Effects of Caffeine on Pyrimidine Biosynthesis and 5-Phosphoribosyl 1-pyrophosphate Metabolism in Chinese Hamster Cells*

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Caffeine, at doses which enhance killing by UV light, inhibits the biosynthesis of pyrimidines in Chinese hamster ovary cells (K1) in culture. This inhibition was measured as a decrease in [3H]TTP and [3H]CTP accumulation after a 3-h incubation with [14C]aspartate or [14C]orotate and a similar decrease in Urd-A cells (which lack the first three enzymes of the pathway) using [14C]orotate as substrate. There was no such inhibition in Urd-C cells (which lack the last two enzymes) using [14C]aspartate as substrate and measuring accumulation of orotate. There is some inhibition of the fifth enzyme of the pathway, orotate phosphoribosyltransferase by caffeine in vitro and this is most striking at low 5-phosphoribosyl 1-pyrophosphate concentrations. The level of 5-phosphoribosyl 1-pyrophosphate is decreased in Chinese hamster ovary K1 cells by about 20% after 3 h and by about 70% after 16 h in the presence of caffeine.

It is suggested that inhibition of pyrimidine biosynthesis by caffeine over a 16-h period may be due mainly to decreased intracellular 5-phosphoribosyl 1-pyrophosphate levels but that in the decrease in pyrimidine accumulation over 3 h, direct inhibition of orotate phosphoribosyltransferase by caffeine may also play a role.

Caffeine (1,3,7-trimethyl-2,6-deoxypurine) has been shown to have a number of effects on DNA metabolism including enhancement of the damage caused by various types of radiation (1) and chemicals (2). These effects are generally attributed to the inhibition of cellular repair processes. The mechanisms of repair in mammalian cells are not well understood and little is known of how caffeine inhibits them. There are reports that caffeine binds to damaged DNA (3), inhibits DNA synthesis (4), inhibits cellular transport of certain molecules including precursors of both nucleic acids and proteins (6, 7), and interferes with the elongation of DNA in UV-irradiated cells (5).

This last observation may reflect the need for an adequate supply of nucleotide bases for efficient repair. Waldren and Patterson (8) found that caffeine had an inhibitory effect on

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purine biosynthesis and concluded that this inhibition was at an early step in de novo biosynthesis. They also found a decrease in the conversion of radiolabeled hypoxanthine, adenine, and guanine into their respective di- and triphosphates. They suggested that the substrate, 5-phosphoribosyl 1-pyrophosphate may be involved in this caffeine effect as it is the only substrate utilized in both the de novo biosynthesis and reutilization of purines.

If the effect of caffeine on purine biosynthesis is mediated through an effect on PRPP metabolism or utilization, there should also be an inhibition of pyrimidine biosynthesis since PRPP is (i) a positive allosteric effector of carbamoyl-phosphate synthetase (EC 6.3.5.5), the first enzyme in pyrimidine biosynthesis, and (ii) a substrate for orotate phosphoribosyltransferase (EC 2.4.2.10), the fifth step in the pathway. These two enzymes appear to be most important in the regulation of pyrimidine biosynthesis. Moreover, an inhibitory action of caffeine on pyrimidine biosynthesis would be further evidence that an insufficient supply of nucleotides may be significant in the inhibitory action of caffeine on DNA repair (23).

Therefore, we have investigated the effect of caffeine on pyrimidine biosynthesis and have also examined intracellular levels of PRPP during incubation of CHO-K1 cells with caffeine and the effect of caffeine upon PRPP synthetase in cell-free extracts.

MATERIALS AND METHODS

Medium—Stock cultures were maintained in medium F12 (9) supplemented with 10% fetal calf serum. This medium was supplemented with uridine (3 X 10⁻⁴ M) for the maintenance of uridine-requiring mutants.

Cells—The parental cell CHO-K1 and the uridine auxotrophs, Urd A and Urd C, have been fully described previously (10–12). Urd A has been identified as lacking the first three enzymes of pyrimidine biosynthesis, carbamoyl-phosphate synthetase (EC 6.3.5.5), aspartate transcarbamoylase (EC 2.4.2.1), and dihydro-orotate (EC 5.3.2.1), aspartate transcarbamoylase (EC 2.4.2.10) and OMP decarboxylase (EC 4.1.1.23), and there is some evidence that these also reside on a single polypeptide (15, 16).

Whole Cell Labeling—The possible effects of caffeine on pyrimidine biosynthesis were investigated using uridine auxotrophs defective in steps in the pathway. Using appropriate radiolabeled precursors and measuring end products accumulated by various mutants, it is possible to divide pyrimidine biosynthesis into early and late stages. Urd C lacks the last two enzymes of UMP biosynthesis. By incubating Urd C in the presence of [14C]aspartate, a substrate of aspartate transcarbamoylase, and caffeine and measuring the accumulation of orotate (Fig. 1), the effect of caffeine on the early part of the pathway can be assessed. Urd A lacks the first three enzymes. With [14C]orotate as substrate, effects on the last two enzymes of the pathway

1 The abbreviations used are: PRPP, 5-phosphoribosyl 1-pyrophosphate; CHO, Chinese hamster ovary.
can be examined by measuring UTP and CTP accumulation. These experiments were also carried out with wild type CHO-K1 cells, to see if the mutants and the parental cells have similar regulation and activity. The final concentrations of radioactive precursors used with each cell line were [U-14C]aspartic acid, 0.05 mM (200 mCi/mm01, New England Nuclear), [6-14C]orotic acid, 0.3 mM (50 mCi/mm01, New England Nuclear).

The cells were removed from plates with trypsin, washed 2 times with media, counted, and resuspended in incubation medium (F12 plus 5% fetal calf serum) without aspartate and asparagine, with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4) at 10^6 cells/assay and appropriate amounts of caffeine. The cells were preincubated for 15 min at 37 °C in a shaking water bath. Then the radioactive precursor was added to give a total incubation mix of 1 ml. The cells were incubated for 3 h. The mix was then transferred to centrifuge tubes and the cells were pelleted at 4 °C, washed 2 times with ice cold media, and extracted with 100 μl of 80% ethanol at room temperature for 30 min.

Debris was removed by centrifugation, and 20 μl of the extract spotted onto polyethyleneimine cellulose thin layer chromatography plates. For the separation of orotate, 0.5 M LiCl was used as the solvent for chromatography, and for the isolation of UTP and CTP, a formate buffer system, pH 3.4, was used. The chromatogram was developed in 0.5 μl buffer for 2 cm, 1 μl buffer for 7 cm, and 4 μl buffer for 16 cm.

Exposure to Cronex 4 x-ray film was used to visualize the appropriate radioactive spots which were cut out and the radioactivity counted in a liquid scintillation counter.

**Measurement of Orotate Phosphoribosyltransferase and OMP Decarboxylase**—The effect of caffeine on orotate phosphoribosyltransferase and OMP decarboxylase was measured in a crude cell-free extract.

CHO-K1 cells were removed from plates by trypsinization, washed 2 times with saline, counted, and resuspended in 60 ml Tris-HCl, pH 7.6, with 2 mM diithiothreitol at a concentration of 10^6 cells/ml (19). The cells were subjected to 3 cycles of freeze-thawing, the debris was removed by centrifugation, and the supernatant was used for enzyme assays.

Orotate phosphoribosyltransferase was measured in a 100-μl reaction mixture containing 27 mM Tris-HCl, pH 7.6, 0.9 mM dithiothreitol, 5 mM MgCl2, 0.1 mM [6-14C]orotic acid (46.9 mCi/mm01, New England Nuclear), and 25 μl of cell extract. The PRPP concentration was varied between 0.25 and 2 mM, and caffeine was added to a final concentration of either 10 or 20 mM.

The reaction mix was incubated for 15 min at 37 °C, the reaction was stopped with 200 μl of 95% ethanol, and the tubes were placed on ice. The debris was removed and 20 μl of the supernatant spotted onto a polyethyleneimine TLC plate. The plate was developed using 0.35 M LiCl as solvent.

Radioactive spots corresponding to OMP and UMP were located using Cronex 4 x-ray film and cut out, and the radioactivity was counted using a liquid scintillation counter. Since there was no accumulation of OMP which would suggest OMP decarboxylase inhibition, and since orotate phosphoribosyltransferase is considered the rate-limiting step of the fifth and sixth steps of pyrimidine biosynthesis (26), the radioactivity in the areas of the chromatogram corresponding to OMP and UMP was summed to give orotate phosphoribosyltransferase activity.

To verify that OMP decarboxylase was not inhibited by caffeine, the enzyme was assayed directly by the release of ^14CO2 from [1-^14C]orotic acid in the presence of caffeine (12).

**Measurement of Intracellular PRPP**—CHO-K1 cells were plated at a density of 5 × 10^3 cells/100-mm plates in medium F12 plus 10% fetal calf serum 16 h before the experiment. Caffeine was added to the plates (final concentration 15 mM), and they were placed in a standard incubator at 27 °C. After 1, 2, 3, 4, and 16 h, medium was removed from the plates (3 plates/time point). The cells were washed with Hank’s solution and the plates placed on dry ice. The resultant frozen cells were scraped off and volume made up to 300 μl PRPP was extracted according to the method of Gudas et al. (17). 80 μl of 1.5 M perchloric acid was added to the cell suspension, which was mixed for 30 s. Then the suspension was neutralized with 1.2 M KOH.

The extract was clarified by centrifugation at 10,000 × g for 5 min and the supernatant used for the determination of PRPP.

PRPP was measured by a modification of the method of May and Krotz (18). This measures the PRPP-dependent release of ^14CO2 from [1-^14C]orotic acid in the presence of yeast orotate phosphoribosyltransferase and OMP decarboxylase. In a volume of 200 μl, the reaction mix contained 100 mM Tris-HCl, pH 8.0, 12.5 mM MgCl2, 88 μM [1-14C]orotic acid (51.1 mCi/mm01), 0.2 unit of yeast orotate phosphoribosyltransferase and OMP decarboxylase (Sigma), and 100 μl of extract. The ^14CO2 produced was trapped by an absorbent (NCS, Amersham Corp.) held in a center well in a closed tube.

The reaction mix was incubated for 2 h, and the reaction was stopped by the addition of 66 μl of 2 N perchloric acid, with the tubes on ice. The mix was reincubated for 30 min at 37 °C to complete the absorption of liberated ^14CO2. The tubes were then placed on ice, and the center wells with NCS were removed, and the absorbed ^14CO2 was counted in a liquid scintillation counter.

In this assay, 1 nmol of PRPP corresponds to the release of 0.35 nmol of ^14CO2. 0.05 nmol of PRPP was readily detected with good reproducibility. PRPP synthetase was assayed by a modification of the method of Danks and Scholer (20).

**RESULTS**

Fig. 2 shows a decrease in the accumulation of UTP and CTP in the presence of caffeine in CHO-K1 cells. This decrease reaches a maximum at a caffeine concentration of 5 mM and is cumulative over a period of 3 h (data not shown). This decrease is seen using either [U-14C]aspartate or [6-14C]orotic acid as substrate. Therefore, caffeine does inhibit pyrimidine biosynthesis and the inhibition seen using [6-14C]orotic acid as substrate suggests that the block is at the fifth or sixth step in the pathway, orotate phosphoribosyltransferase or OMP decarboxylase.

Urd-C, which lacks orotate phosphoribosyltransferase and OMP decarboxylase, shows no inhibition of orotate accumulation with [U-14C]aspartate or [6-14C]orotic acid as substrate; in fact, there is a marked increase in orotate accumulation with exposure to caffeine (Fig. 3).

Urd A, which lacks the first 3 enzyme activities in pyrimidine biosynthesis, shows a decrease in UTP and CTP accumulation with [6-14C]orotic acid as substrate (Fig. 4). This observation, coupled with the observation that orotate accumulation is not inhibited by caffeine in Urd-C cells, is strong support for the hypothesis that caffeine inhibits the last part of de novo pyrimidine synthesis.

Measurement of the effects of caffeine on the fifth and sixth steps of the pathway showed no accumulation of OMP and...
Effects of Caffeine on Pyrimidine Biosynthesis and PRPP

FIG. 2. Effect of caffeine on [14C]UTP and [14C]CTP accumulation in CHO-K1. Substrates used were [6-14C]orotate (●) and [U-14C]aspartate (○). 100% accumulation of label into UTP and CTP in 3 h was 1500 cpm with [6-14C]orotate and 500 cpm with [U-14C]aspartate.

FIG. 3. Effect of caffeine on 14C-orotate accumulation in Urd-C. Substrate used was [U-14C]aspartate. 100% accumulation of label into orotate was 1400 cpm.

FIG. 4. Effect of caffeine on [14C]UTP and [14C]CTP accumulation in Urd-A. Substrate used was [6-14C]orotate. 100% accumulation of label into orotate was 1400 cpm.

that caffeine had no effect on OMP decarboxylase (data not shown) in a cell-free extract. Caffeine inhibits orotate phosphoribosyltransferase but this inhibition is only striking at low PRPP concentrations (Fig. 5). At 0.2 mM PRPP, the approximate concentration of PRPP in CHO-K1, the inhibition is 54%. Although conclusions from kinetic data on crude extracts must be tentative, it appears that caffeine may have a direct effect on orotate phosphoribosyltransferase at low PRPP levels. During a 3-h exposure to caffeine, intracellular PRPP levels decreased by 20-30% in CHO-K1 cells. Over a 16-h period, however, this decrease in PRPP levels reached about 70% (Fig. 6). Generally, caffeine is added for 16 h in experiments, assessing its inhibitory effects on DNA repair in CHO-K1 cells (7, 8).

Even at 40 mM, caffeine only inhibits PRPP synthetase 10–20% (data not shown). This is probably not a sufficient effect to account for the decrease in intracellular PRPP. So the supply of substrates for PRPP synthesis may be a site of caffeine action on PRPP metabolism.

DISCUSSION

These experiments demonstrate that caffeine has an inhibitory effect on pyrimidine biosynthesis in CHO-K1 cells. This, together with the observations of Waldren and Patterson in 1979 (8), suggests that caffeine can markedly curtail the availability of purine and pyrimidine nucleotides at concentrations which enhance UV killing in these cells. It seems reasonable that this decrease in nucleotide availability may well be important in the inhibition of DNA repair which is considered to be a major effect of caffeine. As well as accentuating damaging effects on DNA of agents such as UV and chemicals, caffeine may change the balance that exists between DNA damage and repair, which could be important in the long term, in the initiation and development of degenerative processes.

Waldren and Patterson (8) suggested that alterations in PRPP metabolism may be involved in the mechanism of action of caffeine since caffeine affects both the de novo biosynthesis and reutilization of purines and PRPP is the only substrate utilized in both of these processes. This study shows that intracellular PRPP levels are significantly reduced after a 16-h exposure to caffeine (Fig. 6), an observation consistent

\[ \text{H. Kato, unpublished observations.} \]
with this suggestion. Therefore, the inhibition of pyrimidine biosynthesis by caffeine over a 16-h period may be due mainly to decreased intracellular PRPP levels. Similarly, the previously reported (8) inhibition of purine nucleotide synthesis may also be due to the caffeine-induced decrease in intracellular PRPP levels.

It is likely that interference with metabolism of a molecule like PRPP which plays such an important role in cellular metabolism would have serious and varied consequences such as those observed after treatment of cells with caffeine. However, it seems unlikely that all of the effects of caffeine on cells can be explained by interference in PRPP metabolism. For example, it seems unlikely that the decrease in pyrimidine accumulation over the first 3 h is due solely to this effect and a direct inhibition of orotate phosphoribosyltransferase, the fifth enzyme in pyrimidine biosynthesis by caffeine, may also play a role. Thus, caution should be used in attributing other effects of caffeine on cells to interference in PRPP metabolism without direct experimental verification.

There is evidence that some of the toxic effects of adenosine on mammalian cells may be similar to those caused by caffeine since the effects of adenosine are due, at least in part, to the inhibition of pyrimidine biosynthesis, and since the site of inhibition is either orotate phosphoribosyltransferase or OMP decarboxylase. This inhibition is accompanied by a decrease in PRPP concentration (17). This could be due to: (i) the inhibitory effect of AMP on PRPP synthetase (24), and (ii) in part to a decrease in synthesis of ribose 5'-phosphate, a substrate of PRPP synthetase (25). Caffeine itself does not inhibit PRPP synthetase to a significant extent (data not shown). The effect of caffeine on the biosynthesis of ribose 5'-phosphate has not yet been examined.

Before a precise mechanism of action for all of the effects of caffeine can be formulated, information is required on the cellular metabolism of caffeine. There is evidence that, in cultured Chinese hamster V79 cells, caffeine is not metabolized to any detectable extent at concentrations of up to 5 mM (8, 21, 27). In preliminary studies using the techniques of Tu et al. (27), we have demonstrated metabolism of caffeine by rat liver cells but none by CHO-K1 cells. Thus, the effects of caffeine on these hamster cells are likely to be due to the action of caffeine itself. However, there has been a report of nucleic acid bases produced from caffeine in certain mouse and human cell lines (22). Rat hepatocytes clearly do metabolize caffeine (21, 27). Thus, the effects of caffeine on different cell types may be different depending on the ability of each cell type to metabolize caffeine. Therefore, it becomes important to assess this possibility in each cell type studied. Moreover, the findings presented here and previously (8) predict that treatment of CHO-K1 cells with caffeine should cause detectable alterations in cellular nucleotide metabolism. Such studies are currently in progress.

REFERENCES