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Original Contribution

The neuronal nitric oxide synthase inhibitor NANT blocks acetaminophen toxicity and protein nitration in freshly isolated hepatocytes

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ABSTRACT

3-Nitrotyrosine (3NT) in liver proteins of mice treated with hepatotoxic doses of acetaminophen (APAP) has been postulated to be causative in toxicity. Nitration is by a reactive nitrogen species formed from nitric oxide (NO). The source of the NO is unclear. iNOS knockout mice were previously found to be equally susceptible to APAP toxicity as wildtype mice and iNOS inhibitors did not decrease toxicity in mice or in hepatocytes. In this work we examined the potential role of nNOS in APAP toxicity in hepatocytes using the specific nNOS inhibitor NANT (10 μM)(N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N′-nitroguanidinetris (trifluoroacetate)). Primary hepatocytes (1 million/ml) from male B6C3F1 mice were incubated with APAP (1 mM). Cells were removed and assayed spectrofluorometrically for reactive nitrogen and oxygen species using diaminofluorescein (DAF) and Mitosox red, respectively. Cytotoxicity was determined by LDH release into media. Glutathione (GSH, GSSG), 3NT, GSNO, acetaminophen-cysteine adducts, NAD, and NADH were measured by HPLC. APAP significantly increased cytotoxicity at 1.5–3.0 h. The increase was blocked by NANT. NANT did not alter APAP mediated GSH depletion or acetaminophen-cysteine adducts in proteins which indicated that NANT did not inhibit metabolism. APAP significantly increased spectroflurometric evidence of reactive nitrogen and oxygen formation at 0.5 and 1.0 h, respectively, and increased 3NT and GSNO at 1.5–3.0 h. These increases were blocked by NANT. APAP dramatically increased NADH from 0.5–3.0 h and this increase was blocked by NANT. Also, APAP decreased the Oxygen Consumption Rate (OCR), decreased ATP production, and caused a loss of mitochondrial membrane potential, which were all blocked by NANT.

1. Introduction

Acetaminophen (paracetamol, APAP; N-acetyl-p-aminophenol) is the most widely used analgesic/antipyretic drug in the world. APAP is believed to be safe at therapeutic doses but may produce hepatic centrilobular necrosis when an overdose occurs [1]. In the United States approximately 500 deaths occur annually from APAP overdose [2]. The initial metabolic events in APAP toxicity have been well described. Hepatic metabolism of APAP by cytochrome P450 to the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) is the initial event [3]. Glutathione (GSH) efficiently detoxifies NAPQI after a therapeutic dose but when an overdose occurs GSH is depleted [4,5] and the metabolite covalently binds with hepatic cellular proteins to form 3-(cystein-S-yl)-acetaminophen (APAP-Cys) adducts [6]. The covalent binding of the hepatic proteins correlates with hepatic toxicity. The mode of cell death induced by APAP toxicity is necrosis. Previously, we reported the presence of nitrated tyrosine (3-nitrotyrosine) in hepatic proteins of APAP-treated mice, which coincided with the presence of APAP-Cys adducts in centrilobular hepatocytes and developing necrosis [7]. It was postulated that 3-nitrotyrosine was formed by

Abbreviations: APAP, Acetaminophen; NAPQI, N-Acetyl-p-benzoquinone imine; GSH, Reduced glutathione; APAP-cys, 3-(cystein-S-yl)-acetaminophen; NO, Nitric Oxide; iNOS, Inducible nitric oxide synthase (NOS2); nNOS, Neuronal nitric oxide synthase (NOS1); GSNO, *S*-Nitrosoglutathione; GSSG, Oxidized glutathione; NANT, N-[(4S)-4-amino-5-[(2-aminoethyl) amino] pentyl]-N′-nitroguanidinetris (trifluoroacetate); MPT, Mitochondrial Permeability Transition; LDH, Lactate Dehydrogenase; OCR, Oxygen Consumption Rate; 3-NT, 3-Nitrotyrosine

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nitration of tyrosine by peroxynitrite, a highly reactive species generated from superoxide and nitric oxide (NO) [8–10]. Peroxynitrite is a nitrating and oxidizing agent. Peroxynitrite is detoxified by GSH [11] which is depleted in APAP toxicity [4]. A number of major proteins have been reported to be nitrated in murine APAP toxicity, including MnSOD [12], mitochondrial aldehyde dehydrogenase, glutathione peroxidase, ATP synthase, and 3-ketoacyl-CoA thiolase [13]. Importantly, Abdelmegeed et al., found that inhibition of APAP toxicity by N-acetylcysteine treatment decreased protein nitration [13].

Kon et al. [14] and Reid et al. [15] reported that mitochondrial permeability transition (MPT) occurs in APAP toxicity in hepatocytes. MPT is an abrupt increase in the permeability of the inner mitochondrial membrane to ions and small molecular weight solutes. It is promoted by oxidative stress as well as nitrosative stress. It results in the inability of the mitochondria to produce adenosine 5′- triphosphate (ATP) and is a lethal event for cells [16,17]. APAP induced MPT has been postulated to be mediated by increased peroxynitrite resulting in nitration of proteins [18]. Since inducible nitric oxide synthase (iNOS; NOS2) is present in hepatocytes and Kupffer cells, [19] it was initially postulated to be the source of NO. However, both iNOS knockout and wild-type mice were found to be equally sensitive to APAP toxicity [20,21]. Decreased APAP hepatotoxicity was not observed in mice treated with pharmacological inhibitors of iNOS [18,22], suggesting iNOS was not involved in APAP toxicity. However, the neuronal nitric oxide synthase (nNOS; NOS1) inhibitor 7-nitroindazole did inhibit APAP toxicity and nitration when added in the late phase of toxicity and nNOS has been previously reported to be present in hepatocytes [23].

In this work experiments were performed to develop a greater understanding of early events important in APAP toxicity in hepatocytes. A highly selective inhibitor of nNOS (Ki = 120 nM), N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N′-nitroguanidinetris(trifluoroacetate) (NANT) was utilized to determine its effect on APAP toxicity. The chemical structure of NANT suggested that it would not inhibit CYP (cytochrome P450), enabling reactive nitrogen formation as it relates to APAP toxicity to be examined.

2. Materials and Methods

2.1. Chemicals and suppliers

Acetaminophen (APAP; 4-acetamidophenol), HEPES, heparin sodium salt grade I-A from porcine intestinal mucosa, penicillin G sodium salt, RPMI-1640 modified media with L-glutamine and without sodium bicarbonate and phenol red, Percoll, 0.4% Trypan blue solution, GSH, GSSG, GSNO, 3NT, NAD, NADH were obtained from sigma chemical company (St louis, MO, USA). N-[(4S)-4-amino-5-[(2-aminoethyl)amino] pentyl]-N′-nitroguanidinetris (trifluoroacetate) (NANT) was obtained from Santa Cruz biotechnology Inc. (Santa Cruz, CA, USA). Collagenase A from clostridium histolyticum was obtained from Roche Diagnostics (Indianapolis, IN, USA). 4-amino-5-Methylamino-2′,7′-Difluorofluorescein Diacetate (DAF-FM), MitoSOX red and JC1 were purchased from life technologies (Eugene, OR, USA). Cytotoxicity detection kit (LDH) was obtained from Roche diagnostic corporation (Indianapolis, IN, USA). ATP Bioluminescent assay kit was purchased from sigma-Aldrich (St. louis, MO, USA).

2.2. Animals

Male six-week old B6C3F1 mice were obtained from Harlan Laboratories (Indianapolis, IN, USA). All animal experimentation and protocols were approved by University of Arkansas for Medical Sciences Animal Care and Use Committee. Experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health. Mice were acclimated one week prior to the experiments and fed *ad libitum* until the time of sacrifice.

2.3. Hepatocyte isolation and incubations

Freshly isolated hepatocytes were obtained from mice by collagenase perfusion as previously described [18]. Hepatocytes yielding >40 million cells and cell viability $>90\%$ as determined by Trypan blue exclusion were used for the experiments. The hepatocytes were incubated at a concentration of 1 million cells/ml in RPMI 1640 media (supplemented with 25 mM HEPES, 10 IU heparin/ml, and 500 IU penicillin G/ml) in 125 ml Erlenmeyer flasks at 37 °C under an atmosphere of 95% O_2-5% CO_2. APAP (1 mM) was added to experimental hepatocytes [18]; no APAP was added to the control flasks. Other experimental and control flasks contained 10 μM NANT. The toxicity data were obtained from three to four separate incubations that were performed on separate mice on different days.

2.4. Toxicity assays

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane. Briefly, the hepatocytes were separated from the media by centrifugation. To each sample media (100 μl), reaction mixture from the kit (100 μl) was added followed by incubation in a heating bath at 37 °C for 30 mins. During this incubation period the mixture was protected from light. The absorbance of samples was determined spectrophotometrically in a Bio-rad 550 plate reader at a wavelength of 490 nm and cytotoxicity values determined.

2.5. Fluorescence assays

Increased fluorescence of MitoSOX Red was used as an assay for reactive oxygen as previously described [24]. Even though the increased fluorescence is commonly used as an assay for superoxide, the assay is not specific. The increased fluorescence may also be catalyzed by hydrogen peroxide plus a peroxidase, or other intracellular processes [25]. Thus, the increased fluorescence is an assay for reactive oxygen. 4-Amino-5-Methylamino-2′,7′-Difluorofluorescein Diacetate (DAF- FM) was used to assay for reactive nitrogen (NO) [25]. Briefly, 1 ml aliquots of hepatocytes were centrifuged at 140 *g* for 2 min and supernatant discarded. The hepatocytes were resuspended with DAF-FM (10 μM) or MitoSOX (5 μM) in 2 ml of phosphate-buffered saline and incubated for 20 min at 37 °C in atmosphere of 95% O_2-5% CO_2. Following incubation, cells were centrifuged and washed free of excess of dye, resuspended in 2 ml of phosphate-buffered saline, and excited/emitted at 495/515 nm for DAF-FM and 510/580 nm for MitoSOX respectively using SpectraMax M2^e fluorescence spectrophotometer.

2.6. HPLC assays

High-performance liquid chromatography (HPLC) was used to quantify the reduced as well as oxidized glutathione, *S*-nitrosoglutathione (GSNO) and 3-nitrotyrosine (3-NT). Approximately 2 million hepatocytes were homogenized in ice-cold phosphate-buffered saline. To precipitate proteins, 10% metaphosphoric acid was added to the homogenate and incubated for 30 min on ice. The samples were then centrifuged at 18,000 *g* at 4 °C for 15 min, and 20 μl of the resulting supernatants were