Toxicity of Acetaminophen, Salicylic Acid, and Caffeine for First-Passage Rat Renal Inner Medullary Collecting Duct Cells

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ABSTRACT

Chronic excess ingestion of nonsteroid anti-inflammatory drugs causes renal medullary necrosis. Previously, using an immortalized line of mouse inner medullary collecting ducts cells (mIMCD3), we found that acetaminophen, salicylic acid, and caffeine are toxic, and the effects of acetaminophen and caffeine are strongly additive. Furthermore, toxicity was greater in proliferating than in nonproliferating cells. Important limitations were that mIMCD3 cells do not readily tolerate the high concentrations of salt and urea normally present in renal inner medullas and proliferate much more rapidly than inner medullary cells in vivo. Thus, these cells may not serve as an appropriate model for the in vivo IMCD. The present studies address these limitations by using passage-1 rat inner medullary collecting duct (p1rIMCD) cells, which tolerate high salt and urea and become contact inhibited when confluent. At 640 mOsmol/kg (the lowest normal inner medullary osmolality), the drugs, singly and in combination, reduce the number of proliferating (i.e., subconfluent) p1rIMCD cells more than they do confluent cells. Effects of acetaminophen and caffeine are strongly additive. Addition of as little as 0.1 mM caffeine significantly enhances the toxicity of acetaminophen plus salicylic acid. With confluent cells at 640 mOsmol/kg and very slowly growing cells at 1370 mOsmol/kg, combinations of drugs that include acetaminophen increase proliferation, accompanied by DNA damage and apoptosis. We conclude that these drugs are toxic to renal inner medullary collecting duct cells under the conditions of high osmolality normally present in the inner medulla, that combinations of the drugs are more toxic than are the drugs individually, and that the toxicity includes induction of proliferation of these cells that are otherwise quiescent in the presence of high osmolality.

Excessive consumption of mixtures of nonsteroidal anti-inflammatory drugs (NSAIDs) over a long period of time can cause renal disease, characterized by papillary necrosis and scarring ([No Authors Listed], 1984). The patients have progressive renal failure and are susceptible to the subsequent development of uroepithelial tumors. Combination of NSAIDs, often including caffeine, and chronic antiuretic are toxic, and the effects of acetaminophen and caffeine are strongly additive. Furthermore, toxicity was greater in proliferating than in nonproliferating cells. Important limitations were that mIMCD3 cells do not readily tolerate the high concentrations of salt and urea normally present in renal inner medullas and proliferate much more rapidly than inner medullary cells in vivo. Thus, these cells may not serve as an appropriate model for the in vivo IMCD. The present studies address these limitations by using passage-1 rat inner medullary collecting duct (p1rIMCD) cells, which tolerate high salt and urea and become contact inhibited when confluent. At 640 mOsmol/kg (the lowest normal inner medullary osmolality), the drugs, singly and in combination, reduce the number of proliferating (i.e., subconfluent) p1rIMCD cells more than they do confluent cells. Effects of acetaminophen and caffeine are strongly additive. Addition of as little as 0.1 mM caffeine significantly enhances the toxicity of acetaminophen plus salicylic acid. With confluent cells at 640 mOsmol/kg and very slowly growing cells at 1370 mOsmol/kg, combinations of drugs that include acetaminophen increase proliferation, accompanied by DNA damage and apoptosis. We conclude that these drugs are toxic to renal inner medullary collecting duct cells under the conditions of high osmolality normally present in the inner medulla, that combinations of the drugs are more toxic than are the drugs individually, and that the toxicity includes induction of proliferation of these cells that are otherwise quiescent in the presence of high osmolality.

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; SA, salicylic acid; APAP, acetaminophen; mIMCD3, mouse inner medullary collecting duct 3; COX, cyclooxygenase; NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide; p1rIMCD, passage-1 rat inner medullary collecting duct; DMEM, Dulbecco’s modified Eagle’s medium; DPBS, Dulbecco’s phosphate-buffered saline; LSC, laser scanning cytometer; PI, propidium iodide; PGE, prostaglandin E; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; DMSO, dimethyl sulfoxide.
cell death with both oncosis (swollen cells and nuclei) and apoptosis. APAP is reported to inhibit the synthesis of DNA (Hongsho et al., 1990) and cause chromosomal aberrations (Brunborg et al., 1995) due to inhibition of ribonucleotide reductase. Such effects of APAP might account for renal medullary cell death in vivo and development of uroepithelial tumors from surviving cells that have chromosomal aberrations.

mIMCD3 cells have important limitations for such studies, however, which led us to develop for the present studies a more realistic model for studying toxicity of drugs for renal inner medullary cells. mIMCD3 cells are immortalized by expression of SV40 (Rauchman et al., 1993). SV40 drives mIMCD3 cells to proliferate rapidly, even when they are confluent, which might sensitize them to toxicity of NSAIDs and caffeine, especially since, as noted above, subconfluent cells are more susceptible than are slower growing confluent cells. In addition, the mIMCD3 cells are routinely cultured in medium in which osmolality is 300 mosmol/kg, whereas the osmolality in the inner medulla varies over a range of 600 to 1700 mosmol/kg or more (Bankir, 1996), depending on the species and diuretic state. mIMCD3 cells are killed by acute increases in osmolality to 650 mosmol/kg or above (Santos et al., 1998; Michea et al., 2000). Furthermore, proliferation affects the tolerance of mIMCD3 cells for high NaCl. When osmolality is increased from 300 to between 500 and 600 mosmol/kg by adding NaCl, p53 activity increases (Dmitrieva et al., 2000). If p53 is suppressed, fewer cells survive (Dmitrieva et al., 2000) because the premature onset of renewed DNA replication is harmful (Dmitrieva et al., 2001).

Because of these considerations, we have reinvestigated the effects of SA, APAP, and caffeine using passage-1 rat inner medullary collecting duct (p1iIMCD) cells, which tolerate much higher concentrations of NaCl and urea than do mIMCD3 cells and proliferate more slowly, particularly when they are confluent.

Materials and Methods

Culture of mIMCD3 Cells. mIMCD3 cells (Rauchman et al., 1993) were grown on 100-mm Falcon plastic dishes and used between passages 16 and 20. Cells were fed with 1:1 DMEM-Ham's F-12 medium (Irvine Scientific, Santa Ana, CA) that contained 2 mM L-glutamine and 10% fetal bovine serum at 37°C under continuous agitation (300 rpm) in a humidified incubator (5% CO₂/95% O₂) in 50 mL of DMEM/F-12 without phenol red (Invitrogen), containing 100 mg of collagenase B (Roche, Indianapolis, IN) and 35 mg of hyaluronidase (Worthington Biochemical, Lakewood, NJ). The resulting cell suspension was centrifuged at 160g for 1 min, washed three times in prewarmed DMEM/F-12 without added enzymes, and resuspended in the tissue culture medium containing 50% DMEM with HEPES, 2 mM L-glutamine (Invitrogen), penicillin G (100 U/mL), streptomycin sulfate (100 mg/mL), 50 nM hydrocortisone, 5 mM 3,3,5-triiodo-L-thyronine, 10 nM sodium selenate, 5 mg/mL transferrin, 10% fetal bovine serum (v/v), and added NaCl and urea, as above. The cells from four kidney medullas were plated on a 6 or 10-cm (300 or 640 mosmol/kg) or 3.5-cm (1370 mosmol/kg) plastic Petri dish (Corning Incorporated, Corning, NY). Cells were fed each day.

Both subconfluent and confluent cells were studied at 640 mosmol/kg. The cells were harvested from the dishes by trypsinization in Ca²⁺/Mg²⁺-free DPBS. To study subconfluent cells at 640 mosmol/kg, 7,000 were seeded in each of eight chambers on a plastic slide (Nalge Nunc International, Naperville, IL) or human fibronectin- or collagen type-I-coated glass, eight-chamber slides (BD Biosciences, San Jose, CA). Then, after 6 h for attachment, experimental media were substituted. To study confluent cells at 640 mosmol/kg, 90,000 cells were seeded in each chamber. The cells were confluent after 48 h, then experimental media were substituted. Twenty-five thousand cells were seeded in each chamber at 1,370 mosmol/kg and allowed to attach overnight before substituting the experimental media. At 1,370 mosmol/kg, proliferation was very slow, and the cells generally did not become confluent. The osmolality of all media was verified with a freezing-point osmometer (Advanced Instruments Inc., Norwood, MA).

Laser Scanning Cytometry (LSC) and Photography. Cells that had been fixed and stained were examined through an epifluorescence microscope with a 20× objective (Olympus BX50; Melville, NY), analyzing them with an LSC (Compucyte Corp., Cambridge, MA) or photographing them with a Kodak Digital Science DC 120 Zoom digital camera (Eastman Kodak, Rochester, NY).

To characterize the cell type, they were fixed with 500 µl of methanol at −20°C for 2 h, washed 3 times (5 min each) with 500 µl of TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) at room temperature, and incubated for 1 h in 5% bovine serum albumin (fraction V; Sigma-Aldrich, St. Louis, MO) in TBST. The cells were then incubated with mouse monoclonal anti-alpha-smooth muscle actin conjugated with Cy5 (1:100 dilution, clone 1A4; Sigma-Aldrich) and/or mouse monoclonal anti-pan cytokeratin (1:100 dilution, clone C11; Sigma-Aldrich). The nuclei were counterstained with propidium iodide (5 µg/mL; Sigma-Aldrich) in DPBS containing ribonuclease A (1 µg/mL; Sigma-Aldrich). The cells were finally mounted in SlowFade or ProLong antifade solution (Molecular Probes, Inc., Eugene, OR), photographed, and analyzed by LSC.

To quantitate the cell number and analyze the cell cycle, cells grown on the eight-chamber permanox slides were fixed in 100% methanol at −20°C for 20 min, permeabilized with 0.1% Triton X-100, incubated with 1 mg/ml ribonuclease A (Sigma-Aldrich) for 15 min, stained with 20 µg/ml propidium iodide (PI) for 5 min, mounted with 150 µl of SlowFade antifade solution (Molecular Probes, Inc.), and analyzed by LSC. The LSC was used to count the number of cells (nuclei) and quantify the PI staining in each nucleus. The integral of PI fluorescence in each nucleus (total PI fluorescence), which is proportional to DNA content, was used to determine position in the cell cycle (G₀/G₁, S, or G₂/M). The data are displayed as cytograms, plotting the number of cells versus the integral of DNA content, and were analyzed using WinCyte software (Compucyte Corp., Cambridge, MA).

Cyclooxygenase Activity. Cells were incubated in control medium or medium containing indomethacin for 2 days. Arachidonic acid (5 µM) (lot 122844; Cayman, Ann Arbor, MI) was added during the last 24 h. PGE₂ in the medium was measured by immunoassay (Cayman), according the manufacturer’s instructions. Briefly, 50 µl of medium or serial dilutions of PGE₂ standards were incubated at...
room temperature for 18 h in wells coated with PGE$_2$, antiseraum and acetyl-cholinesterase-labeled tracer. The reaction mixture was de-
canted, and the wells were rinsed with wash-buffer, and then 200 µl of Ellman’s reagent, containing substrate for the acetylcholinester-
ase. The enzyme reaction was carried out for 90 min with slow
shaking at room temperature. Then, using a Labsystems Multiskan
MCC/340 microplate scanning spectrophotometer (Helsinki, Fin-
land), the plates were read at a 415 nm. The PGE$_2$ concentration is
expressed in nanograms per milliliter per milligram of cell protein
(bicinchoninic acid protein assay; Pierce, Rockford, IL).

**Analysis of Mitosis by Immunostaining with Anti-Phospho-
Histone H3 Antibody.** After fixation in 100% methanol at −20°C
for 45 min, the cells were washed 3 times for 5 min each with 0.1%
Triton X-100 in PBS, followed by blocking buffer (3% bovine serum
albumin-0.1% Triton X-100). Then they were incubated with anti-
phospho-histone H3 (mitosis marker) (no. 06-570; Upstate Biotech-
nology, Lake Placid, NY) antibody, washed with 0.1% Triton X-100 in
PBS, incubated for 1 h with secondary antibody (Alexa 488 goat-anti-
rabbit IgG, no. A-11034; Molecular Probes), stained with 0.7 µg/ml
PI and mounted with 150 µl of antifade (S-7461; Molecular Probes).
The slides were analyzed by LSC. Green fluorescence integral was
recorded to measure anti-phospho-histone H3 antibody binding (P-
histone H3 content). Red fluorescence was recorded to measure PI
binding (DNA content). Bivariate distributions of cells showing P-
histone H3 content versus DNA content were obtained, and the
percentage of P-histone H3 positive (mitotic) cells was determined
(Juan et al., 1998).

**Analysis of Proliferating Cell Nuclear Antigen (PCNA)
Abundance and of Histone H2AX Phosphorylation by Immu-
nostaining.** The immunostaining procedure was the same as for
anti-phospho-histone H3, except that the primary antibodies were
anti-phospho-H2AX(Ser139) (no. 07-164; Upstate Biotechnology) or
anti-PCNA (PC10) (Santa Cruz Biotechnology, Santa Cruz, CA).
Using LSC, the cells were analyzed for green fluorescence to measure
anti-PCNA or anti-phospho-histone H2AX antibody binding and red
fluorescence to measure PI binding (DNA content). Bivariate distri-
butions of cells showing maximal intensity of green fluorescence in
a nucleus (Green Max Pixel) versus DNA content were obtained.
The limits of the region of the cytoplasm containing cells with high of
Green Max Pixel (PCNA or phospho-H2AX positive cells) was deter-
memed by eye based on the control sample and the percentage of cells
in that region (PCNA or phospho-H2AX-positive cells) was calcul-
ated.

**Hoechst Staining.** p1rIMCD cells were plated in an eight-cham-
ber slide and allowed to grow for 2 days to reach confluence and then
treated with 2 mM of the indicated NSAIDs for 18 or 24 h. The cells
were fixed in 10% formalin (Fisher Scientific, Fair Lawn, NJ) for 15
min and washed 3 times with PBS followed by staining with 10 µg/ml
Hoechst-33258 DNA dye (Molecular Probes). After staining, the
slides were mounted with antifade solution (Molecular Probes).

**Drugs.** APAP, caffeine, SA, and indomethacin were from Sigma-
Aldrich. All drugs were directly dissolved in medium except indo-
methacin, which was dissolved in dimethyl sulfoxide (DMSO) and
then diluted with medium to a final DMSO concentration of 0.1% or
less. The same concentration of DMSO was added to control cells, as
appropriate.

**Statistical Methods.** In each individual experiment, each condi-
tion was analyzed in triplicate (three separate chambers on slides).
Then the results from the individual experiments were averaged and
are presented as the mean ± S.E.M. (n = number of individual
experiments). Significance was analyzed with the GraphPad Instat
program (GraphPad Software, San Diego, CA), using analysis of
variance completed by Dunnett’s multiple comparisons post-test,
according to the number of experiments. A p value <0.05 is consid-
ered significant.

**Results**

**Characterization of p1rIMCD Cultures from Rat Renal Inner Medulla.** Renal inner medullas contain several different types of cells, including epithelial cells in the loop of Henle and collecting ducts and myofibroblasts. No specific procedure was applied to select between cell types in the cultures. The types of cell that proliferated depended on the osmolality of the medium and on the surface that they were grown on (Fig. 1). Epithelial cells were identified by the expression of cytokeratin and myofibroblasts by expression of smooth muscle actin. When the osmolality of the medium used to prepare and grow the cells is 300 mOsmol/kg and the cells are grown on permanox tissue culture plastic, the proportion of epithelial cells and myofibroblast cells is approxi-
mately equal, but when the surface is glass, coated with fibronectin or collagen, epithelial cells predominate (Fig. 1). When the osmolality of the medium used to prepare and grow the cells is elevated by adding NaCl and urea to mimic the normal composition of the interstitial fluid in the inner me-
dulla, most of the cells are epithelial, regardless of the sur-

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**Fig. 1.** Expression of the epithelial cell marker, cytokeratin, and the myofibroblast marker, smooth muscle actin, by rat renal inner medullary cells in culture. Cultures were stained with PI (nuclear DNA marker, red), fluorescein isothiocyanate-labeled anti-cytokeratin antibody (green), and Cy3 labeled anti-smooth muscle actin antibody (orange). A (20×) and B (40×), show cells at 640 mOsmol/kg; C (20×) and D (40×), show cells at 1370 mOsmol/kg; E, LSC was used to quantify the propor-
tion of cells expressing the epithelial marker (cytokeratin; Cyt+) and the
fibroblastic marker (smooth muscle actin; SMA+). Cells were prepared
and grown at 300, 640, or 1370 mOsmol/kg on permanox slides or glass
slides coated with fibronectin or collagen type I. Mean ± S.E.M (n = 3).
face on which they are grown (Fig. 1E). At 640 mOsmol/kg, more than 80% of these epithelial cells express aquaporin-2, as previously illustrated by Chou et al. (2000), which identifies them as IMCD cells.

LSC was used for cell cycle analysis of subconfluent and confluent mIMCD3 in 300 mOsmol/kg medium and of p1rIMCD cells prepared and grown in media at 300, 640, or 1370 mOsmol/kg (Table 1). Proliferating cells are in the S or G2/M phases of the cell cycle. Approximately 34% of confluent mIMCD3 cells are in S and G2/M, consistent with continued proliferation that persists even after the cells become confluent. In contrast, only approximately 14 to 25%, depending on the osmolality, of confluent rat primary inner medullary cells are in S and G2/M, consistent with more effective contact inhibition of proliferation. Also, the percentage of confluent cells in S, i.e., those that are replicating DNA, is much less for p1rIMCD (2 to 6%) than for mIMCD3 cells (20%). Furthermore, confluent mIMCD3 cells tend to pile up in multiple layers after they are confluent, whereas the primary rat inner medullary cells do not (not shown). We conclude that the percentage of confluent cells that are in the process of replicating DNA and proliferating is much smaller for p1rIMCD cells than for mIMCD3 cells.

**Effect of APAP, SA, and Caffeine on Subconfluent p1rIMCD Cells at 640 mOsmol/kg.** When p1rIMCD cells are prepared and grown at 640 mOsmol/kg, 4 days of SA, APAP, or caffeine significantly reduces the number of subconfluent p1rIMCD cells (Fig. 2A). A higher concentration of SA or APAP is required to reduce the number of subconfluent p1rIMCD cells than previously observed for subconfluent mIMCD3 cells (Rocha et al., 2001). SA or APAP (1.0 to 2.0 mM) reduces the subconfluent p1rIMCD cell number by approximately 50%, whereas only 0.5 mM is required to reduce the mIMCD3 cell number to the same extent. On the other hand, less caffeine is necessary to reduce the subconfluent p1rIMCD cell number than to reduce the mIMCD3 cell number. More than 1.0 mM caffeine is required to reduce the subconfluent mIMCD3 cell number significantly, whereas 0.5 mM caffeine reduces the subconfluent p1rIMCD cell number significantly.

The effects of APAP and caffeine on subconfluent p1rIMCD cells are additive. APAP (0.5 mM) plus caffeine (0.5 mM) reduces the p1rIMCD cell number significantly more than either drug alone (Fig. 2B). In contrast, adding SA to APAP and/or caffeine does not further reduce the cell number. The previous results with subconfluent mIMCD3 cells were qualitatively similar (Rocha et al., 2001). As little as 0.1 mM caffeine adds significantly to the effect of 0.5 mM SA plus 0.5 mM APAP (Fig. 2C). Reduction of the cell number was associated with a decrease in the percentage of cells with damaged DNA (P-H2AX staining; Fig. 2D). The percentage of confluent cells in mitosis and APAP + caffeine or all drugs reduce mitosis further (p < 0.05). E, effect of 0.5 mM of drugs for 19 h on the percentage of cells with damaged DNA, identified by immunostaining with anti-phospho-histone H3 antibody. APAP reduces the percentage of cells in mitosis and APAP + caffeine or all drugs reduce mitosis further (p < 0.05). F, drugs, 0.5 mM each, added for 1 day or more reduce the net rate of proliferation.

**TABLE 1**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>mOsmol/kg</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>mIMCD3 subconfluent</td>
<td>300</td>
<td>44.7 ± 2.2</td>
<td>27.3 ± 0.9</td>
<td>27.8 ± 1.6</td>
</tr>
<tr>
<td>mIMCD3 confluent</td>
<td>300</td>
<td>68.5 ± 5.6</td>
<td>19.8 ± 2.7</td>
<td>14.2 ± 2.4</td>
</tr>
<tr>
<td>p1rIMCD subconfluent</td>
<td>640</td>
<td>77.0 ± 0.8</td>
<td>11.5 ± 0.4*</td>
<td>11.3 ± 0.8</td>
</tr>
<tr>
<td>p1rIMCD confluent</td>
<td>300</td>
<td>75.4 ± 4.4</td>
<td>5.7 ± 1.8*</td>
<td>18.9 ± 2.9</td>
</tr>
<tr>
<td>p1rIMCD confluent</td>
<td>640</td>
<td>70.2 ± 4.3</td>
<td>5.7 ± 0.8*</td>
<td>13.9 ± 0.7</td>
</tr>
<tr>
<td>p1rIMCD subconfluent</td>
<td>1370</td>
<td>87.6 ± 0.4</td>
<td>1.9 ± 0.7*</td>
<td>10.6 ± 0.4</td>
</tr>
</tbody>
</table>

*Significantly different from mIMCD3 subconfluent (p < 0.05).
Effect of APAP, SA, and Caffeine on Confluent p1rIMCD Cells at 640 mOsml/kg. When the p1rIMCD cells are confluent at 640 mOsml/kg, 4 days of 2.0 mM SA or caffeine does not affect the cell number significantly (Fig. 3A), similar to the lack of effect of SA and caffeine, singly or combined, on confluent mIMCD3 cells (Rocha et al., 2001).

When p1rIMCD cells are confluent at 640 mOsml/kg, APAP has a strikingly different effect (Fig. 3) from that on confluent mIMCD3 cells (Rocha et al., 2001) and that on subconfluent p1rIMCD cells (Fig. 2A). Whereas 2.0 mM APAP reduces the number of subconfluent p1rIMCD cells and does not significantly affect number of confluent mIMCD3 cells, it significantly increases the number of confluent p1rIMCD cells at 640 mOsml/kg (Fig. 3A). The increase is even larger when both APAP and caffeine are added (Fig. 3A).

DNA replication and cellular proliferation generally are accompanied by expression of PCNA (Fairman, 1990; Iatroupolos and Williams, 1996). APAP increases the percentage of cells expressing PCNA (Fig. 3B), consistent with increased proliferation. DNA damage is generally accompanied by increased phosphorylation of histone H2AX (Redon et al., 2002). Associated with increased proliferation of p1rIMDC cells, APAP increases the percentage of cells expressing P-H2AX (Fig. 3C), indicative of DNA damage. Nevertheless, there is no apparent change the appearance of the nuclei stained with the Hoechst reagent (Fig. 3D).

When p1rIMCD cells are confluent at 640 mOsml/kg, 2.0 mM SA plus 2.0 mM caffeine or a combination of APAP, SA, and caffeine at 2.0 mM each significantly reduces the cell number (Fig. 3A), similar to the lack of effect of SA and caffeine, singly or combined, on confluent mIMCD3 cells (Rocha et al., 2001). However, when both APAP and SA are added, the increase in the cell number is evident after 2 days (Fig. 4B). It is accompanied by elevated expression of PCNA (Fig. 4C), consistent with increased cellular proliferation, and elevated P-H2AX (Fig. 4D), consistent with increased DNA damage. When both APAP and SA are added, the increase in the cell number (Fig.
4A) is accompanied by nuclear condensation and fragmentation (Fig. 4E), consistent with apoptosis.

In contrast to the increase in the cell number with APAP and mixtures of drugs containing APAP, at least 1.0 mM caffeine or at least 1.0 mM each of caffeine and SA significantly decreases the cell number and is accompanied by the appearance of cells with condensed and fragmented nuclei (Fig. 4F), characteristic of apoptosis.

The effect of indomethacin was tested to determine how inhibition of COX might affect the pIRIMCD cells. COX activity in subconfluent pIRIMCD cells at 640 mOsmol/kg was estimated from the appearance of PGE$_2$ in the medium (Fig. 5). Indomethacin (10 μM) almost completely inhibits COX activity without any effect on the cell number, which excludes the possibility that the effects of the other drugs under these conditions involve COX inhibition. Similar lack of effect of COX inhibition, per se, was previously observed with mIMCD3 cells (Rocha et al., 2001).

**Discussion**

Direct Effects of SA, APAP, and Caffeine on pIRIMCD Cells. The present studies differ from our previous ones with mIMCD3 cells (Rocha et al., 2001) in that pIRIMCD could be readily tested under conditions of high NaCl and urea like those normally found in inner medullas. Similar to the effects of the drugs on subconfluent mIMCD3 cells at 300 mOsmol/kg (Rocha et al., 2001), SA, APAP, and/or caffeine reduce the number of pIRIMCD cells when they are subconfluent at 640 mOsmol/kg (Fig. 2, A and B). The concentration of SA or APAP needed to reduce the pIRIMCD cell number (Fig. 2A) is approximately twice that needed to produce a comparable decrease in the mIMCD3 cell number (Rocha et al., 2001), but the concentration of caffeine is approximately half as great. With both kinds of cells, the drug effects are additive, particularly the combination of APAP and caffeine (Fig. 2) (Rocha et al., 2001). It is particularly striking that as little as 0.1 mM caffeine significantly increases the effect of APAP plus SA to reduce the pIRIMCD cell number (Fig. 2), which supports the possibility that the caffeine, taken in excess, could add to the renal medullary toxicity of the combination of APAP plus SA.

SA and caffeine have a greater effect on mIMCD3 cells when they are subconfluent at 300 mOsmol/kg than when they are confluent (Rocha et al., 2001). The same trend is seen with pIRIMCD cells at 640 mOsmol/kg. SA significantly reduces the number pIRIMCD cells when they are subconfluent (Fig. 2, A and B) but not when they are confluent (Fig. 3A). Nevertheless, 2.0 mM SA plus 2.0 mM caffeine or 2.0 mM of all three drugs combined reduces the number of confluent pIRIMCD cells (Fig. 3A). This is consistent with our previous conclusion that these agents are more toxic to proliferating cells than to quiescent ones and that the drugs might be more toxic in combination than singly. Few cells normally are proliferating in rat inner medullas (Sheikh-Hamad et al., 2001; Zhang et al., 2002). Targeting of toxicity to those few cells is consistent with the slow onset and progression of analgesic-associated renal disease ([No Authors Listed], 1984).

An unexpected novel result of the present studies is that APAP, alone and in combination with the other drugs, strikingly increases the number of pIRIMCD cells when they are confluent at 640 mOsmol/kg (Fig. 3) or growing very slowly at 1370 mOsmol/kg (Fig. 4) in striking contrast to reduction of the number of pIRIMCD cells that are subconfluent at 640 mOsmol/kg (Fig. 2). Caffeine alone does not affect the number of confluent cells at 640 mOsmol/kg, but it adds greatly to the increase in the cell number caused by APAP (Fig. 3A).

The effect of APAP to increase the cell number is even more striking at 1370 mOsmol/kg. APAP (0.5 mM or more), alone, or 0.1 mM or more of APAP combined with 0.1 mM or more of SA and/or caffeine greatly increases the number of cells that otherwise are proliferating very slowly at 1370 mOsmol/kg (Fig. 4A). As discussed above, there is little proliferation of renal inner medullary cells in vivo, and proliferating inner medullary cells have reduced tolerance for hyperosmolality. APAP-induced proliferation appears to sensitize the cells to toxicity, as evidenced by appearance of DNA damage (increased P-H2AX expression; Figs. 3C and 4D) and apoptosis when APAP is added in combination with SA (Fig. 4E).

APAP was previously reported either to increase or decrease proliferation of various cells. The reports of increased proliferation include that 0.1 to 0.3 mM APAP produces significant growth stimulation of several human tumor cell lines and one of two normal fibroblast cell lines (Schonberg and Skorpen, 1997). It causes a brief burst of mitosis in a glioma cell line, which transiently increases the cell number, followed by apoptosis which reduces the cell number (Casper et al., 2000). It also stimulates proliferation (3H-dT incorporation) of breast cancer cells (Harnagea-Theophilus et al., 1999) and increases bromodeoxyuridine incorporation in livers of hamsters but not of rats (Hiruma et al., 2001). Treating mice with increasing doses of APAP for 8 days increases hepatocellular proliferation 4-fold (Shayiq et al., 1999). Reports of decreased proliferation include that APAP produces cell cycle block in Hepa 1-6 cells (Boulares et al., 1999) and HL-60 cells (Wiger et al., 1997), reduces replicative DNA synthesis in mouse mammary tumor cells (Hongslo et al., 1990), and reduces proliferation of HepG2 cells (Dai and Cederbaum, 1995) It has not been clear what factors deter-

![Graph](image-url)
mine whether APAP will increase or decrease the cell number, but the rate of proliferation and DNA replication apparently is important in p1IMCD cells. It would be of interest to know whether high levels of APAP increase the otherwise slow rate of proliferation of renal inner medullary cells in vivo.

**Mechanisms of the Effects of APAP and Caffeine.** NSAIDs inhibit proliferation of some tumorigenic cells and kill them (Baron and Sandler, 2000), which has led to numerous studies of the mechanisms involved. Both COX-dependent and independent mechanisms have been identified (Chan et al., 1998; Baron and Sandler, 2000). Since we did not find COX inhibition per se to be toxic (Fig. 5), we will concentrate on COX-independent mechanisms.

Acute overdoses of APAP cause acute and sometimes fatal liver damage (Perry and Shannon, 1998). The toxicity is caused by a minor metabolic product, N-acetyl-p benzoquinone imine (NAPQI) that attaches to the hepatic cell membranes and injures the lipid bilayer if not neutralized by an antioxidant. Hepatic glutathione appears to be the primary antioxidant that conjugates and neutralizes NAPQI. The resulting oxidative stress in the cell may ultimately lead to its demise. NAPQI also binds to cell macromolecules, which can cause cell death (Mirochnitchenko et al., 1999). However, APAP, has an additional toxic effect that may be more pertinent to the toxicity that we observe. The drug directly inhibits ribonucleotide reductase, which reduces cell growth by stopping DNA replication (Hongso et al., 1990). Then, the relative number of cells in S phase increases (Hongso et al., 1990), as we observed following exposure of mIMCD3 cells to APAP (Rocha et al., 2001). In the process DNA is damaged, leading to sister chromatid exchange and chromosomal aberrations. APAP also inhibits nucleotide excision repair (Brunborg et al., 1995). Histone H2AX is phosphorylated on Serine 139 in response to double-strand breaks (Rogakou et al., 1998), which is one of the first steps in DNA repair (Paull et al., 2000). That APAP induces this phosphorylation (Figs. 3C and 4D) supports the conclusion that, under these conditions, the drug induces DNA double-strand breaks, causing toxicity.

Caffeine has long been known to have numerous actions (Serafin, 1996), including 1) inhibition of phosphodiesterases, thereby increasing intracellular cyclic AMP, 2) direct effects on intracellular calcium concentration, 3) indirect effects on intracellular calcium concentrations via membrane hyperpolarization, and 4) antagonism of adenosine receptors. In addition, it has recently become apparent that caffeine also influences multiple pathways involved in the cellular response to DNA damage. It reduces DNA damage-induced cell cycle arrest in G2, S, and G2/M, abolishing the G2/M checkpoint by inhibiting ATM kinase activity (Zhou et al., 2000). Caffeine also blocks p53 activation in response to DNA damage and blocks the repair of DNA damage (Murnane, 1995). The result is that caffeine potentiates the lethal effects of ionizing radiation. We now observe that caffeine strongly potentiates the toxicity of APAP. The mechanism could be related to the cumulative effects of the two drugs on DNA damage and repair. The effects of caffeine start at concentrations close to those that inhibit ATM/ATR kinases (Sarkaria et al., 1999). These kinases are activated by DNA damage and coordinate a DNA damage response, involving accurate DNA repair accompanied by cell cycle arrest (Zhou and Elledge, 2000). Thus, inhibition of ATM/ATR kinases by caffeine is a mechanism that might impair repair of the DNA damage caused by APAP, increasing its toxicity.

Why might these drugs be particularly toxic to renal inner medullary cells? One factor is that drugs can be concentrated in the inner medulla as a result of the urinary concentrating mechanism. Thus, during antidiuresis, APAP is 4 times higher in the renal medulla than in peripheral plasma (Duggin, 1980). We do not know whether this is also true of caffeine, being unaware of any measurements of caffeine concentration in renal medullas. A second factor is that the high osmolality present in renal medullas could sensitize the cells to the drugs. We previously showed that proliferation sensitizes renal inner medullary cells to osmotic stress (Zhang et al., 2002), and the cells die mostly in the S phase of cell cycle, during DNA replication (Dmitrieva et al., 2001). High levels of NaCl, like those that occur in the renal inner medulla, damage DNA (Kultz and Chakravarty, 2001), particularly during DNA replication, and inhibit repair of this damage (Dmitrieva et al., 2003), which possibly adds to DNA damage caused directly by APAP. In addition, at high osmolality, APAP increases the number of PCNA positive cells, evidence that it causes the cells to enter S phase under these conditions, which could sensitize them to the osmotic stress.

**References**


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