SUPPLEMENT

DOWNREGULATION OF HEPATIC UREA SYNTHESIS BY OXYPURINES: XANTHINE AND URIC ACID INHIBIT HEPATIC N-ACETYLGLUTAMATE SYNTHASE

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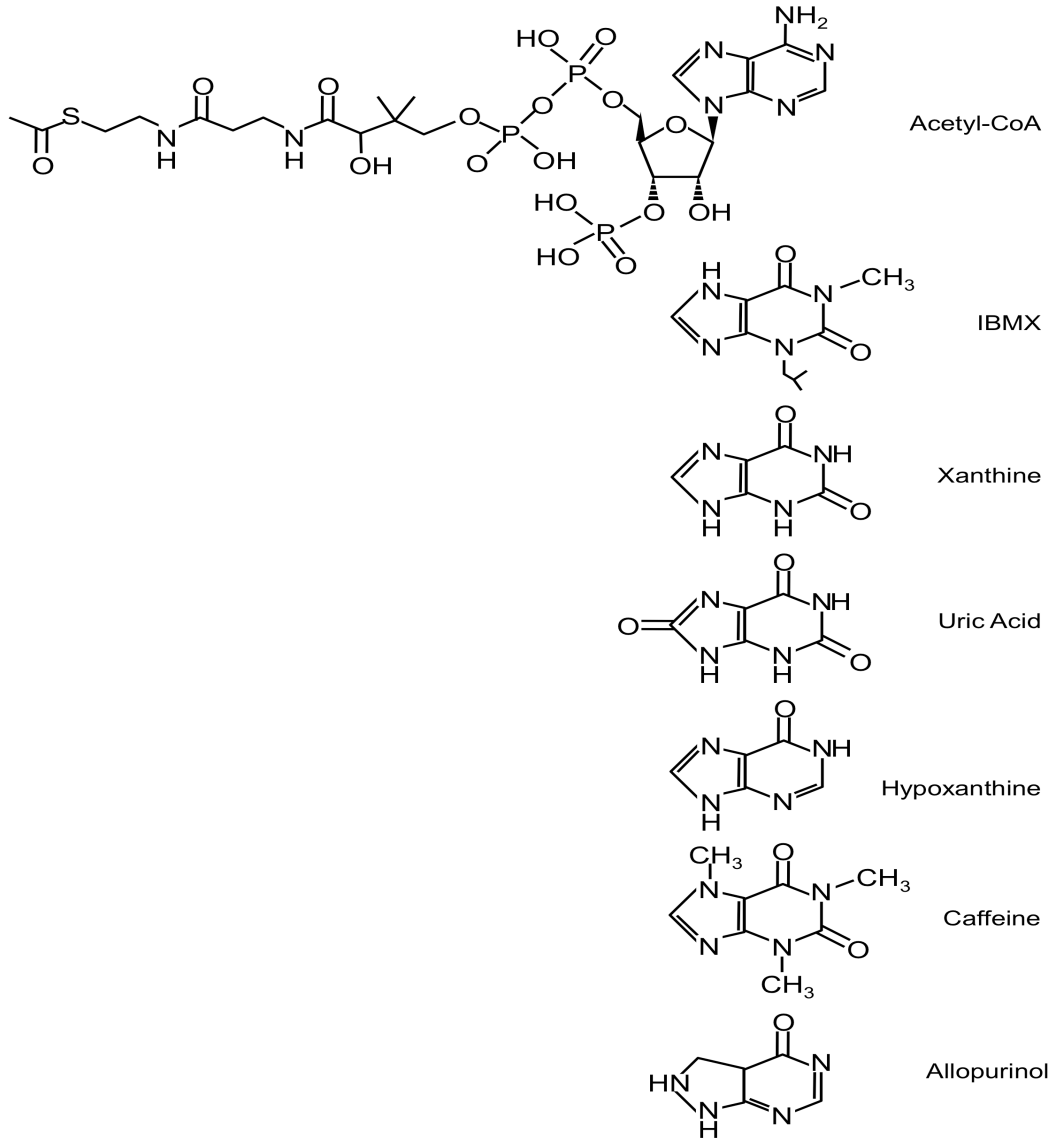
Figure 1S:

The chemical structure of acetyl-CoA, IBMX and other oxypurines tested for action on* N-acetylglutamate synthetase *activity: These oxypurines are selected as a representative of those physiologically present (xanthine, uric acid and hypoxanthine), drugs (allopurinol) or diet (caffeine).



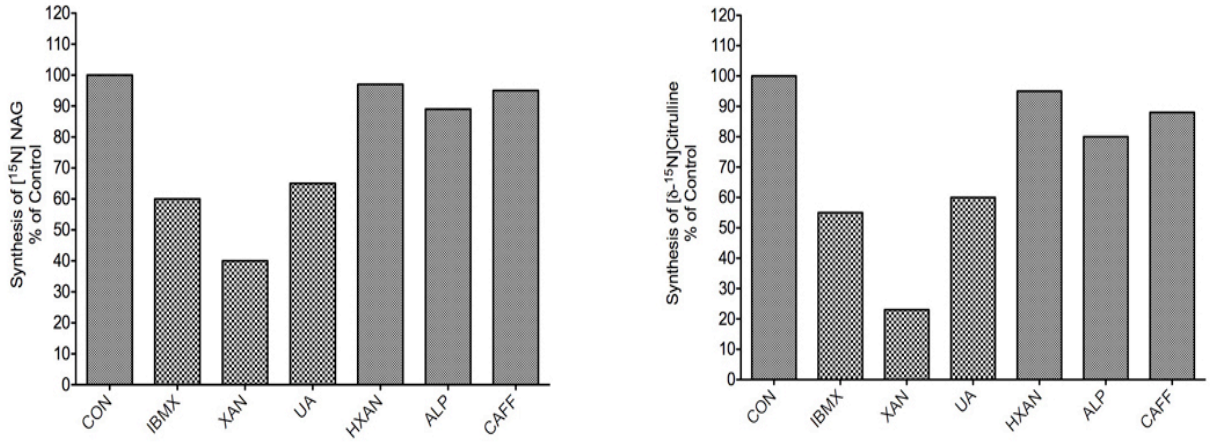


Figure 2S: ***The effect of selected oxypurines on N-acetyglutamate and citrulline synthesis:*** Experiments were carried out with mitochondrial lysates (2 mg protein) incubated for 20 minutes with medium containing (mM): Tris (50), EDTA (1), KCl (5), $MgCl_2$ (5), $KHCO_3$ (15) and KH_2PO_4 (5), pH 7.4, ornithine (10), MgATP (5), $^{15}NH_4Cl$ (1), [^{15}N]glutamate (10), ACoA (0.5) and either 2 mM IBMX (dissolved in DMSO), 1 mM xanthine (XAN), hypoxanthine (HXAN), uric acid (UA), allopurinol (ALP) or caffeine (CAFF). An equal amount of DMSO was also added to incubations without IBMX. The histograms are means of two independent experiments. The average 100% (CON) value for [^{15}N]NAG synthesis was about 4.5 nmol/mg and for [δ-^{15}N]citrulline synthesis was about 30 nmol/mg mitochondrial protein.

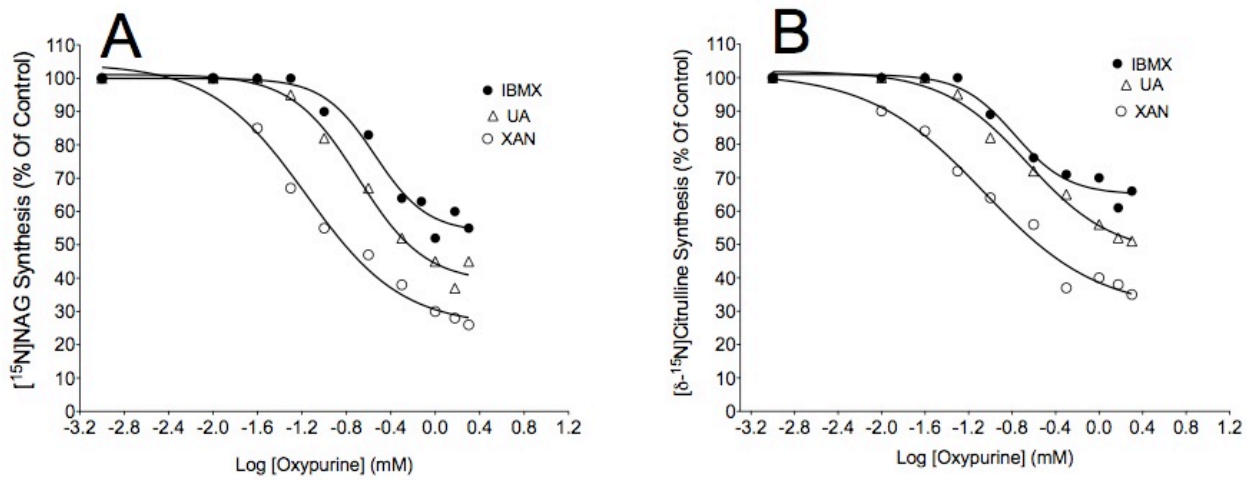


Figure 3S: ***Percent inhibition of ^{15}N-labeled N-acetyglutamate and citrulline synthesis versus Log [oxypurine]:*** Experiments were carried out with mitochondrial lysates (2 mg protein.ml^{-1}), incubated for 20 minutes with basic medium consisting of (mM): Tris (50), EDTA (1), KCl (5), $MgCl_2$ (5), $KHCO_3$ (15) and KH_2PO_4 (5), pH 7.4, plus (mM): MgATP (5), ornithine (5), acetyl-CoA (0.5), [^{15}N]glutamate (10), $^{15}NH_4Cl$ (1), 5% DMSO and increasing concentrations of oxypurine as indicated. ***Panel A*** represents the inhibition of NAGS activity as determined by synthesis of [^{15}N]NAG. ***Panel B*** represents the inhibition of [δ-^{15}N]citrulline synthesis as a proxy for flux through CPS1. The lines are the best fit to a sigmoidal dose-response obtained by Prism-5 software. These curves were used to determine the relative Inhibition Constant (IC_{50}) for inhibition of the flux through CPS1 or NAGS by the indicated oxypurine. The mean 100% value for [^{15}N]NAG synthesis was about 4.12 ± 0.12 nmol/mg, and for [δ-^{15}N]citrulline synthesis was about 25 ± 8 nmol/mg. Data points are mean of 3-4 independent experiments.

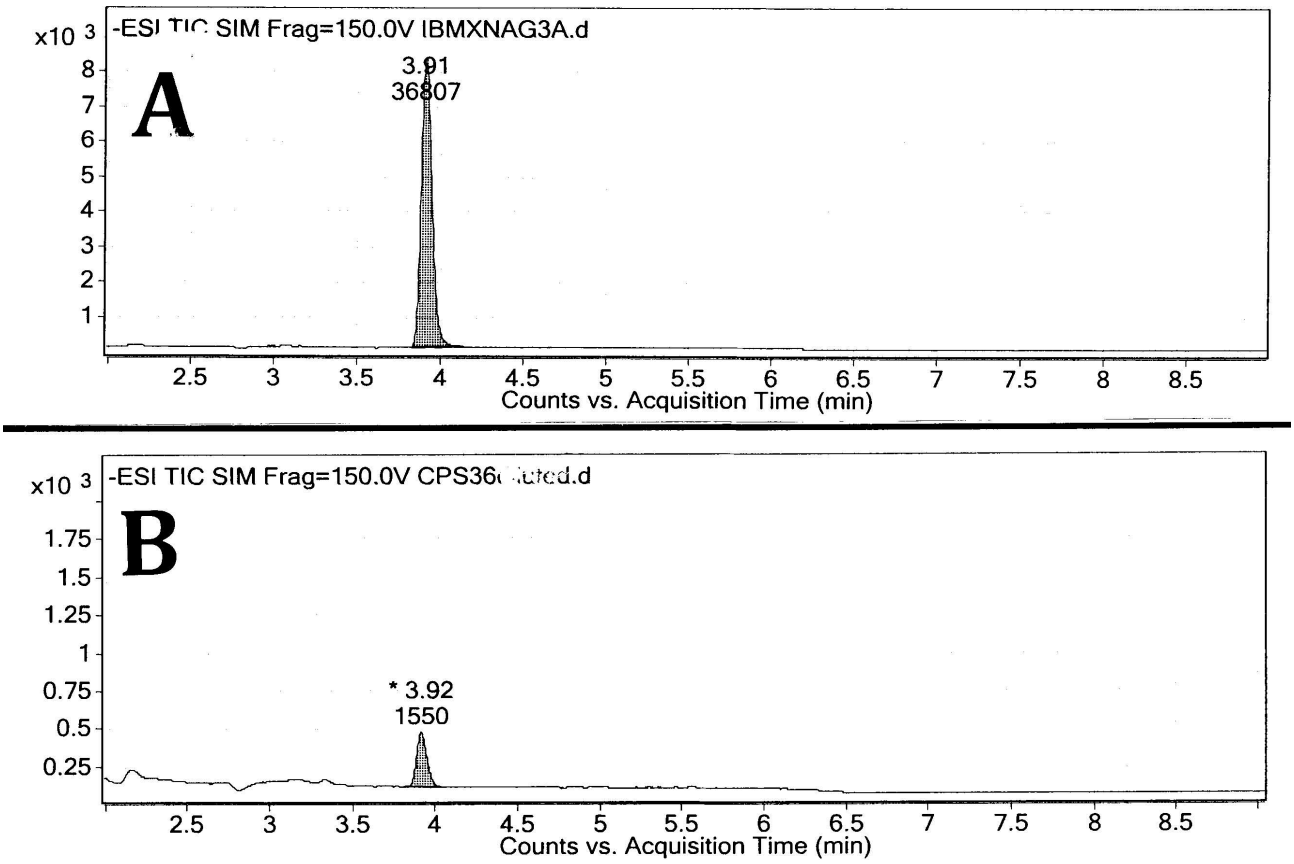


Figure 4S*: *Liquid Chromatography-Mass Spectrometry measurement of IBMX removed from mitochondria preincubated with 2 mM IBMX. Intact mitochondria were preincubated for 20 minutes at room temperature with medium consisting of (mM): Tris (50), EDTA (1), KCl (5), $MgCl_2$ (5), $KHCO_3$ (15) and KH_2PO_4 (5) and IBMX (2 mM) dissolved in 5% DMSO. Thereafter, mitochondria were separated from incubation medium by centrifugation (6000xg for 15 min), and mitochondrial pellets were washed with isolation medium containing 5% DMSO by a second round of centrifugation. The supernatant fraction was diluted 1:100 in 5% DMSO for LC-MS analysis. A lysate was prepared from mitochondrial pellets and diluted 1:100 in 5% DMSO for LC-MS analysis. ***Panel A*** represents the LC-MS chromatogram of IBMX that was removed from intact mitochondrial preincubated with 2 mM IBMX. 2 μl of the supernatant fraction (indicated above) was injected into LC-MS system. The area of IBMX peak was 36807 (arbitrary units). ***Panel B*** represents the LC-MS chromatogram of mitochondrial lysate. 2 μl of the diluted mitochondrial lysate (indicated above) was injected into LC-MS system. The area under the peak of IBMX in Panel B is about 3% of the area under the peak of panel A.

This chromatogram of IBMX was obtained by an Agilent 1290 Infinity liquid chromatography system combined with Agilent 6410 Triple-Quad mass spectrometer with ESI ionization source. The column used is Agilent Zorbax Poroshell 120 EC-C18 50 mm x 4.6 mm 2.7–Micron. The mobile phase was composed of solution A (water with 0.1% formic acid) and solution B (acetonitrile with 0.1% formic acid and 0.01% TFA). Both solutions are LC-MS grade. The separation Gradient used: 0 min 0 % B, 1 min 25% B, 2 min 50%B, 4 min 75% B, 5 min 100% B, 10 min 100% B, 11 min 0% B, 12 min 0% B. Data were acquired in single ion monitoring mode monitoring the mass 221 m/z with negative polarity (Fragmentor 150 and dwell time 200). Retention time of IBMX peak is 3.91-3.92 min.

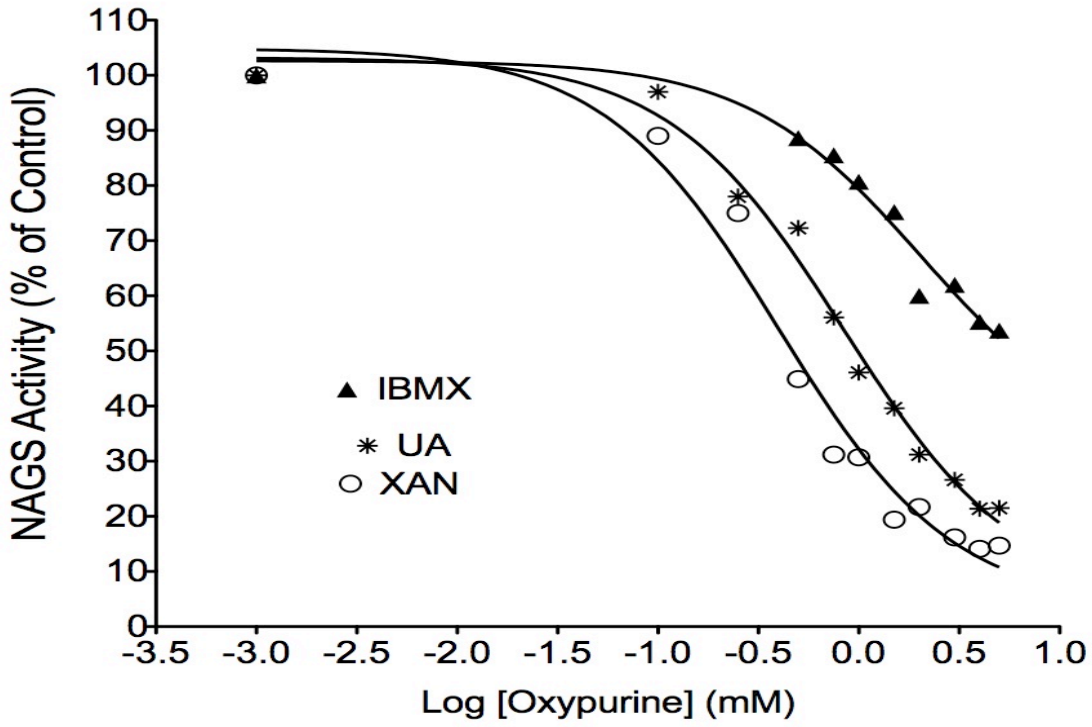


Figure 5S: ***Percent activity of the recombinant N-acetyglutamate synthase (NAGS) versus Log [oxypurine]:*** Assays were performed with fixed 1 mM ACoA and 10 mM [^{15}N]glutamate. The reaction was carried out for 5 min, at 30^{0}C in 250 μl of 50 mM Tris–HCl buffer, pH 8.5 containing about 2.5 μg of enzyme protein and 1 mM arginine. Results are expressed as a percentage of the enzyme activity without oxypurine (control). The 100 % activity was about 25 nmole.μg^{-1}.min^{-1}. The lines are the best fit to a sigmoidal dose-response obtained by Prism-5 software. These curves were used to determine the relative Inhibition Constant (IC_{50}) for inhibition of the NAGS activity by the indicated oxypurine.