered extrarenal or proximal tubular cell NGAL mRNA. This additionally underscores fundamental differences in the biological activity of FMX compared with the two other iv Fe formulations.

Given the apparent absence of FMX toxicity in each of the above experiments, we hypothesized that FMX would exert a...
lesser effect on in vivo HO-1 and MCP-1 expression than FeS and FeG (39, 41, 42–45). Indeed, this was the case. Whereas FeS or FeG each increased plasma MCP-1 and renal cortical MCP-1 mRNA levels, FMX had no effect. Furthermore, unlike FeS and FeG, FMX failed to raise renal cortical HO-1 mRNA. Thus these data are again entirely consistent with the hypothesis that FMX has a lower prooxidant potential than the other two test Fe formulations. The degree to which the plasma MCP-1 elevations reflected intrarenal vs. extrarenal production remains unknown.

In each of the above studies, direct Fe effects were assessed. However, it should also be noted that iv Fe formulations can impact tissue damage by altering superimposed injury responses. As one example, if mice are pretreated with either FeS or FeG, increased susceptibility to subsequent Gram-negative sepsis or LPS-mediated cytokine generation results (41, 43–45). Thus we next questioned whether FMX might lack this “LPS-sensitizing” effect. As shown in Fig. 12, this was the case: whereas both FeS and FeG pretreatment sensitized to LPS-driven TNF-α production, FMX did not alter this LPS response. A second pathway by which Fe may impact subsequent tissue injury is by evoking cytoprotective stress proteins, which then protect against “downstream” tissue damage. One of the best examples of this principle, so-called “acquired resistance,” is Fe-mediated HO-1 production, which then protects against subsequent oxidant attack (25, 26). Recently, it has been suggested that NGAL also possesses cytoprotective effects (24). Because FeS, but not FMX, increased renal cortical HO-1 and NGAL expression, we hypothesized that the former, but not the latter, would protect against the glycerol model of rhabdomyolysis-induced ARF. As shown in Fig. 11, FeS preconditioning did indeed, attenuate glycerol-induced renal injury, as denoted by ~50% reductions in both azotemia and glycerol-induced HO-1 induction. Conversely, FMX pretreatment conferred no cytoprotective effect. These data raise a compelling clinical question: might it be possible to use prooxidant Fe compounds to precondition patients, and thus, confer renal protection against procedures or treatments that have a high risk of inducing ARF (e.g., cardiovascular surgery, radiocontrast administration, or cisplatin therapy)? If so, based on the present findings, it would appear that either FeS or FeG would have greater utility in this regard compared with compounds with lesser prooxidant effects (e.g., FMX).

Finally, for a number of reasons, we would like to stress that the present results cannot be readily extrapolated to the clinical arena. First, in this study, we used identical amounts of, and time exposures to, FeS, FeG, and FMX to assess relative degrees of prooxidant and cytotoxic effects. However, in clinical practice, marked differences in Fe dosing regimens exist. For example, FMX is typically administered as a single 510-mg bolus. Conversely, FeS and FeG are infused more slowly (e.g., 30–60 min), in lower amounts (~100–125 mg/treatment), but with repetitive dosing. Whether these dosing differences ultimately impact potential parenteral Fe effects remains unknown. Second, although FeS and FeG have greater intrinsic prooxidant potentials than FMX, it is intriguing to speculate that following their initial administration, FeS and FeG upregulate HO-1 and NGAL, which, via the cytoprotective properties, might serve to negate subsequent adverse effects. This may be particularly germane in light of the fact that iv Fe formulations are given on a long-term basis rather than acutely as done in this study. Third, it is critical to note that the present study did not assess the risk of acute allergic reactions to iv Fe compounds, and the latter represent the major recognized complication of iv Fe therapy; and fourth, the present study, for the most part, utilized Fe dosages that were in great excess to those that are clinically deployed. This general approach is by no means unique to this study, since virtually all rodent studies of nephrotoxicity require extremely high drug concentrations to model clinically observable toxic effects. However, in this regard, it is notable that even at a dose of 1 μg/ml FeS (~5% of plasma concentrations following iv injection in patients), HK-2 cell HO-1 mRNA induction was observed. That Agarwal et al. (1) noted differential Fe compound toxicity in humans, using proteinuria as an endpoint, further suggests that clinical correlates to the current study may well exist. Hopefully, the present results will stimulate future trials to evaluate this possibility in the clinical arena, particularly in light of the fact that parenteral Fe administration is increasingly being administered to patients with renal disease.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Research Grant DK38432 and a grant from AMAG Pharmaceuticals.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES