Inhibition of caffeine biosynthesis in tea (*Camellia sinensis*) and coffee (*Coffea arabica*) plants by ribavirin

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Received 3 September 2003; revised 30 September 2003; accepted 14 October 2003

First published online 27 October 2003

Edited by Marc Van Montagu

Abstract The effects of ribavirin, an inhibitor of inosine-5′-monophosphate (IMP) dehydrogenase, on [8-^{14}C]inosine metabolism in tea leaves, coffee leaves and coffee fruits were investigated. Incorporation of radioactivity from [8-^{14}C]inosine into purine alkaloids, such as theobromine and caffeine, guanine residues of RNA, and CO_2 was reduced by ribavirin, while incorporation into nucleotides, including IMP and adenine residues of RNA, was increased. The results indicate that inhibition of IMP dehydrogenase by ribavirin inhibits both caffeine and guanine nucleotide biosynthesis in caffeine-forming plants. The use of IMP dehydrogenase-deficient plants as a potential source of good quality caffeine-deficient tea and coffee plants is discussed.

Key words: Coffee; Caffeine; Inosine; Purine alkaloid; Ribavirin

1. Introduction

Inosine-5′-monophosphate (IMP) dehydrogenase (IMPDH, EC 1.1.1.205) is an enzyme which catalyses the NAD^+-dependent conversion of IMP to xanthosine-5-monophosphate (XMP) at the metabolic branch point in the de novo purine nucleotide biosynthetic pathway [1–3]. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a potent inhibitor of IMPDH, is phosphorylated by cellular enzymes, such as adenosine kinase [4], forming ribavirin-monophosphate (RMP), and this compound inhibits IMP dehydrogenase at the IMP–XMP ligand site [4–7]. The structures of ribavirin, RMP, IMP and XMP are shown in Fig. 1. In mammals, IMPDH increases significantly in cancer cells and is therefore considered a sensitive target for cancer chemotherapy and IMPDH inhibitors are used as anti-tumour agents [8]. Ribavirin is also widely used as an anti-viral agent [6,7,9], but there are no reports of its effects on metabolism in plant tissues. In purine alkaloid-forming plants, IMP is a precursor for AMP, GMP and caffeine biosynthesis [10]. The major route for caffeine biosynthesis in coffee and tea plants is an IMP → XMP → xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway (Fig. 2) [1,11]. IMP may be produced from (i) adenosine released from the *S*-adenosyl-L-methionine cycle [12] and/or (ii) purine nucleotide biosynthesis de novo [13]. Since IMP appears to synergistically improve the flavour of amino acid-based taste in humans [14], Koshiishi et al. [15] recently suggested that blocking IMPDH and enhancing IMP levels may be a key step to produce a caffeine-deficient tea with enhanced flavour.

In the present study, we first examined caffeine biosynthesis from [8-^{14}C]inosine in tea and coffee plants. Inosine was efficiently taken up by the tissues and used for both caffeine and RNA synthesis in tea and coffee. Using this experimental system, we investigated the effect of ribavirin on purine metabolism in caffeine-producing plant tissues. The data obtained indicate that IMPDH catalyses a crucial step in the caffeine biosynthesis pathway.

2. Materials and methods

2.1. Plant materials

Flash shoots of tea (*Camellia sinensis*) were obtained from the Tokyo Metropolitan Agricultural Experimental Station, Tachikawa, Tokyo, Japan. The most recently emerged young leaves (30 mm in length, ca. 40 mg fresh weight) were used for the experiments. Fresh leaves of coffee (*Coffea arabica*) (40 mm in length, 100 mg fresh weight) were collected from plants grown at Ochanomizu University, Tokyo, Japan. Immature arabica coffee fruits, cv. Guatemalan typica (4 mm in length, ca. 40 mg fresh weight), were obtained from the Kunia Station, Hawaii Agricultural Research Center, USA.

2.2. Administration of [8-^{14}C]inosine

Segments of samples (c. 100 mg fresh wt) were placed in the main compartment of a 30 ml Erlenmeyer flask that contained 2 ml of incubation medium comprising 30 mM potassium phosphate buffer (pH 5.6), 10 mM sucrose, and 500 μM ribavirin (Sigma, St. Louis, MO, USA). For control incubations, ribavirin was omitted. A glass tube containing a piece of filter paper impregnated with 0.1 ml of 20% KOH was fitted into the centre well of the flask. After a 1 h preincubation of samples in an oscillating water bath operated at 100 strokes/min at 27°C, 9 μM [8-^{14}C]inosine (1.96 MBq/μmol, Moravek Biochemicals, Brea, CA, USA) was added to the incubation medium in each flask. The preliminary time-course experiments demonstrated that an incubation time of 18 h with tea and coffee samples resulted in significant incorporation of label into purine alkaloids and was therefore suitable for studying the biosynthesis of caffeine. Thus, data shown in this paper were obtained with 18 h incubations.

2.3. Analysis of ^{14}C metabolites

At the completion of the incubation period, the filter paper was removed from a centre well and placed in a 50 ml flask that contained 10 ml distilled water. $KH^{14}CO_3$ absorbed by the filter paper during the incubation was allowed to diffuse into the distilled water overnight and aliquots of the resultant solution (0.5 ml) were used to estimate the amount of $^{14}CO_2$ released by the plant tissues. The radioactivity

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0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)01213-4

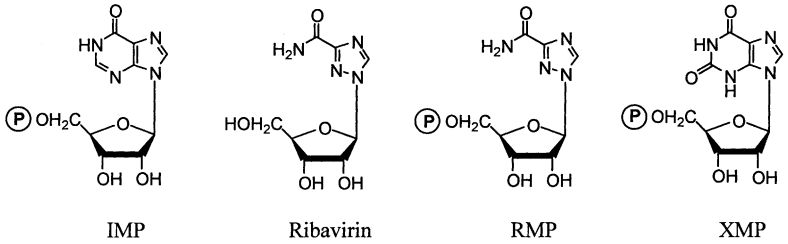

Fig. 1. Structures of IMP, ribavirin, RMP and XMP.

was measured with a liquid scintillation spectrometer. Leaf and fruit segments were separated from the incubation medium by filtering through a tea strainer, washed with distilled water, frozen in liquid N_2 and stored at −80°C prior to extraction. Cellular metabolites of frozen tissues were extracted with cold 6% perchloric acid (PCA). The tissue homogenate was centrifuged at 12 000×*g* for 10 min and the supernatant and pellet were separated. The pellet was re-suspended in 3 ml of 6% PCA and re-centrifuged. After two extractions, the supernatant fractions containing the PCA-soluble metabolites were combined to yield a total volume of c. 6 ml. The PCA fraction was neutralised by 20% KOH. After centrifugation, radioactivity in aliquots, typically 0.1 ml of the supernatant, was measured with a liquid scintillation counter. The neutralised PCA-soluble fraction was frozen and reduced to dryness in a lyophiliser. The PCA-insoluble fraction was washed twice with diethylether:ethanol (1:1, v/v) for 15 min at 50°C to remove lipids, and then dissolved in 6% PCA (4 ml). Nucleic acids were hydrolysed at 100°C for 15 min. After centrifugation at 12 000×*g* for 5 min, this 'hot PCA' fraction containing purine nucleobases derived from RNA was neutralised and re-centrifuged after which a 0.1 ml aliquot of supernatant was taken for estimation of radioactivity by liquid scintillation counting. The remainder of the supernatant was lyophilised. Both the freeze-dried PCA-soluble cellular metabolites and 'hot PCA-soluble' nucleobases derived from RNA were dissolved in small amounts of 50% ethanol prior to analysis by thin layer chromatography (TLC) on 20×20 cm cellulose sheet (Merck, Darmstadt, Germany) using solvents of *n*-BuOH/OHAc/H_2O (4:1:2, v/v) and *n*-BuOH/MeOH/H_2O/NH_3 (60:20:20:1, v/v). Radioactivity of ^{14}C on the TLC sheet was determined using a Bio-Imaging Analyser (Type, FLA-2000, Fuji Photo Film Co., Tokyo, Japan).

3. Results and discussion

3.1. Tea leaves

In young tea leaves large amounts of caffeine are produced [16,17] and in the present study more than 60% of the radioactivity from [8-^{14}C]inosine taken up by the leaf segments was incorporated into the purine alkaloids theobromine (12.9%) and caffeine (47.2%) after an 18 h incubation (Fig. 2). The remaining radioactivity was associated with nucleotides (6.1%), adenine (4.0%), guanine residues of RNA (6.3%) and CO_2 (15%), and to a lesser extent the purine degradation products hypoxanthine, xanthine and allantoic acid. These results suggest that most of [8-^{14}C]inosine was first converted to [8-^{14}C]IMP by inosine kinase (EC 2.7.1.73) and/or non-specific nucleoside phosphotransferase (EC 2.7.1.17). The latter enzyme has been detected in tea leaf extracts [18]. In young tea leaves, IMP is converted primarily to XMP by IMPDH, an enzyme which is also found in tea leaves [10], and then enters the main route of caffeine biosynthesis via xanthosine and 7-methylxanthosine (Fig. 2). The remainder of IMP was converted to AMP or GMP, and phosphorylated to ATP or GTP. The resultant purine nucleoside triphosphates were utilised for RNA synthesis. Inosine is unlikely to be converted directly to xanthosine because tea leaves do not contain inosine dehydrogenase activity [18].

Ribavirin significantly reduced the incorporation of radioactivity from [8-^{14}C]inosine into theobromine, caffeine, and guanine residues of RNA while there was an increase in ^{14}C associated with nucleotides, inosine, xanthine, allantoic acid and adenine residues of RNA (Fig. 3). It is noteworthy that in ribavirin-treated tea leaves, 5% of the total radioactivity was associated with IMP, while there was no detectable incorporation in control leaves.

Major metabolic changes induced by ribavirin, such as de-

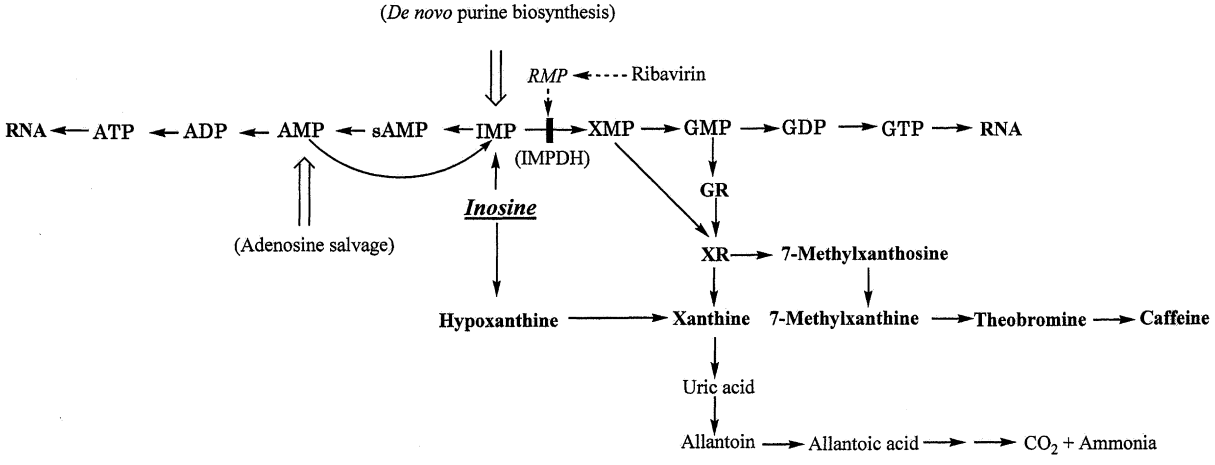

Fig. 2. Possible metabolic fate of inosine in tea and coffee plants in the absence and presence of ribavirin. sAMP, adenylosuccinate. Conversion of ribavirin to RMP and its inhibition site is shown in dotted arrows.

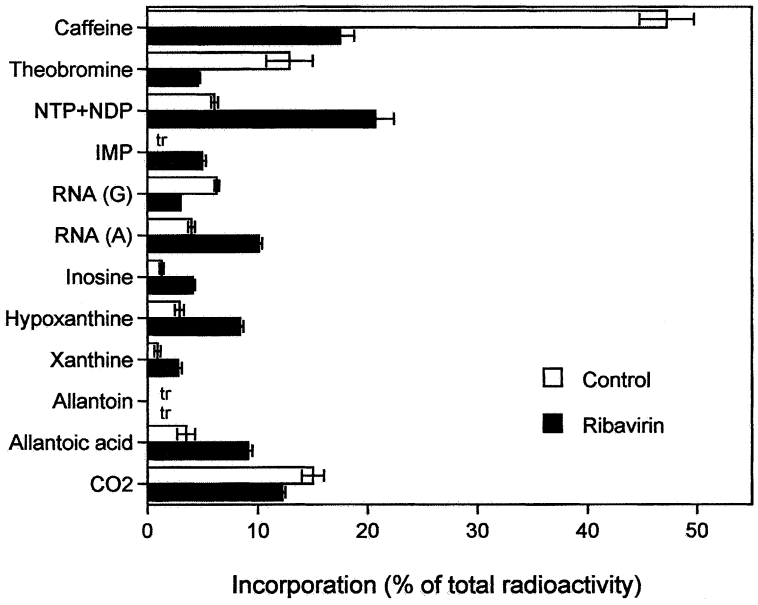

Fig. 3. Metabolic fate of 9 μM [8-^{14}C]inosine (1.96 GBq/mmol) in tea leaves incubated for 18 h with and without ribavirin. Incorporation of radioactivity into individual metabolites is expressed as a percentage of total radioactivity taken up by the samples ± S.D. ($n = 3$). Total uptake of radioactivity by the control and ribavirin-treated tissues was 6.5 ± 0.3 and 5.2 ± 0.4 kBq/100 mg fresh weight, respectively. tr, trace.

creased radioactivity in guanine nucleotides and caffeine and increased incorporation into IMP, can be explained by an inhibition of IMPDH activity. Guanine nucleotides and caffeine are derived from XMP which is produced from IMP in a reaction catalysed by IMPDH. IMP is located at the branch point of the pathways for AMP and GMP synthesis. Thus, metabolism may shift to AMP synthesis if IMPDH activity is inhibited by ribavirin (Fig. 2) and, in turn, this may result in increased incorporation of [^{14}C]ATP into RNA. In practice, however, incorporation of radioactivity from [8-^{14}C]inosine into the total RNA fraction was higher in ribavirin-treated tissues. This suggests that the IMPDH inhibitor brought about a partial rather than a complete depletion of the guanylate (GMP, GDP, GTP and dGTP) pools in tea leaves.

Increased incorporation of radioactivity into hypoxanthine, xanthine and allantoic acid in ribavirin-treated leaves may be due to enhanced inosine catabolism. Enhanced IMP catabolism may accompany the increased synthesis of ATP and IMP. As shown in other species, AMP deaminase (EC 3.5.4.6) may be activated by the increase in ATP [19,20], and the near-equilibrium reaction catalysed by nucleoside phosphotransferase may shift from IMP to the formation of inosine, and as a result, catabolism of AMP via IMP and inosine may be stimulated.

3.2. Coffee leaves and fruits

The effect of ribavirin on [8-^{14}C]inosine metabolism in coffee leaves was examined (Fig. 4A). Approximately 20% and 13% of the total radioactivity from [8-^{14}C]inosine taken up by the control leaf segments was incorporated into theobromine and caffeine, respectively. Compared with young tea leaves, biosynthesis of caffeine, especially in the final step, was slower in coffee leaves. This trend has been observed in previous studies [13,21]. In coffee leaves, incorporation of radioactivity from [8-^{14}C]inosine into RNA (21%) is c. two-fold higher than in tea leaves (10%), and more than 10% of total radioactivity was associated with CO_2. The metabolic changes induced by ribavirin in coffee leaves are similar to those observed in tea leaves. It is unclear why $^{14}CO_2$ release from [8-^{14}C]inosine was inhibited by ribavirin. One possible explanation is that, in addition to IMPDH, ribavirin may inhibit the activity of purine catabolism enzyme(s).

The metabolism of [8-^{14}C]inosine by young small coffee fruits is summarised in Fig. 4B. Here, 44% of total radioactivity was incorporated into purine alkaloids, and 28% into RNA. Thus, inosine salvage activity is extremely high in young coffee fruits. In contrast, [8-^{14}C]inosine is degraded to $^{14}CO_2$ much more extensively by other plant species. For example, 96% of the radioactivity from [8-^{14}C]inosine was released as $^{14}CO_2$ in white spruce cells during an 18 h incubation [22,23].

Ribavirin treatment of coffee fruit inhibited the incorporation of radioactivity into theobromine, caffeine, guanine residues of RNA and CO_2, and increased radioactivity associated with IMP, other nucleotides, mainly nucleoside triphosphate, xanthine, adenine residues of RNA and allantoic acid. These trends are similar to those observed with tea leaves.

3.3. Concluding remarks

The results obtained in this study suggest that blocking of IMPDH activity with ribavirin inhibited caffeine biosynthesis. By blocking the conversion of IMP to XMP, ribavirin may lead to a decrease of the free guanylate pools, but a concomitant inhibition of nucleic acid synthesis was not observed. Two isoforms of human IMPDH, designated type I and type II, have been identified and the nucleotide sequence of genes is known. Type I is constitutively expressed and is the predominant isoform in normal cells, while type II is selectively up-regulated in neoplastic and replicating cells [8]. No isoforms of plant IMPDH have been reported, but seasonal variation of the activity of IMPDH in young tea leaves indicated that caffeine-forming leaves collected in April had 23

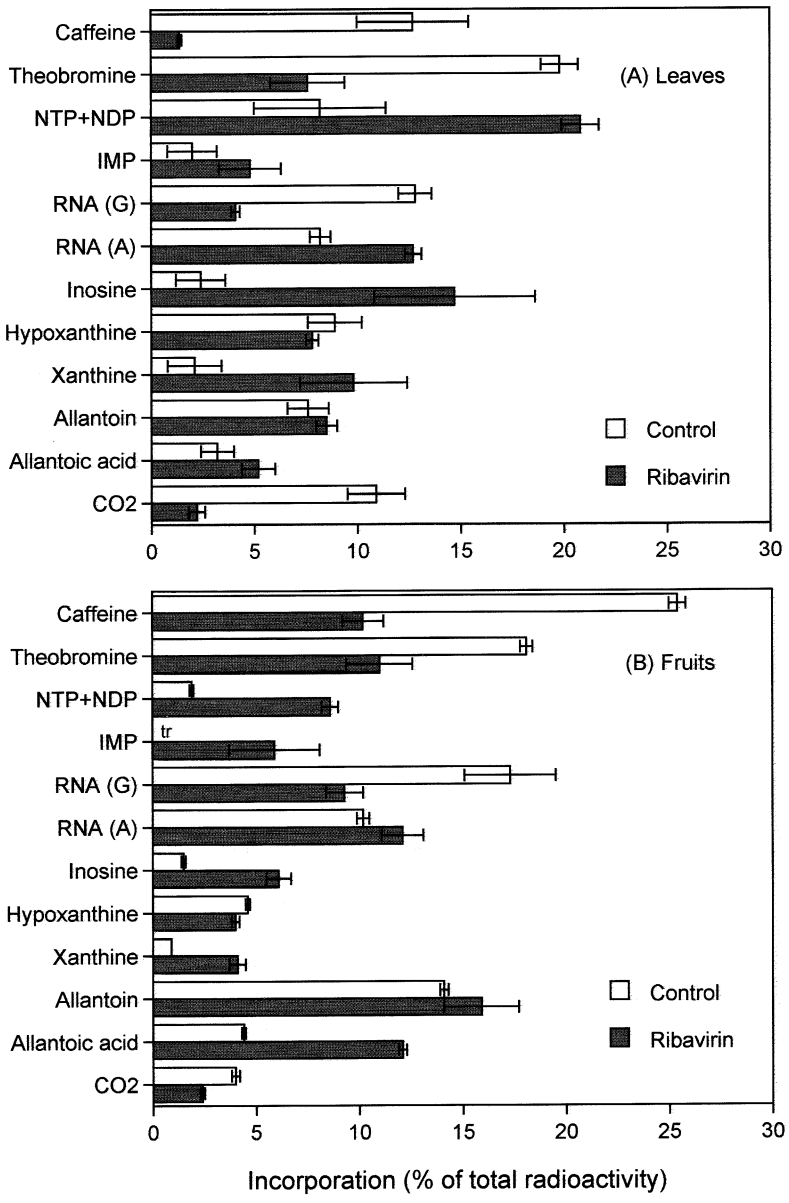

Fig. 4. Metabolic fate of 9 μM [8-^{14}C]inosine (1.96 GBq/mmol) in coffee leaves (A) and young coffee fruits (B) incubated for 18 h with and without ribavirin. Incorporation of radioactivity into individual metabolites is expressed as a percentage of total radioactivity taken up by the samples ± S.D. ($n = 3$). Total uptake of radioactivity by the control and ribavirin-treated tissues was (A) 6.6 ± 1.1 and 2.7 ± 0.5 kBq/100 mg fresh weight and (B) 9.6 ± 1.1 and 7.0 ± 0.1 kBq/100 mg fresh weight, respectively. tr, trace.

times higher activity (320 pkat/g fresh weight) than older non-caffeine-forming leaves of the same tree collected in December (14 pkat/g fresh weight). Thus, it is possible that an IMPDH isoform related to caffeine biosynthesis may be present in young tea leaves.

Recently, genes encoding *N*-methyltransferases for caffeine biosynthesis have been cloned [24–27] and transgenic caffeine-deficient coffee plants were made [28]. Suppression of IMPDH gene expression may be an alternative route for the production of decaffeinated transgenic tea and coffee plants. Koshishi et al. [15] have shown that nucleotide profiles of tea leaves are similar to those of other plant species. It is currently believed that the tea-specific amino acid, theanine, is an important factor contributing to umami taste. IMP may also participate as it has been shown that nucleotide seasonings interact synergistically with amino acid-based taste. Nucleoside tri- and diphosphates in fresh tea leaves may be converted to IMP during commercial processing [15]. If so, metabolic engineering to accumulate IMP and related nucleotides may be of value. The data presented in this report indicate that caffeine synthesis will be reduced and free purine nucleotides, including IMP, may accumulate when IMPDH activity is blocked. In the circumstances, transgenic tea plants with reduced IMPDH activity offer the intriguing prospect of yielding a beverage with a low caffeine content coupled with enhanced flavour quality.

Acknowledgements: We thank Dr Yuko Yoshida (Tokyo Metropolitan Agricultural Experimental Station) and Dr Chifumi Nagai (Hawaii Agricultural Research Center) for their generous gifts of plant materials.

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