Structure and activity of N-Nitroso Compounds (NOC) and overview on endogeneous nitrosation (bio)chemistry

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History

More than 50 years ago N-nitrosodimethylamine (NDMA) has been reported by Peter Magee and his colleague M.J. Barnes (1956) to induce primary liver tumours in rats. This finding originated from an investigation into the potential causes of hepatotoxicity in workers in the rubber industry, where the compound had been explored for suitability as a solvent in the vulcanization process (Barnes and Magee, 1954). Sporadic case reports on accidental (Freund, 1937) or intentional fatal intoxications (Cooper& Kimbrough, 1980; Fussgänger & Ditschuneit,1980) by NDMA published before or later described human postmortem findings, especially acute liver necrosis and haematological toxicity similar to those seen in experimental animals at lethal dosage.

NDMA is a quite polar, low molecular weight water soluble compound, significantly different from the lipophilic carcinogens of higher molecular mass known at that time such as polycyclic aromatic hydrocarbons (PAH), aromatic amines or pyrrolizidines. The seminal finding by Magee & Barnes (1956) was confirmed by Schmähl & Preussmann (1959) who also showed the next homologue, N-nitrosodiethylamine (NDEA) to be at least as potent a carcinogen as NDMA (Schmähl et al., 1960). Subsequently, research into biological activities of N-nitroso compounds (NOC) rapidly gained momentum and a wide range of NOC, including certain nitrosamides such as N-methyl-N-nitrosourea or N-methyl-N-nitrosorurethane (Schoental, 1960; Druckrey et al., 1961), were identified as potent carcinogens. The group headed by Hermann Druckrey in Freiburg, Germany published a seminal and comprehensive study on structure-activity and dose-response relationship (Druckrey et al., 1967) that soon became a “Citation classic”. A specific feature of NOC early on gained much attention, namely their propensity to selectively induce tumours in organs of various animal species, irrespective of the application route. This was systematically extended by William Lijinsky and his colleagues (summarized 1992) and provided the basis for the establishment of highly reproducible experimental tumour models for clinical therapy studies.

The pivotal step in bioactivation of many NOC consists in metabolic generation of electrophilic ultimate carcinogens, in general through cytochrome P450 (CYP) dependent α-hydroxylation, as first shown by Magee and coworkers (1962).

Research on biological effects of NOC in the subsequent years was driven by trying to understand mechanisms of biological and molecular events responsible for the biological effects observed.

Exposure to preformed NOC

Already in the early sixties, the suspicion was raised that NOC might be formed in the human environment at certain conditions (Druckrey & Preussmann, 1962). Ender et al. (1964) in Norway described poisoning of sheep, with clear indication of liver toxicity, after feeding nitrite-treated fish meal. Although appropriate analytical methods were not at hands yet, the group identified NDMA, obviously present at rather high concentrations, as the most probable causative agent. This triggered an avalanche of analytical research to uncover presence of NOC in food.

Formation of NOC: basic considerations

Virtually any situation where nitrosating agents encounter N-nitrosatable amino compounds can give rise to NOC. The classical situation would reflect the reaction of an amine with nitrous acid, in aqueous solution (Ridd, 1961; Mirvish, 1975). Nitrite and nitrous acid under proton catalysis generate the actual nitrosating species, dinitrogen trioxide (N_2O_3) or tetroxide (N_2O_4) or nitrous acidium ion $NO^+ \bullet H_2O$. The acidity of the medium determines the relative prevalence of the nitrosating species. In addition, the basicity of the nitrosatable amine precursor is of great influence for nitrosation in aqueous solution, because only non-protonated nitrogen atoms are available for nitrosation. Therefore, strongly basic amines like simple dialkylamines (pka < 9,5) are not considered to exhibit nitrosation rates that favour substantial NOC formation in aqueous media. Because acid-catalyzed nitrosation is inappreciable at pH > 5, NOC in food or certain consumer products or in specific working place situations most likely arise from exposure to gaseous NO_x.

Such conditions are to be met, for instance, in a variety of food production situations, including smoke treatment or direct drying and kilning technology. Addition of nitrate/nitrite, e.g. in the curing of meat is also of some relevance. Carbonyl compounds present or generated during food treatment can act as nitrosation catalysts as shown for formaldehyde, a reaction discovered by Keefer and Roller (1973).

Nitrosamines most frequently found in food are NDMA, N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP) and N-Nitrosothiazolidine (NTHZ). Nonvolatile N-nitroso compounds consist mainly of N-nitrosated amino acids, including the N-nitroso products of sarcosine (NSAR), 3-hydroxyproline and proline (NPRO), thiazolidine-4-carboxylic acid (NTCA), oxazolidine-4-carboxylic acid (NOCA) and N-Nitroso-2-methyl-nitroso thiazolidine-4-carboxylic acid (NMTCA) as well as the oxazolidine analog (NMOCA). NPRO and NTCA are most frequently found in foods, the other compounds have been reported only sporadically. Of note, with the exception of NSAR, which is a relatively weak carcinogen, N-nitrosated amino acids are not mutagenic and not carcinogenic. Average NOC exposure has been calculated for various countries, mostly from dietary surveys (Table 1).

Table 1: Dietary intake of volatile NOC: Germany 1980-1990 (Janzowski et al., 2000)

NDMA µg/person/day	**NPyr + NPIP** µg/person/day	**∑ NOC**	**Year (Country)**	**ng/kg b.w.**
0.1 – 1.0	0.1 – 0.4	0.2 - 1.4	before 1989/90 (D, NL, FIN, UK, F, SW)	1.7 – 23.3
0.2 – 0.3	0.03	0.2 - 0.3	1989/90 (D)	1989/90

A margin of exposure (MOE) may be calculated to arrive at some basis for priority decisions concerning risk management. The MOE describes the ratio between human exposure and a dose level inducing a certain tumour response. As benchmark either the T25, reflecting the dose rate in mg/kg/d which will give 25% tumours at a specific site, or the lower limit of the 95% confidence interval of a dose resulting in a 10% tumour response is used. The thus calculated MOE of dietary NOC exposure from these relatively old data is 5,400-8,200. According to EFSA, a MOE of 10,000 or higher would be of low concern for risk management (EFSA Opinion, 2005).

Formation of NOC in vivo:

Amine contents in foods have been studied in a comprehensive study by Pfundstein et al. (1991). According to these data, the mean daily per capita intake of amines from food was about 30-37 mg of primary and 6-8 mg of secondary amines. Thus in principle, NOC can be expected not only to form under environmental, technical or household conditions favouring the reaction of nitrosatable amines in food with nitrosating agents, but also after ingestion, e. g. during stomach passage.

In the aqueous-acidic medium of the stomach, the nitrosation rate is primarily governed by the protonation of the amine nitrogen and the pH dependent availability of N_2O_3. Therefore the pK-value of the amine determines the rate of NOC formation at a given acidic pH. Mirvish (1975) has investigated the kinetics of amine nitrosation under such conditions, reflecting significant differences in rates. Thus, a weakly basic amine is nitrosated at a given pH-value at a rate about 5 orders of magnitude faster than a strongly basic one, such as piperidine or diethylamine (pK > 11). Accordingly, no tumour formation was observed after feeding nitrite and such secondary amines to rats. In contrast, when weakly basic amines like N-methylbenzylamine and morpholine were given together with nitrite, the same tumours were induced as observed with the corresponding nitrosamines, as first shown by Sander & Bürkle (1969).

The drug amidopyrine (AP) had been withdrawn from the European market in the eighties for various reasons, including its inherent risk of NDMA formation. AP has been found to give rise to liver tumours when fed to rats with nitrite at quite low concentrations (Lijinski, 1975).

Biomarkers of exposure to NOC formed in vivo

In human volunteers, an increased risk for endogenous formation of NDMA after oral intake of 1 tablet of AP (500 µg) and of nitrate (200 mg), taken up in a vegetable juice, had been demonstrated by Spiegelhalder (1990). NDMA excretion in the urine became detectable by blocking it's metabolism

with concurrent application of ethanol (500 ml beer). Earlier studies had shown that concurrent ethanol administration (in 500 ml beer containing 60 ppb NDMA) results in / up to 2.5% NDMA being excreted unmetabolized in urine. From this, an in-vivo formation up to 1.8 mg NDMA was estimated in 1/3 volunteers. In probands saliva the drug was present in concentrations reflecting plasma values, together with nitrate that was reduced in part in the oral cavity to nitrite. When saliva samples were acidified to model the situation after being swallowed into the acidic stomach, NDMA rapidly formed at substantial rates (Spiegelhalder, 1990; Tricker, 1997). Urinary excretion of N-nitrosated metabolites was also used to measure exposure at working places or as a result of anthelmintic medication in the case of piperazine (Bellander, 1990; Tricker et al., 1991)

Although it has been clearly demonstrated that carcinogenic NOC might easily be formed when the appropriate precursors are being taken up, endogenous formation of carcinogenic NOC has not really been evaluated adequately as a process of relevance to human cancer. Apart from selected drugs of high reactivity towards nitrosation endogenous formation of NOC occuring under normal nutritional and physiological conditions needs to be adequately addressed. Nitrosated amino acids such as NPRO, because they are noncarcinogenic and practically quantitatively excreted in the urine, have often been utilized as surrogate biomarkers for overall endogenous nitrosation. It is well known, that there is endogenous formation of nitrosating agents and that certain disorders, such as inflammatory diseases, bacterial, viral or parasite infections and the like can substantially increase endogenous formation of nitrosating agents, thus enhancing the risk of forming carcinogenic NOC in vivo. It is still not clearly established yet, whether NPRO is a valid biomarker for endogenous formation of NOC other than those arising from nitrosation of amino acids. Within nitrosated amino acid derivatives, evidence from data presented by Shuker (Shuker & Margison, 1997; Harrison et al., 1999) suggests that for example N-nitrosoglycocholic acid (and other nitrosated glycine derivatives) form several DNA adducts, including O^6-carboxymethylguanine and, concomitantly = O^6 methylguanine. It is thus very important to develop in the future appropriate biomarkers that might allow a realistic estimate of in vivo formation of carcinogenic NOC. A possible way forward may be to exploit gene expression responses in animals and humans in an exposure related way. This might open new research avenues towards a better understanding of nitrate / NO_x -related biological effects with respect to both, potential health benefits and risks.

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