Azathioprine Acts upon Rat Hepatocyte Mitochondria and Stress-Activated Protein Kinases Leading to Necrosis: Protective Role of \(N\)-Acetyl-L-cysteine

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ABSTRACT

Azathioprine is an immunosuppressant drug widely used. Our purpose was to 1) determine whether its associated hepatotoxicity could be attributable to the induction of a necrotic or apoptotic effect in hepatocytes, and 2) elucidate the mechanism involved. To evaluate cellular responses to azathioprine, we used primary culture of isolated rat hepatocytes. Cell metabolic activity, reduced glutathione, cell proliferation, and lactate dehydrogenase release were assessed. Mitochondria were isolated from rat livers, and swelling and oxygen consumption were measured. Mitogen-activated protein kinase pathways and proteins implicated in cell death were analyzed. Azathioprine decreased the viability of hepatocytes and induced the following events: intracellular reduced glutathione (GSH) depletion, metabolic activity reduction, and lactate dehydrogenase release. However, the cell death was not accompanied by DNA laddering, procaspase-3 cleavage, and cytochrome c release.

The negative effects of azathioprine on the viability of hepatocytes were prevented by cotreatment with \(N\)-acetyl-L-cysteine. In contrast, 6-mercaptopurine showed no effects on GSH content and metabolic activity. Azathioprine effect on hepatocytes was associated with swelling and increased oxygen consumption of intact isolated rat liver mitochondria. Both effects were cyclosporine A-sensitive, suggesting an involvement of the mitochondrial permeability transition pore in the response to azathioprine. In addition, the drug’s effects on hepatocyte viability were partially abrogated by c-Jun N-terminal kinase and p38 kinase inhibitors. In conclusion, our findings suggest that azathioprine effects correlate to mitochondrial dysfunction and activation of stress-activated protein kinase pathways leading to necrotic cell death. These negative effects of the drug could be prevented by coincubation with \(N\)-acetyl-L-cysteine.

Azathioprine (Aza) is an immunosuppressant widely used in the treatment of autoimmune diseases (Nash and Sutherland, 2001) and in organ transplantation (Ponticelli et al., 1999). It has even been reported that 3.5% of 173 inflammatory bowel disease patients treated with Aza developed hepatitis as a consequence of treatment (Rietdijk et al., 2001).

Hepatotoxicity is thus an unpredictable side effect of the drug, whose pathogenic mechanism remains unknown. Recently, it has been proposed that Aza toxicity is related to its cellular biotransformation (Lee and Farrell, 2001). The transformation of azathioprine to its active form, 6-mercaptopurine, depends on the availability of reduced glutathione (GSH) (Kaplowitz, 1977). Azathioprine metabolism in rat hepatocytes leads to GSH depletion, mitochondrial injury, decreased ATP levels, and cell death (Lee and Farrell, 2001).

Currently, three GSH hepatoprotection models have been proposed: the GSH/glutathione peroxidase system, which

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ABBREVIATIONS: Aza, azathioprine; GSH, reduced glutathione; GST, glutathione S-transferase; TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; LDH, lactate dehydrogenase; EDF, epidermal growth factor; PMSF, phenylmethysulfonyl fluoride; DMSO, dimethyl sulfoxide; CsA, cyclosporin A; 6-MP, 6-mercaptopurine; PBS, phosphate-buffered saline; NBT, nitro blue tetrazolium; TCA, trichloroacetic acid; NAC, \(N\)-acetyl cysteine; MPTP, mitochondrial permeability transition pore; SAPK, stress-activated protein kinase; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive species; NO, nitric oxide; XO, xantine oxidase; GSSG, oxidized glutathione; ASK-1, apoptosis signal-regulating kinase 1.
buffers H2O2 produced in drug oxidation (Fernández-Checa et al., 1998); the GSH/GST system, which acts as an inactivating mechanism through which glutathione regulates compartmentalization of the toxic compounds (Strange et al., 2000) or even their efflux (Sheehan et al., 2001); and the GSH/GST/thioredoxin system, which acts as sensor of intracellular changes in redox potential regulating different types of cell death (Fernández-Checa, 2003). The GSH depletion sensitizes hepatocytes for cell death induced by TNF-α (Matsumaru et al., 2003) or arsenic trioxide (Davison et al., 2003). Recently, it has been observed that mitogen-activated protein kinases (MAPKs) may serve as regulators of cell death induced by endogenous and exogenous stresses. Three members of the MAPK family have been implicated as regulators of cellular response to toxic injury: extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun N-terminal kinase (JNK). The development of specific inhibitors of these pathways involves the intracellular MAPK pathways.

Reagents and Animal Model.

Materials and Methods

Reagents and Animal Model. Insulin, EGF, GSH, o-phtaldialdehyde, MTT, 1-methoxyphenazine methosulfate, lithium 1-lactate, 6-mercaptopurine, rotenone, dimethyl sulfoxide (DMSO), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma Chemical Co. (Madrid, Spain). Cyclosphorine A (CsA) was a gift from Novartis (Basel, Switzerland). p38 inhibitor 4-(4-fluorophenyl)-2-((4-methylsulfonylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), JNK inhibitor 1,9-pyrazoloanthrone (SP600125), and ERK inhibitor 2-amino-3-methoxyphenylazo (PD980598) were from Calbiochem (Barcelona, Spain). Antibody against procaspase-3 (p20 reactive fragment) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against bcl-2 and cytochrome c were from Calbiochem, and antibodies against phospho-p44/42 MAPK, phospho-p54/46 JNK, and phospho-p38 MAPK were from Cell Signaling Technology Inc. (Beverly, MA). All other chemicals were of the highest purity commercially available.

Male Wistar rats (200–220 g b.wt.) were allowed free access to food and tap water before surgery. Animals were handled and caged according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences.

In all experiments, Aza and 6-MP were dissolved in DMSO, and cells treated with DMSO alone were used as control.

Hepatocyte Preparation. Hepatocytes were isolated “in situ” two-step collagenase perfusion (Seglen, 1976). Cell viability estimated by trypan blue exclusion was >90%. The isolated cells were plated (2 × 10^6 cells/ml) in 24-multwell plastic dishes (1 ml volume) in medium E containing 10% bovine fetal serum and 1% antibiotic/antimycotic solution in a humidified atmosphere of 5% CO2 and 95% air at 37°C.

Hepatocyte GSH Content. Intracellular GSH levels were determined according to the method described previously with modifications (Hissin and Hilf, 1976). Briefly, after treatment, a cell homogenate in 0.1 M sodium phosphate-5 mM EDTA buffer (pH 8) was treated with 14% potassium perchlorate to precipitate proteins. After centrifugation, GSH was estimated by monitoring the fluorescence intensity of the o-phtaldialdehyde-glutathione adduct at 420 nm with the excitation wavelength set at 350 nm.

Metabolic Activity. The MTT reduction assay was performed as described previously (Alley et al., 1988) with modifications. After incubation with the corresponding drug, MTT stock solution in PBS buffer was added to the cell culture to obtain a final concentration of 0.5 mg/ml MTT in the medium. Cells were incubated for a further 2.5 h at 37°C. The formazan crystals were dissolved in 0.1 M HCl in isopropanol, and absorbance was measured using a microplate reader at a test wavelength of 570 nm and reference wavelength of 690 nm.

LDH Release. Hepatocyte LDH activity was determined as described previously (Abe and Matsuki, 2000). Briefly, after treatment, the supernatant and cells were separated. The cells were solubilized with 0.2% Triton X-100, and 50 μl of samples was transferred to 96-well culture plates and mixed with 50 μl of LDH substrate (prepared by adding 2.5 mg of lactate and 2.5 mg of NAD+ to 1 ml of 0.2 M Tris-HCl buffer (pH 8.2) containing 0.1% (v/v) Triton X-100, 100 μM 1-methoxyphenazine methosulfate, and 600 μM MTT in PBS). The formazan formed was measured using a microplate reader as noted above.

Measurement of Superoxide Radicals. The NBT reduction assay was determined as described previously (Sharma and Morgan, 2001) with modifications. After incubation with azathioprine (150 μM), NBT stock solution in PBS buffer was added to the cell culture to obtain a final concentration of 0.5 mNBT in the medium. Cells were incubated for a further 1 h at 37°C. At the end, incubation medium was removed, and cells were lysed and removed in pyridine-water (1:1). The absorbance of reduced NBT (formazan) was measured at a wavelength of 630 nm using a mixture of pyridine and water (1:1) as blank.

DNA Synthesis. DNA synthesis was determined as described previously (Rodríguez-Henche et al., 1998) by estimating the incorporation of [3H]-thymidine into trichloroacetic acid (TCA)-precipitable material. Briefly, 2 μCi of [3H]-thymidine was added to the cells, and after 24 h of incubation, the cells were washed twice in PBS, and 0.5 ml of 5% TCA was added. After 20 min at 4°C, TCA was removed, and the precipitate was treated with 0.2 ml of 2 N KOH for 60 min at room temperature and then neutralized with 0.25 ml of 2 N HCl. The plate content was harvested into a glass fiber filter, and radioactivity was counted.

DNA Fragmentation. The cells were collected and lysed as described previously (Román et al., 1998) in 1 ml of buffer STE (250 mM sucrose/5 mM Tris-HCl, pH 8.0, 1 mM EDTA), 3 ml of TE (10 mM Tris, pH 8.0, 1 mM EDTA) and 0.5 ml of 25% SDS and incubated overnight at 40°C. RNase A was added at a concentration of 50 μg/ml, and incubation was continued for 1 h at 37°C. Potassium acetate 8 M was added to obtain a final concentration of 1.33 M, and the lysate was extracted twice with an equal volume of chloroform/isomyl alcohol (24:1) and centrifuged at 1000g for 5 min. DNA was precipitated in the aqueous phase with 2 volumes of absolute ethanol overnight at −20°C. DNA samples (5 μg/ml) were analyzed by electrophoresis in 2% agarose gel. Gels were stained with 0.5 μg/ml ethidium bromide.

Western Blotting of Procaspase-3, bcl-2, Cytochrome c, ERK 1/2, JNK 1/2, and p38 Kinase. Total cell protein was extracted using a Tris (50 mM), pH 7.4, buffer containing β-mercaptoethanol (10 mM), EDTA (5 mM), EGTA (1 mM), PMSF (1 mM),...
soybean trypsin inhibitor (10 μg/ml), leupeptin (5 μg/ml), and aprotinin (5 μg/ml). The cells were scraped into a suspension with a rubber cell lifter and lysed by sonication. Cytosol and mitochondrial pellet were separated as described previously (Di Paola et al., 2000) to study the cytochrome c release. Homogenized proteins were determined by the Bradford method. Twenty-five micrograms of protein per lane was run on 15% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane overnight at 25 V. Membranes were blocked with 5% nonfat dried milk in PBS-Tween 20 and probed with a polyclonal antibody against pro-caspase-3, cytochrome c, or bel-2 and a monoclonal antibody against phospho-p44/42 MAPK (ERK 1/2), phospho-JNK 1/2, or phospho-p38 kinase. After blotting, the immunoreactive bands were detected by enhanced chemiluminescence using LumiGlo and peroxide reagent by Cell Signaling Technology Inc. The gel autoradiography scans were quantified by densitometry using the Scion Image software from Scion (Frederick, MD).

Isolation of Rat Liver Mitochondria. Liver mitochondria were prepared by the differential centrifugation technique (Di Paola et al., 2000). Livers from adult male Wistar rats were excised, finely minced, and homogenized in 10 volumes of cold isolation medium containing 0.25 M sucrose, 0.1 mM EGTA, and 0.25 mM PMSF in 10 mM Tris-Cl (pH 7.4). The homogenate was centrifuged at 1200 g for 10 min. The remaining supernatant was centrifuged at 9500 g for 10 min, and the pellet was resuspended in the isolation medium and centrifuged again as described above. The final pellet was resuspended in the isolation medium and the concentration of EGTA was lowered to 0.01 mM, at a protein concentration of 50 to 60 mg/ml as determined by the biuret method. All the above-mentioned operations were carried out at 0–4°C.

Mitochondrial Swelling. Mitochondrial swelling was determined as described previously (Di Paola et al., 2000) using 50 mM HEPES (pH 7.4) buffer in a reaction mixture containing 0.25 M sucrose, 1 μg/ml rotenone, and 10 mM succinate at 25°C, in the presence of 150 μM Aza or 150 μM 6-MP with or without 1 μM CsA. The reaction was initiated by adding the mitochondria (0.25 mg/ml), and changes in absorbance were recorded at 540 nm in a Beckmann DU 7400 spectrophotometer, equipped with a thermostat controlled and magnetically stirred automatic sampling unit.

Mitochondrial Oxygen Consumption. The respiratory activity of rat liver mitochondria was measured polarographically in a Rank Brothers oxygen at 25°C following a previously described procedure (Di Paola et al., 2000) with minor modifications, by suspending mitochondria at 0.1 mg/ml in a basic reaction mixture containing 75 mM sucrose, 30 mM KCl, 30 mM Tris-Cl, pH 7.4, 2 mM KH2PO4, 10 μM EGTA, 10 mM succinate, and 1 μg/ml rotenone. After 5 min to reach equilibrium, 150 μM Aza or 150 μM 6-MP with or without 1 μM CsA was added. From beginning to end of the experiments, ADP (1 mM) and carbonylcyanide-m-chlorophenylhydrazone (0.3 μM) were added to check the respiratory chain.

Statistical Analysis. All data represent the results of at least three independent experiments performed on a minimum of three separate hepatocyte or mitochondrial isolates. Analysis of variance was used to compare three or more samples. The level of statistical significance was set at p < 0.05.

Results

Effect of Azathioprine on Cultured Rat Hepatocytes’ Metabolic Status (GSH Concentration, MTT Reduction, LDH Release, and Superoxide Concentration). Treatment of cells with Aza (150 μM) in the presence of mitogens (0.1 μM insulin plus 20 ng/ml EGF) induced the following sequential events: 1) intracellular GSH depletion (t1/2 = 0.5 h), 2) decreased metabolic activity determined by the MTT reduction assay (t1/2 = 2.5 h), and 3) increased cellular LDH release (t1/2 = 4.5 h) (Fig. 1). The time dependence of these effects of Aza on metabolic status variables could indicate a cause-effect relationship.

The Aza effects on metabolic activity (Fig. 2A) were abolished by coincubating the hepatocytes for up to 24 h with Aza (150 μM) and NAC (1 mM). In parallel, the negative effect of Aza (150 μM) on GSH levels was partially reversed by cotreatment with NAC (1 mM), indicating that the half-restoration of GSH intracellular level (Fig. 2B) was enough to maintain the MTT reduction ability of the cell.

Figure 2C shows the concentration dependence of thiopurine-induced effects on metabolic activity of rat hepatocytes incubated with the drugs during 24 h. Increased Aza concentrations (0–150 μM) led to a steady decrease in cellular metabolic activity (IC50 = 83 μM). Under the same conditions no effect on MTT reduction assay was shown when cells were treated with 6-MP (150 μM). The different effects of both drugs (Aza and 6-MP) on MTT reduction activity correlate well with their impact on the intracellular GSH content (Fig. 2D). These data demonstrate that Aza-induced negative effects are related to GSH depletion. Figure 3A shows the effect of Aza (150 μM) on superoxide radicals. The incubation of hepatocytes with Aza (150 μM) for 0 to 3 h increases slightly the superoxide concentrations with respect the control values (DMSO-treated cells). This period of time has been chosen because Aza depleted GSH content, reaching a steady state after 3 h of treatment.

Effect of Azathioprine on [methyl-3H]Thymidine Incorporation into DNA. Treatment of cells with Aza (150 μM) for 24 h, in the presence of insulin (0.1 μM) plus EGF (20 ng/ml), induced a significant decrease in [methyl-3H]thymidine incorporation into DNA (Fig. 3B). The inhibitory effect of Aza on DNA synthesis was not affected by NAC (1 mM). In basal conditions (without mitogens) about a 10% [methyl-3H]thymidine incorporation into DNA with respect to control (DMSO-treated cells) this period of time has been chosen because Aza depleted GSH content, reaching a steady state after 3 h of treatment.

Effect of Azathioprine on Apoptosis Indicators (DNA Fragmentation, Procaspase-3 Cleavage, and Cytochrome c Release). Treatment of rat hepatocytes with Aza (150 μM) in the presence of mitogens (0.1 μM insulin plus 20 ng/ml EGF) for the times indicated, gave rise to a DNA fragmentation profile similar to the control (DMSO-treated cells).
cells) (Fig. 4A). Rat hepatocytes incubation with Aza (10–150 μM) for 24 h gave rise to a DNA fragmentation profile similar to the control (DMSO-treated cells) (data not shown). As a positive control, DNA laddering of hepatocytes was measured in the presence of etoposide (150 μM) for 24 h, resulting in a typical apoptotic profile.

Because a direct cause-effect relationship between GSH depletion and caspase-3 activation has already been established by others, we went on to evaluate whether Aza treatment of the cells could result in procaspase-3 cleavage. Western blot analysis (Fig. 4B) indicated no procaspase-3 cleavage after Aza treatment (2, 4, or 6 h). Likewise, immunoblot analysis (Fig. 4C) against cytochrome c revealed no release of this mitochondrial protein at 4 h of treatment with azathioprine. With all these
data and the DNA laddering profile, the possibility of generalized apoptotic induction by the drug is excluded.

**Azathioprine Induces Swelling and Increase of Oxygen Consumption in Rat Liver Mitochondria.** Azathioprine (150 μM) and 6-MP (150 μM) accelerated the swelling of isolated mitochondria (Fig. 5A) relative to controls (in the presence of DMSO). Figure 5B shows that under nonphosphorylating conditions, succinate-supported respiration increased at roughly the same time as the onset of mitochondrial swelling. The presence of Aza (150 μM) or 6-MP (150 μM) led to a faster oxygen consumption rate. These results suggest that the drugs effects on mitochondrial swelling and respiration are related in some way. When mitochondria were incubated with Aza or 6-MP in the presence of CsA (1 μM), both mitochondrial swelling and respiration rate transitions were inhibited (Fig. 5, C and D). Given that CsA is a very specific inhibitor of the mitochondrial permeability transition pore (MPTP), these results strongly suggest that Aza and 6-MP provoke directly the above-described mitochondrial effects by MPTP transient opening.

**Effect of Azathioprine on Mitochondrial GSH, Pro-caspase-3, and bcl-2.** Incubation of isolated rat liver mi-
mitochondria with 150 μM Aza for 45 min produced no significant change in mitochondrial GSH content (28.8 ± 4.8 nmol GSH/mg mitochondrial proteins) with respect to the control (25.6 ± 1.6 nmol GSH/mg mitochondrial proteins). Mitochondrial procaspase-3 and bcl-2 levels remained unchanged during Aza (150 μM) treatment (0, 15, 30, or 45 min) (Fig. 6), indicating no leakage of these proteins from mitochondria.

**Effects of NAC, JNK, and p38 Kinase Inhibitors on the Viability of Rat Hepatocytes Treated with Azathioprine.** Cotreatment of rat hepatocytes with Aza (150 μM) and NAC (1 mM) fully blocked the metabolic activity loss induced by Aza alone (Fig. 7A). Cotreatment with Aza and CsA (1 μM) partially inhibited the effect of Aza, a result consistent with the effect of CsA on the inhibition of swelling in isolated mitochondria (Fig. 7A). SAPK and ERK are signaling proteins known to be involved in cell death induced by GSH depletion. In this set of experiments, we examined the effects of pharmacological inhibitors of SAPK and ERK on the metabolic activity of Aza-treated hepatocytes. Figure 7B shows that treatment of hepatocytes with SP600125 (a JNK inhibitor) or SB203580 (a p38 kinase inhibitor) intensely inhibits the loss of metabolic activity elicited by Aza. On the other hand, PD98059 (ERK inhibitor) treatment failed to affect the loss of metabolic activity induced by Aza (Fig. 7B).

**Azathioprine Induces ERK, JNK, and p38 Kinase Phosphorylation in Cultured Rat Hepatocytes.** Treatment of cells with Aza (150 μM) in the presence of mitogens (0.1 μM insulin plus 20 ng/ml EGF) induced the phosphorylation of p44/42 MAPK (ERK 1/2) after 3 h of incubation (Fig. 8A). This effect was reversed by cotreatment with CsA (1 μM) or NAC (1 mM), indicating the involvement of the mitochondria in the MAPK activation induced by Aza. The capacity to detect α-tubulin structural protein was used as a load control. In a further set of experiments, we explored the Aza effect on JNK and p38 kinase phosphorylation. Treatment of cultured rat hepatocytes with Aza (150 μM) led to increased levels of phosphorylated JNK and p38 kinase (Fig. 8, B and C). Aza/NAC (1 mM) cotreatment inhibited JNK and p38 phosphorylation and, similarly, diminished the cell metabolic activity loss induced by Aza (Fig. 7A). However, CsA (1 μM) was unable to block the JNK phosphorylation provoked by Aza. Interestingly, CsA was able to inhibit the p38 kinase phosphorylation induced by Aza. Moreover, in the absence of Aza, PD98059 (an ERK inhibitor) treatment gave rise to increased JNK phosphorylation (positive test). In conclusion, Aza (150 μM) was shown to induce the early phosphorylation of JNK and p38 kinase, the effect being blocked by cotreatment with NAC (1 mM). As a protein loading control, Western blots were used to detect total JNK and p38 kinase.

**Discussion.**

The most important finding emerging from our results was that Aza causes the necrosis of rat hepatocytes through several pathways, including disabling the mitochondria and activating MAPKs (ERK, JNK, and p38 kinase). These effects could be reversed by NAC, a GSH precursor (Lawrence et al., 2002). The treatment of rat hepatocytes with Aza leads to necrotic cell death indicated by cytosolic GSH depletion followed by a decrease in metabolic activity (measured as an MTT reduction) and subsequent increase in LDH leakage. No changes were observed in the biochemical factors characteristic of apoptosis (Hauoui et al., 2000) such as cytochrome c release, procaspase-3 proteolysis, and DNA fragmentation. In programmed cell death (apoptosis), a complex is formed among cytochrome c, apoptotic protease-activating factor-1, and ATP or dATP (Cain et al., 2002), which activates procaspase-9; activated caspase-9, in turn, activates procaspase-3, leading to apoptosis. In the present experimental model, the intracellular ATP depletion induced by Aza (Lee and Farrell, 2001) and the lack of cytochrome c release from mitochondria precludes an apoptotic process. The bioener-
genetic failure provoked by Aza, among other factors, requires the induction of the MPTP. The treatment of liver-isolated mitochondria with Aza for short periods increases both mitochondrial swelling and the oxygen consumption rate in a CsA-sensitive manner, which suggests MPTP involvement in the Aza effect. However, the Aza response is independent of mitochondrial GSH pool depletion, which remains unchanged during drug treatment. Moreover, 6-mercaptopurine (an Aza-related compound without GSH depleting activity) showed a minor but significant effect on MPTP. All these data suggest that the purine moiety (present in both drugs) is able to bind to an MPTP domain, increasing the transition frequency between their open and close states, leading to transient mitochondrial swelling (Minamikawa et al., 1999). Transient opening of the MPTP seems to be its physiological mode of behavior, as demonstrated previously (Husser and Blatter, 1999; Petronilli et al., 1999). It is likely that transient MPTP opening does not induce the release of the markers of outer mitochondrial membrane integrity: cytochrome c, bel-2, and procaspase-3, but it could inhibit ATP synthesis. Long-term treatment of hepatocytes with Aza shows a dramatic effect on mitochondria, which is consistent with the necrotic state and is characterized by the appearance of giant mitochondria showing a loss of mitochondrial cristae and outer membrane disruption (Lee and Farrell, 2001). It has been suggested that ROS production by mitochondria could damage membranes and macromolecules at this level (Lee and Farrell, 2001), although there are no convincing results supporting this hypothesis. We suggest a minor contribution on behalf of ROS in Aza toxicity, given the discrete increase in the superoxide anion. Other ROS species (as hydroxyl radical) could be involved in Aza toxicity due to cytosolic GSH depletion. Furthermore, it has been established that Aza fails to increase thiorbituric acid-reactive substances (TBARS, as an indicator of lipid peroxidation) in vitro (Lee and Farrell, 2001). The discrete increase of superoxide anion and the absence of lipid peroxidation in this model could be due to the fact that GSH is not depleted completely, remaining 25% GSH after 12 h of drug treatment. Moreover, Aza does not change GSH pool in isolated mitochondria. In the same way, in microglial cells, NO produced by an inducible NO synthase isofrom was able to deplete cytosolic GSH pool without modifications in mitochondrial GSH (Roychowdhury et al., 2003). Also, in the same model, buthionine sulfoximine was able to decrease cytosolic GSH, whereas etachyrnic acid was able to deplete completely both mitochondrial and cytosolic GSH (Roychowdhury et al., 2003); Aza effect on cellular GSH could resemble buthionine sulfoximine and NO behavior. Moreover, in a rat in vivo model (Raza et al., 2003), Aza hepatotoxicity involves NO- and GSH-dependent mechanisms; it has not been found a good correlation between GSH depletion and TBARS production, because Aza plus NAC cotreatment produces a major increase in TBARS than Aza treatment alone. All these facts suggest an extramitochondrial origin for ROS induced by Aza because NAC promotes cytosolic drug metabolism. We must not forget that 6-mercaptopurine produced by the participation of GSH is oxidized by xanthine oxidase (XO), which could generate ROS and subsequently trigger mitochondrial swelling (Colell et al., 2004). Only high doses of cytosolic inhibitors such as allopurinol (an inhibitor of cytosolic XO) and Trolox (a water-soluble vitamin E analog) can partially reverse the Aza effect on the viability of rat hepatocytes (Lee and Farrell, 2001). Collectively, these data do not completely account for Aza toxicity. It has been recently suggested that cytosolic GSH and redox-sensitive proteins (thioredoxin and GST) could regulate cell death pathways by modulating the redox state of specific thiol residues of target proteins (stress kinases, transcription factors and caspases) (Fernández-Checa, 2003). GST-M1 has been shown to protect primary hepatocytes against transforming growth factor-β1-induced apoptosis by blocking ASK-1, a ubiquitous mitogen-activated protein kinase kinase kinase that mediates JNK and p38 kinase activation (Gilot et al., 2002). In addition, thioredoxin oxidation by GSSG formation promotes ASK-1 activation, which mediates apoptosis induced by TNF-α or hydrogen peroxide (Saitoh et al., 1998). At the cellular level, JNK is kept inactive through its association with GST-Pi, forming a GST-π-JNK complex.
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complex. GSSG formation provoked by cell insult gives rise to GST-Pi oligomerization and the release of active JNK (Adler et al., 1999). Thus, GSTs and reduced thioredoxin act as sensors of intracellular changes in the GSH/GSSG ratio; when GSH drops, SAPK (JNK and p38 kinase) is activated, leading to cell death. Consistent with this model, here we report that cytosolic GSH depletion induced by Aza causes JNK and p38 kinase activation, leading to hepatocyte death. The role of both kinases is demonstrated in our model, because the treatment of hepatocytes with specific inhibitors of JNK or p38 kinase induced by menadione (Czaja et al., 2003) or the TNF-α-induced death of RALA hepatocytes (Liu et al., 2002). Classically, ERK has been considered a cell survival factor (Ballif and Benlis, 2001), and, accordingly, its specific inhibition in hepatocytes does not protect the cell from the adverse effects of Aza. We therefore propose that ERK phosphorylation induced by Aza is a compensatory cell effect against its toxicity, a role suggested for menadione in rat hepatocytes (Czaja et al., 2003).

The role played by mitochondria in the Aza-induced activation of MAPK is unclear. ERK and p38 kinase phosphorylation induced by Aza was inhibited by CsA, suggesting that mitochondrial dysfunction could activate these pathways, whereas JNK was insensitive to CsA. Collectively, these data point to a mitochondrial activation pathway for ERK and p38 kinase, whereas JNK has no such mechanism. However, all MAPK (ERK/JNK/p38 kinase) could be sensitive to GSH levels, because MAPK phosphorylations induced by Aza were reversed by NAC. This demonstrates that by restoring GSH, the cells are completely protected from the cell death induced by Aza through both mitochondrial and cytosolic pathways.

Current data correlate well with our proposed Aza toxicity model toward rat hepatocytes and the hepatoprotection induced by NAC (Fig. 9). When Aza enters the cell, it is transformed into 6-MP by an unknown cytosolic isoform of GST using GSH as a cosubstrate, which depletes the cytosolic GSH pool. After this, the following metabolic pathways are proposed for 6-MP: 1) methylation by thiouine-methyltransferase to produce 6-methyl-mercaptopurine (Menor et al., 2001); 2) oxidation by XO to yield thiouric acid (Lewis and Oliiber, 1977); 3) “the salvage pathway”, whereby 6-MP would be incorporated into the intracellular nucleotide pool by the participation of hypoxanthine phosphoribosyl transferase (Aarbakke et al., 1997); or 4) interaction with MPTP. The Aza/6-MP detoxifying role is attributable to thiopurine-methyl-transferase and XO-catalyzed reactions, whereas the antimitic effect observed for both drugs corresponds to their incorporation into the 6-thioguanine nucleotide pool. Here, we show that the necrotic effect of Aza is produced through interaction with the MPTP and JNK and p38 kinase activation and that these negative effects are completely abolished by NAC. In our model, when cytosolic GSH is available (in the presence of NAC), SAPK are inhibited and Aza enters detoxifying and/or antimitotic pathways, without producing necrosis but inhibiting cell proliferation. In a GSH-depleting situation, however, Aza could not be metabolized as indicated above and would interact with mitochondria, producing a bioenergetic crisis caused by ATP depletion, activating the p38 kinase pathway, and contributing to the necrotic process. Moreover, the low levels of GSH would activate JNK, further enhancing the toxic effect of Aza. The restoration of GSH levels by NAC precludes Aza-MPTP interaction and inhibits JNK activation. Nevertheless, NAC does not block the antimitotic effect of Aza, which is a requirement of the immunosuppressant pharmacological effect.

We suggest the addition of NAC to the therapeutic regime of patients suffering hepatotoxicity due to azathioprine treatment could be of interest.

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References

Fig. 9. Model of azathioprine toxicity in hepatocytes. 1) Glutathione S-transferase; 2) xantine oxidase; 3) thiopurine-S-methyltransferase; and 4) hypoxantine phosphoribosyl transferase.


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