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Hum Exp Toxicol 1996 15: 329
DOI: 10.1177/096032719601500409

Human & Experimental Toxicology (1996) 15, 329–334

Bacterial reduction of *N*-oxides of tobacco-specific nitrosamines (TSNA)*

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1 Contrary to established metabolic pattern, a recent investigation of NNK metabolism produced in rat urine higher levels of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) than their *N*-oxides, suggesting that reconversion of *N*-oxides could occur after urine formation.

2 To verify the possible role of bacteria in the reduction of NNK-*N*-oxide and NNAL-*N*-oxide to their respective parent compounds, NNK and NNAL, in smokers with urinary tract infection (UTI), the *N*-oxides were isolated from the urine of rats treated with [5-^{3}H]NNK and individually incubated at 37°C with ten bacterial species in sterile human urine under different pH regimens. After incubation with the bacteria, aliquots of culture media were analyzed by high pressure liquid chromatography (HPLC) with radiochemical detection.

3 *Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae and Proteus mirabilis* possessed varying capacity to regenerate NNK and NNAL from their *N*-oxides while others showed no detectable reductive capability within 24 h.

4 This result constitutes the first experimental evidence that in tobacco users with concomitant UTI, bacterial regeneration of the procarcinogenic NNK and NNAL from their *N*-oxides could occur in the bladder leading to increased carcinogen burden in these individuals.

Keywords: bacterial reduction; bladder cancer; *N*-oxides; tobacco-specific nitrosamine; urinary tract infection

Introduction

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is considered to play a major role in the induction of lung cancer in cigarette smokers.[1] Independent of route of administration, NNK is highly carcinogenic to the rodent lung, inducing adenocarcinoma and squamous cell carcinoma in rats, hamsters or mice. In addition, tumours of the nasal mucosa, liver and exocrine pancreas have been observed in rats treated with NNK. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a major metabolite of NNK induces tumours in the lung of rats and mice and is an especially potent carcinogen for the exocrine pancreas in rats.[1]

NNK and its reduction product, NNAL are procarcinogens requiring metabolic activation before they can react with cellular components.[2] Experiments utilizing human cell tissues, cells expressing cytochrome P450 isoenzymes and a variety of laboratory animals have demonstrated that metabolism of NNK involves three principal pathways, namely, carbonyl reduction, pyridine *N*-oxidation and α-hydroxylation (Figure 1). Carbonyl reduction of NNK produces NNAL while α-hydroxylation of both NNK and NNAL produces 4-oxo-(3-pyridyl) butyric acid (keto acid) and/or 4-hydroxy-(3-pyridyl) butyric acid (hydroxy acid). These acids constitute the major urinary metabolites in experimental animals dosed with NNK.[3–5] Both NNK and NNAL may undergo pyridine *N*-oxidation to produce NNK-*N*-oxide and NNAL-*N*-oxide respectively. NNAL is a substrate for glucuronyl transferase leading to the formation of one (rats and mice) or two (patas monkeys, humans) stereo isomers of [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucopyranosiduronic acid (NNAL-Glu).[3,4,6,7] Essentially, glucuronidation of NNAL and pyridine *N*-oxidation of both NNAL and NNK are considered to be a detoxification process.[2] These reports demonstrated that beside the acids, at low NNK doses, *N*-oxides rather than the parent compounds should be the principal urinary metabolites.

However, in a recent experiment, it was discovered that some rats administered low doses of [5-^{3}H]NNK produced in their urine higher levels of NNK and NNAL than the *N*-oxides.[8] This observation led us to propose that other extraneous factors, possibly of microbial origin must be responsible for the anomaly. To verify this hypothesis, purified

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Received 20 September 1995; revised and accepted 4 December 1995

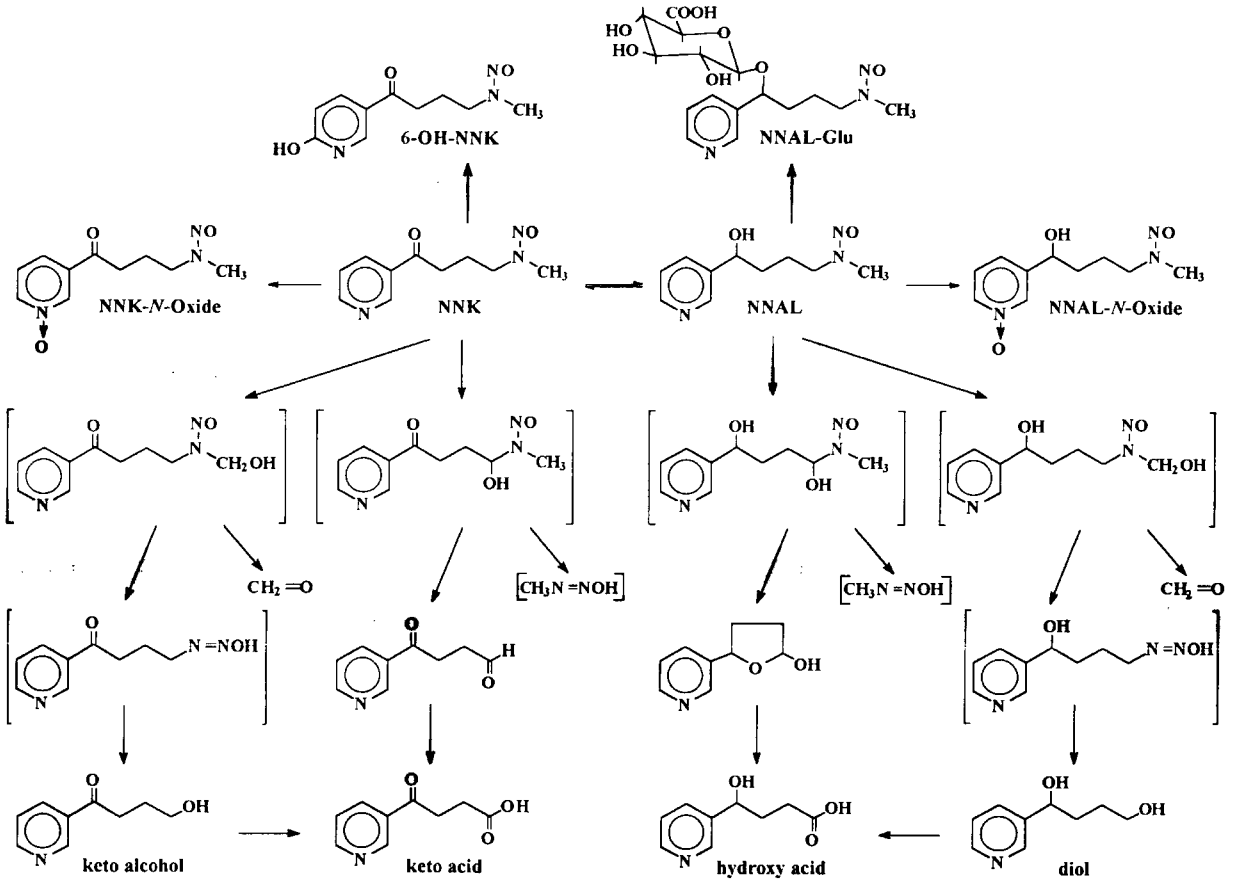

Figure 1 Metabolism of NNK.

NNK-*N*-oxide and NNAL-*N*-oxide were incubated in urine with bacteria known to be associated with diseases of human urino-genital system. In this article, we present the first evidence that some bacterial species as present in the bladder of patients with urinary tract infection (UTI) possess some capacity to reduce *N*-oxides of TSNA to their parent procarcinogenic entities, NNK and NNAL, thus increasing carcinogen burden in these individuals.

Materials

Reagents and solvents were obtained from commercial sources in analytical (Sigma Chemie GmbH, Taufkirchen, Germany) or HPLC grade (Merck, Darmstadt, Germany). [5-^{3}H]NNK (specific activity, 2.89 Ci/mol) was obtained from Chemsyn (Lenexa, USA). NNK-*N*-oxide and NNAL-*N*-oxide were obtained from the urine of [5-^{3}H]NNK-treated rats[8] by repeated HPLC purification and freeze-drying. The identity of the *N*-oxides was confirmed by co-chromatography with NNK metabolite standards generously donated by Dr D Hoffmann (American Health Foundation, Valhalla, NY).

Ten microbial strains, *Enterobacter cloacae, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis, Staphylococcus epidermidis, Staphylococcus aureus, Streptococcus viridans, Enterococcus spp.* and *Candida tropicalis*, 10^{10} counts in 1 mL of physiological saline from the stocks of the Max-von Pettenkofer Institute of Hygiene and Medical Microbiology, Munich, Germany, were kindly donated by Dr V Preac-Mursic.

Methods

Incubation experiments

Morning urine was collected from an apparently healthy human subject who neither consumes tobacco products nor drinks alcohol. The pH was noted and the urine was centrifuged at 3000 r.p.m. for 10 min. The supernatant was filtered through a sterile 0.45 μm membrane filter (Millipore, Eschborn, Germany) into three sterile containers. The pH of one portion was adjusted to 8 while another was adjusted to 6. The pH of the third portion was left unadjusted at 6.9.

Sterile urine (1 mL for each pH level) was incubated in a water bath at 37°C with about 200 μL of the substrate solution corresponding to approximately 33.6 pmol (215 000 d.p.m.) NNK-*N*-oxide and 18.7 pmol (120 000 d.p.m.) NNAL-*N*-

oxide respectively, and 10 μL of bacterial inoculum (count: 10^8) At the initial screening stage, the mixture was incubated at 37°C for 24 h before analysis. In subsequent experiments, aliquots of the incubation media were withdrawn for HPLC analysis after 3, 6 and 9 h of incubation. To exclude formation of NNK and NNAL in the culture media, samples of blank urine were run as control without added bacteria.

HPLC analysis

Aliquots of the incubation mixture 0.3–0.4 mL were stabilized with 0.1 mL phosphate buffer, pH 7.2 (200 mM for normal urine and 500 mM for acidic or basic mixtures). After centrifugation at 3000 r.p.m. for 10 min the supernatant was analyzed immediately or stored at −20°C.

Samples were chromatographed on a 4.6 × 250 mm LiChrosorb^R RP18 selectB column (Merck, Darmstadt, Germany) by elution with a gradient of 100% A for 0.5 min, linear to 80% A/20% B in 20 min and linear to 20% A/80% B in 20 min (A=20 mM phosphate buffer, pH 7.2; B=acetonitrile) at a flow rate of 0.7 mL/min.^{3}H was detected by solid phase radioactivity monitoring (Ramona, Raytest, Straubenhardt, Germany). Radioactive metabolites were identified by co-chromatography with unlabeled reference compounds using UV detection at 254 nm (UVD 160, Gynkotek, Gemering, Germany).

Calculations

The concentration of the *N*-oxides incubated and the TSNA regenerated by bacteria were calculated based on the following relationship: Ten urine samples of NNK-treated rats with known amounts of radioactivity (1034–2253 d.p.m./500 μL) produced a total area of 888–3791 units. The average d.p.m./area unit was 87 ± 7. Based on the specific activity of [5-^{3}H]NNK one area unit corresponds to 13.54 attomol of NNK.

Results

After 24 h incubation, only *Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae and Proteus mirabilis* produced detectable levels of NNK and NNAL under our experimental condition. These bacteria were therefore selected for further investigation while *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus viridans, Candida tropicalis, Enterococcus spp and Pseudomonas aeruginosa* which at this stage regenerated no TSNA were qualitatively considered negative (Tables 1 and 2).

Figure 2 illustrates the complete reconversion of NNK-*N*-oxide and NNAL-*N*-oxide by 24 h incubation of urine with *Proteus mirabilis*. Whereas the control urine without added bacteria had a single peak corresponding to NNK-*N*-oxide or NNAL-*N*-oxide peak (A,B), the test (C,D) had a peak with retention times corresponding respectively to reference NNK and NNAL. The identity of the regenerated [5-^{3}H]NNK and NNAL was confirmed by co-chromatography of these radioactive metabolites with unlabelled reference compounds. This procedure permitted simultaneous monitoring and

Table 1 Qualitative and quantitative studies of NNK formation from NNK-*N*-oxide by bacteria at different pH and incubation period

Bacterial species	*pH Condition**	*pmol of NNK formed (mean±SD) after incubation for:* 3 h	6 h	9 h	12 h
Proteus mirabilis	Acidic	0.35±0.07	0.91±0.11	2.03±0.30	
	Normal	0.96±0.20	4.36±0.29	11.06±0.79	+ve
	Basic	4.40±0.48	10.56±0.78	13.85±0.83	
Klebsiella pneumoniae	Acidic	0.50±0.09	3.83±0.43	4.70	
	Normal	0.53±0.07	3.06±0.39	3.30	+ve
	Basic	1.61±0.14	3.71	4.62	
Enterobacter cloacae	Acidic	nd	nd	1.31±0.11	
	Normal	nd	4.33	1.40±0.15	+ve
	Basic	nd	0.86±0.12	3.01±0.25	
Escherichia coli	Normal	4.32	6.91	10.40	+ve
Staphylococcus aureus	Normal	–	–	–	–ve
Staphylococcus epidermidis	Normal	–	–	–	–ve
Streptococcus viridans	Normal	–	–	–	–ve
Candida tropicalis	Normal	–	–	–	–ve
Enterococcus spp.	Normal	–	–	–	–ve
Pseudomonas aeruginosa	Normal	–	–	–	–ve

–ve= No formation of NNK after 24 h incubation of normal urine with NNK-*N*-oxide during qualitative screening.
+ve= Formation of NNK after 24 h incubation of normal urine with NNK-*N*-oxide during qualitative screening; these bacteria were selected for quantatitive studies.
nd=Not detected.
– = Not determined.
* For definition of pH conditions see methods.

Table 2 Qualitative and quantitative studies of NNAL formation from NNAL-*N*-oxide by bacteria at different pH and incubation period

Bacterial species	*pH Condition**	*pmol of NNK formed (mean±SD) after incubation for:* 3 h	6 h	9 h	12 h
Proteus mirabilis	Acidic	0.92±0.15	1.18±0.15	1.63±0.21	
	Normal	0.73±0.07	2.94±0.41	6.27	**+ve**
	Basic	1.23±0.20	3.97±0.48	6.86	
Klebsiella pneumoniae	Acidic	0.26±0.05	0.80±0.12	1.72±0.12	
	Normal	0.95±0.09	1.19±0.10	1.27±0.11	**+ve**
	Basic	–	0.58±0.06	3.14±0.38	
Enterobacter cloacae	Acidic	0.49±0.08	1.54±0.11	3.37	
	Normal	0.89±0.10	1.07±0.08	1.23	**+ve**
	Basic	nd	nd	**0.22**	
Escherichia coli	Normal	**0.69**	**4.27**	**5.06**	**+ve**
Staphylococcus aureus	Normal	–	–	–	**–ve**
Staphylococcus epidermidis	Normal	–	–	–	**–ve**
Streptococcus viridans	Normal	–	–	–	**–ve**
Candida tropicalis	Normal	–	–	–	**–ve**
Enterococcus spp.	Normal	–	–	–	**–ve**
Pseudomonas aeruginosa	Normal	–	–	–	–ve

–ve= No formation of NNAL after 24 h incubation of normal urine with NNAL-*N*-oxide during qualitative screening.
+ve= Formation of NNAL after 24 h incubation of normal urine with NNAL-*N*-oxide during qualitative screening; these bacteria were selected for quantatitive studies.
nd=Not detected.
– = Not determined.
* For definition of pH conditions see methods.

comparison of the unlabelled compounds by UV detection at 254 nm (UVD 160, Gynkotek, Gemering, Germany) and the regenerated radioactive compounds by solid phase radioactive monitoring.

After 3 h incubation, detectable levels of NNK were produced by *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis* but not *Enterobacter cloacae*. The highest rate of reconversion was found in *Proteus mirabilis* at basic pH. Generally, alkaline pH was most favourable for the regeneration of NNK, while acidic pH was the least favoured. The effect of pH appears to be least dramatic on the regeneration of NNK by *Klebsiella pneumoniae* than in any other species found to possess reductive capacity on NNK-*N*-oxide (Table 1).

Table 2 shows the rate of regeneration of NNAL by tested bacterial species. Like in the reduction of NNK-*N*-oxide, the bacteria are most efficient at reducing NNAL-*N*-oxide at alkaline pH and least efficient at acidic pH except in the case of *Enterobacter cloacae* where the trend appears to be reversed.

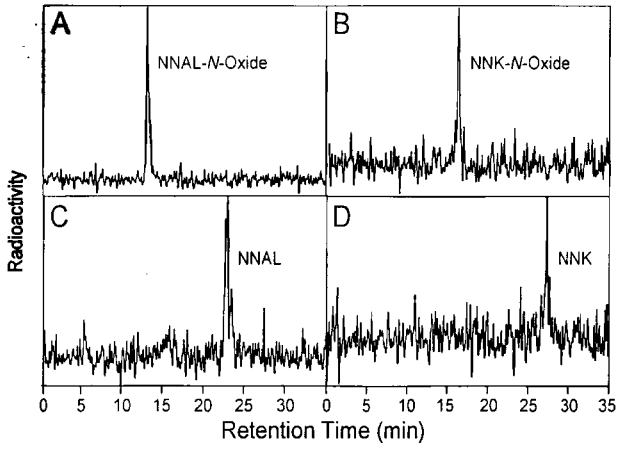

Figure 2 Reduction of NNK-*N*-oxide (**A,C**) and NNAL-*N*-oxide (**B,D**) by *Proteus mirabilis*. HPLC radiochromatograms of human urine (pH 6.9) after 24 h incubation without (**A,B**) or with (**C,D**) added bacteria (count: 10^8/ml).

Discussion

Nitrosamine formation by bacteria in the bladder has been hypothesized to be an associated factor for cancer of the bladder in individuals with UTI.[9–11] Previous studies have established that *Escherichia coli, Klebsiella pneumoniae* and *Proteus mirabilis* are some of the bacterial species that may either catalyze nitrosation of secondary amines or are associated with UTI in which *N*-nitroso compounds have been detected in the urine of patients.[11–13] The present study has demonstrated that these same bacterial species also possess some capacity to regenerate NNK and NNAL from their precursor *N*-oxides. To our knowledge, this is the first report on the possible involvement of bacteria in the reduction of *N*-oxides of TSNA which are primarily considered to be detoxification products, back to their procarcinogenic parent compounds.

Although the bacterial reduction of *N*-oxides of TSNA has not been previously reported, it is not an unexpected observation. The role of the gut flora in

the reduction of *N*-oxide drugs is well established.[14] Regeneration of nicotine and cotinine from corresponding *N*-oxides has been repeatedly reported to occur *in vivo* as well as *in vitro*.[15–17] The reasons for the varying pH-dependent capacities of different bacteria to regenerate NNK and NNAL from their *N*-oxides are not clear and require further investigations. The highest rate of regeneration of the two compounds was observed in *Proteus mirabilis* at alkaline pH. In general, the bacteria tested possessed higher capacity to reconvert NNK-*N*-oxide to NNK than reconversion of NNAL-*N*-oxide to its parent compound.

The low concentrations used in the present investigation, 15–40 nmol/L, are still higher than the concentrations which could be expected to occur in urine of a heavy smoker. NNK uptake is estimated to be on average 300 ng/cigarette.[18] Assuming a 24 h uptake of 12 μg NNK (40 cigarettes/day), and a percentage of *N*-oxide metabolites of maximally 15% as observed in patas monkeys,[4] this would give a concentration of *N*-oxides in urine of $\sim$1.8 nmol/L.

NNK and NNAL are powerful carcinogens with the lung as a major target.[1] Therefore, bacterial reduction of *N*-oxides of these TSNA to their procarcinogenic parent compounds may have some implications: In tobacco users with UTI, regeneration of NNK and NNAL could mean exposure of the bladder walls to active forms of the carcinogens through metabolic activation. Whether the bladder tissue has the capacity to activate NNK and NNAL requires further investigation. The second possibility is that the regenerated NNK and NNAL could be absorbed by the bladder tissues and recirculated through blood to site(s) where they are re-metabolized to produce more ultimate carcinogens. Ability of bladder tissues to absorb other *N*-nitroso compounds into the blood stream has been demonstrated in the rat.[9] In either case, tobacco user with UTI stands higher burden of ultimate carcinogens than the healthy user and might be at higher risk to develop tobacco-related cancers.

Acknowledgements

Dr Atawodi would like to thank Alexander von Humboldt Foundation, Bonn, Germany, for the award of a research fellowship which made this investigation possible. This work was supported by a grant from VERUM.

Abbreviations

NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNK-*N*-oxide,4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanone; NNAL-*N*-oxide,4-(methylnitrosamino)-1-(3-pyridyl-*N*-oxide)-1-butanol; HPLC, high pressure liquid chromatography; TSNA, tobacco-specific nitrosamines; UTI, urinary tract infection.

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