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Nicotinamide N-Oxidation by CYP2E1 in Human Liver Microsomes

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Running Title: Metabolism of nicotinamide by CYP2E1

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Results: 350 words

Discussion: 424 words

Abbreviations: aldehyde oxidase, AOX; alpha-amino-beta-carboxymuconate-epsilon-semialdehyde decarboxylase, ACMSD; flavin-containing monooxygenases; FMOs; N1-methylnicotinamide, MNAM; N1-methyl-2-pyridone-5-carboxamide, 2pyr; N1-methyl-4-pyridone-3-carboxamide, 4pyr; nicotinamide, NAM; nicotinamide N-oxide, NAM N-oxide; nicotinamide N-methyltransferase, NNMT;

Abstract

Excess nicotinamide, a form of vitamin B3, is metabolized through two enzymatic systems and eventually excreted from the body. The first system starts with the methylation of nicotinamide by nicotinamide N-methyltransferase, which can subsequently be oxidized by aldehyde oxidase. The second enzymatic system oxidizes nicotinamide to nicotinamide N-oxide. It is located in the endoplasmic reticulum of hepatocytes but the precise enzyme is unknown. We have used human liver microsomes in combination with selective P450 inhibitors, specific substrates and antibodies to identify CYP2E1 as the main activity producing nicotinamide N-oxide. Our results suggest the potential use of nicotinamide N-oxide as a biomarker of CYP2E1 activity from urine or blood samples.

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Introduction

Nicotinamide (NAM) is one of the forms of vitamin B3. It is a precursor for nicotinamide adenine dinucleotide (NAD), which is best known as an electron carrier in oxidative phosphorylation and as a cofactor for many dehydrogenases (Bogan and Brenner, 2008; Houtkooper et al., 2010). NAM cannot be broken down and excess is excreted. Two enzymatic systems, which remove NAM from the body, have been described. The first is cytoplasmic and starts with the methylation of NAM to N1-methylnicotinamide (MNAM) by the enzyme nicotinamide N-methyltransferase (NNMT) (Cantoni, 1951). MNAM can also be further oxidized by aldehyde oxidase (AOX) to two related compounds, N1-methyl-2-pyridone-5-carboxamide (2-pyr) and N1-methyl-4-pyridone-3-carboxamide (4-pyr) (Fig.1A) (Leifer et al., 1951; Felsted and Chaykin, 1967). The second clearance pathway consists of an unknown microsomal enzyme, most likely a P450 system, which oxidizes NAM to nicotinamide N-oxide (NAM N-oxide) (Fig.1A) (Bonavita et al., 1961; Nomura et al., 1983). Both the methylated and the oxidized forms of NAM can be detected in the blood and urine of humans and rodents. Under normal conditions the cytoplasmic clearance pathway predominates, however pharmacological doses of vitamin B3 increase NAM N-oxide, which can become the most abundant NAM metabolite in mouse blood ((Chaykin et al., 1965; Shibata et al., 1990; Stratford and Dennis, 1992). In humans, NAM N-oxide is detected after therapeutic doses of niacin for the treatment of hyperlipidemia (Menon et al., 2007). Traditionally the NAM clearance metabolites were not considered to have biological activity. However, recent studies suggest that MNAM possesses anti-thrombotic and anti-inflammatory properties in vivo with the precise mechanism of action not well understood (Chlopicki et al., 2007; Bartuś et al., 2008). NAM N-oxide currently has no assigned biological function although high doses have been reported to affect the differentiation of leukemia cells (Iwata et al., 2003).

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In this study, we have used pooled microsomes from human donors to identify CYP2E1 as the main microsomal enzyme oxidizing nicotinamide and we discuss its potential as a natural biomarker of CYP2E1 activity.

Material and Methods

Nicotinamide, nicotinamide N-oxide, NADPH, chlorzoxazone, diethyldithiocarbamate, tranylcypromine, fluconazole, ketoconazole, alpha-napthoflavone, quinidine and orphenadrine were from Sigma (St.Louis, MO). Human liver microsomes (HLM), control, CYP2A6, CYP2B6 , CYP2E1 and FMO3 overexpressing insect cell microsomes (Supersomes) were from BD Biosciences (San Jose, CA). Control anti-mouse IgG (cat#12-371) was from Millipore (Billerica, MA) and anti-CYP2E1 inhibitory antibody (MAB-2E1, cat#458321) was from BD Biosciences (San Jose, CA).

Microsomal assay: Human liver microsomes were diluted to 1mg/ml in 0.1 M NaH_2PO_4, pH 7.4 supplemented with 1mM NAPDH and preincubated at 37^{o}C for 2 min. Total volume was 50 µl. Reaction was started by the addition of substrate NAM, continued for 30 min and stopped by the addition of equal volume of ice-cold methanol. Precipitate was removed by centrifugation. The supernatant was dried under vacuum and resuspended in 50 µl of HPLC mobile phase. Preliminary experiments showed that the formation of NAM N-oxide was linear with time up to 60 min. P450 inhibitors were dissolved in methanol and chlorozoxazone in acetonitrile. The final concentration of the solvent was 0.1% methanol and 1% acetonitrile. At these concentration CYP2E1 activity is not significantly inhibited (Easterbrook et al., 2001). Microsomes were preincubated for 30 min with the inhibitors before the addition of substrate, 2 mM NAM. Concentrations of the inhibitors were at least 10 times the reported Ki for standard substrates (FDA; Kobayashi et al., 1999; Zhang et al., 2001). Final concentrations of inhibitors were: 100

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μM diethyldithiocarbamate, 1 μM tranylcypromine, 100 μM fluconazole, 10 μM ketoconazole, 100 μM alpha-napthoflavone, 1 μM quinidine and 300 μM orphenadrine. For CYP2E1 antibody inhibition, 15 microgram of control mouse antibody or anti-CYP2E1 antibodies were mixed with microsomes, preincubated for 5 min at 37^{o}C before the addition of the 2 mM NAM and processed as described above. Control antibody was dialyzed overnight against excess 25 mM Tris-Cl pH 7.5 in a Slide-A-Lyzer Dialysis Cassette (Cat#66333) from Pierce (Rockford, IL) to remove sodium azide, which inhibits NAM N-oxide activity.
CYP2E1-overexpressing insect microsomes were used essentially as the human microsomes but were diluted 4-fold with control microsomes because of high CYP2E1 activity.

HPLC assay for NAM N-oxide: The samples were analyzed on the Breeze system (Waters, Millford) consisting of in-line degasser, the 1525 binary pump, the UV/Vis detector 2487 and the 717plus autosampler controlled by the Breeze software v3.2. 30 μl of the microsomal extract were injected on a HILIC Atlantis T3 column Waters (Millford, MA) 100 mm, 4.6 mm i.d. protected by a 20 mm guard column of the same material. The compounds were eluted isocratically in 90% Acetonitrile, 0.125% Acetic acid, 10 mM Ammonium Acetate and detected by UV absorbance at 254 nm. NAM N-oxide was identified by coelution with standards and quantified based on a standard curve of known quantities of NAM N-oxide.

Statistics: All results are from triplicate determination and presented as means +/- stdev. Significance was evaluated by the two-tailed Student's t-test $p<0.05$ in JMP Pro v.10 (SAS). Km and Vmax were calculated based on the Michaelis-Menten equation using the nonlinear fit function of JMP Pro v.10 (SAS).

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Results

We developed the HPLC assay to monitor the NAM N-oxide forming activity of human liver microsomes. Robust NAM N-oxide peaks were detected upon incubation with low millimolar concentrations of NAM (Fig.1B). Next, we carried dose response experiments in human liver microsomes to characterize the enzymatic reaction. The apparent Km for NAM N-oxidation under our conditions was 2.98 mM and maximal activity (Vmax) was 60.14 pmoles/mg/min (Fig.2A). In addition to the cytochrome P450 enzymes, human liver microsomes also include flavin-containing monooxygenases (FMOs) , which are similarly capable of broad range oxidations. The two systems have overlapping substrates and both require NADPH (Krueger and Williams, 2005). To gauge the relative contributions of each, we incubated liver microsomes for 5 min at 45^{o}C with and without NADPH. Heat treatment without NADPH is known to abolish FMO activity (Tugnait et al., 1997). However, the NAM N-oxide activity was not significantly affected (Fig.2B). Experiments using insect microsomes overexpressing FMO3 did not show any detectable NAM N-oxidizing activity (not shown) reflecting a likely involvement of P450s. Next, we preincubated the human liver microsomes with selective inhibitors for major hepatic P450 enzymes, ketoconazole (CYP3A4), fluconazole (CYP2C9), alpha-napthoflavone (CYP1A2), tranylcypromine (CYP2A6), quinidine (CYP2D6), orphenadrine (CYP2B6) and diethyldithiocarbamate (CYP2E1). Only the CYP2E1 inhibitor reduced significantly (8-fold) the formation of NAM N-oxide (Fig.2C). Additional experiments using microsomes overexpressing CYP2A6 and CYP2B6 did not show any detectable NAM N-oxidizing activity (not shown).
The large decrease in NAM N-oxide formation in the presence of the CYP2E1 inhibitor indicated that CYP2E1 might be the major enzyme oxidizing NAM in human liver microsomes. To support this idea further, we tested the sensitivity of NAM N-oxidizing activity to CYP2E1 substrate and antibody inhibition. We incubated human liver microsomes with 2 mM NAM in the presence of excess chlorzoxazone (100μM), a standard substrate for CYP2E1 (Peter et al., 1990).

Chlorzoxazone addition decreased NAM N-oxide formation more than 5-fold (Fig.3A). Similarly, preincubation of liver microsomes with specific inhibitory antibody against CYP2E1 decreased NAM N-oxide to less than 8% of control antibody (Fig3B). Finally, insect cell microsomes overexpressing CYP2E1 showed dose-dependent NAM N-oxidizing activity corroborating the inhibitor results (Fig.3C).

Discussion

Clearance of excess NAM is mediated by two enzymatic systems in the liver, cytoplasmic and microsomal (Fig.1A). The cytoplasmic system consists of the enzymes NNMT and AOX. The precise identity of the microsomal system was previously not known. In this report we identify CYP2E1 as the main microsomal enzyme capable of NAM N-oxidation. Using a combination of inhibitors, substrates and antibodies, we show that CYP2E1 is the major and possibly only enzyme producing NAM N-oxide in human liver microsomes, although absolute proof of this statement, at least in mice, would require the use of the CYP2E1 knockout animals.

CYP2E1 metabolizes a broad spectrum of small molecular weight compounds. Among them, pyridine-3-carboxamide, a compound structurally similar to NAM, is also converted by CYP2E1 to pyridine N-oxide (Kim et al., 1988). Metabolism of carcinogens, for example azoxymethane, and industrial chemicals, such as carbon tetrachloride, benzene and acrylamide by CYP2E1 increases their toxicity (Sohn et al., 1991; Ghanayem et al., 2000). Thus variation in CYP2E1 activity might predispose individuals to toxicity due to environmental pollutants, and numerous reports have attempted to correlate genotypic variations in CYP2E1 with various cancers (Neafsey et al., 2009). These studies suffer from limitations because the expression and the activity of CYP2E1 are also influenced by a disease state and are under nutritional control (Peng et al., 1983; Gonzalez et al., 1991; Chalasani et al., 2003). A more direct approach would involve the use of a biomarker to estimate CYP2E1 activity. Published attempts have utilized the hydroxylation of the standard substrate chlorzoxazone after ingestion by volunteers, however

this approach is impractical for large populations (Marchand et al., 1999; Piccoli et al., 2010) . NAM N-oxide is a natural metabolite of NAM and can be assayed in the blood or urine and in historical samples and it could therefore be amenable to large-scale screening. NAM clearance metabolites have recently been proposed as potential biomarkers of PPARα activation by fibrates (Delaney et al., 2005; Zhen et al., 2007). The large increase in NAM metabolites has been attributed to the suppression of the enzyme ACMSD by PPARα, which increases the tryptophan flux through the quinolinic-NAD pathway, giving rise eventually to NAM clearance products (Sanada, 1985; Fukuoka et al., 2002; Shin et al., 2006). Our results suggest that NAM N-oxide might actually be a more direct biomarker of CYP2E1 provided that it shows the required specificity and correlation with CYP2E1 activity. In conclusion, we identified CYP2E1 as the main microsomal enzyme producing the endogenous metabolite nicotinamide N-oxide and raise the potential of this pathway for biomarker development.

Authorship contributions

Participated in research design: Pavlos Pissios

Conducted experiments: Alexander Real, Shangyu Hong

Contributed new reagents or analytic tools: not applicable

Performed data analysis: Pavlos Pissios, Alexander Real, Shangyu Hong

Wrote or contributed to the writing of the manuscript: Pavlos Pissios, Alexander Real, Shangyu Hong

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Footnotes

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Alexander Michael Real and Shangyu Hong contributed equally to the study.

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Figure legends

Fig.1 A. Schematic representation of the nicotinamide clearance pathways. Nicotinamide (NAM) is methylated by nicotinamide N-methyltransferase (NNMT) to N1–methylnicotinamide (MNAM) and subsequently oxidized by aldehyde oxidase to pyridone carboxamides (2-pyr and 4-pyr). Nicotinamide is oxidized to nicotinamide N-oxide by an unknown microsomal activity (most likely P450). **B.** Human microsomes exhibit nicotinamide N-oxidizing activity. HPLC chromatograms of microsomal extracts in the absence of NAM (top panel) and presence of NAM (middle panel). HPLC chromatogram of NAM N-oxide standard (bottom panel). NAM N-oxide peak is indicated by *.

Fig.2 A. Dose response of NAM vs NAM N-oxide production by human liver microsomes, Km=2.98 mM, Vmax=60.14 pmoles/mg/min. **B.** NAM N-oxide production by HLMs is not significantly inhibited by heat in the absence of NADPH (substrate NAM at 2 mM). **C.** Effect of selective P450 inhibitors against major hepatic P450 enzymes on NAM N-oxide production (100 μM diethyldithiocarbamate, CYP2E1; 1 μM tranylcypromine, CYP2A6; 100 μM fluconazole, CYP2C9; 10 μM ketoconazole, CYP3A4; 100 μM alpha-napthoflavone, CYP1A2; 1 μM quinidine, CYP2D6 and 300 μM orphenadrine, CYP2B6). Substrate NAM was at 2 mM. Mean +/- stdev of triplicate determinations, * $p<0.05$ by student's t-test.

Fig.3 A. Inhibition of NAM N-oxide production by 100μM chlorzoxazone (substrate NAM at 2 mM). **B.** Inhibition of NAM N-oxide production by specific anti-CYP2E1 antibody (substrate NAM at 2 mM). **C.** Dose dependent increase in NAM N-oxide production by CYP2E1 overexpressing insect cell microsomes. Mean +/- stdev of triplicate determinations, * $p<0.05$ by student's t-test.

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Figure 1

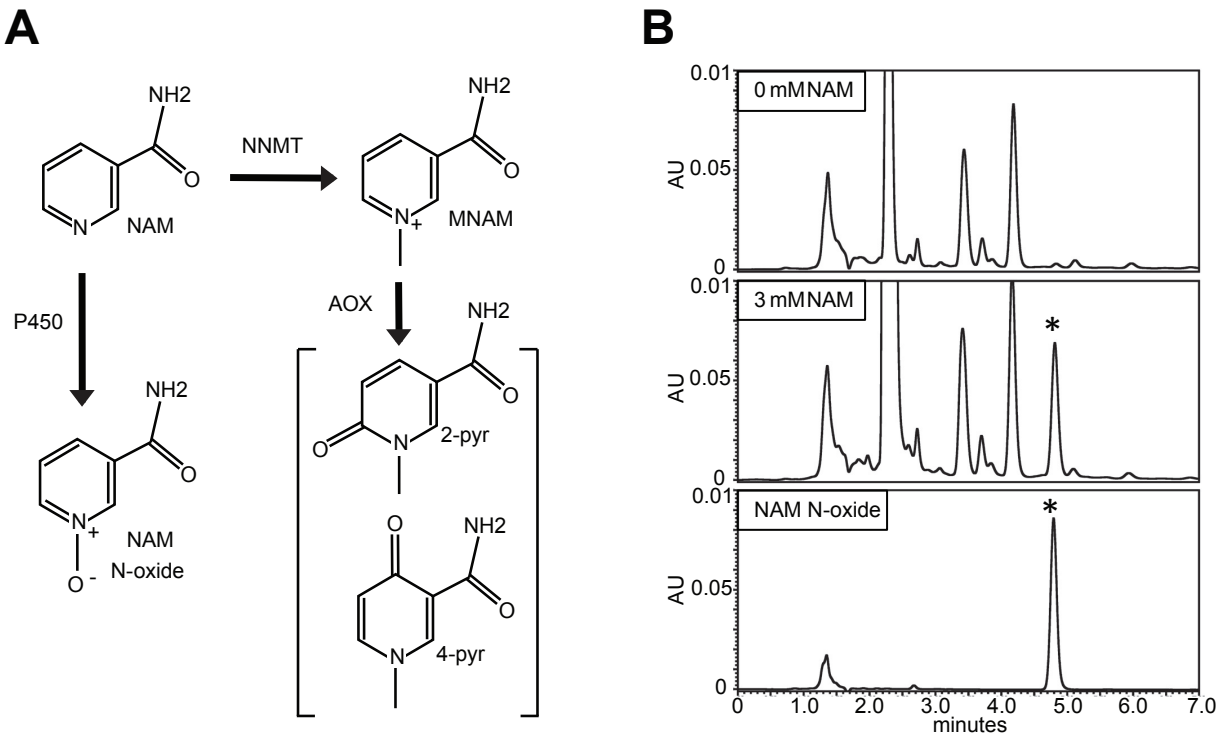

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Figure 2

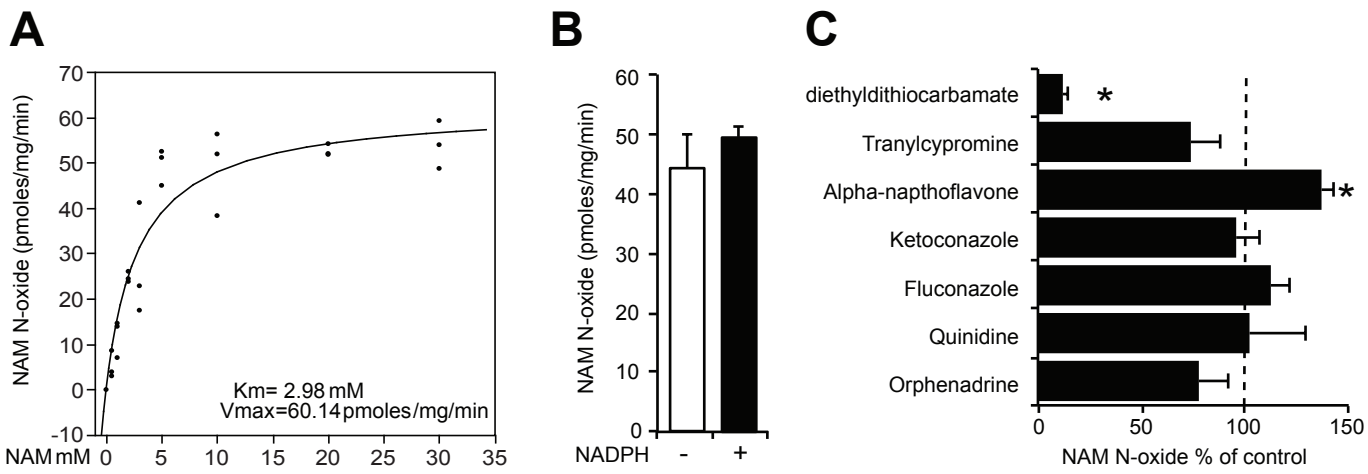

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Figure 3