Acetaminophen, the Active Ingredient of Tylenol, Protects against Peroxynitrite-Induced DNA Damage: A Chemiluminometric and Electron Paramagnetic Resonance Spectrometric Study

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ABSTRACT | This study investigated the hypothesis that acetaminophen at pharmacologically relevant concentrations may act as a protector against peroxynitrite toxicity. Our results showed that acetaminophen inhibited SIN-1 (a peroxynitrite generator)-induced DNA cleavage in a concentration-dependent manner in an in vitro model. With bicarbonate-enhanced luminol-dependent chemiluminesmetry, we further showed a nearly complete blockage of peroxynitrite-derived chemiluminescence by acetaminophen at 25–100 µM with minimal effects on SIN-1-mediated oxygen consumption, suggesting acetaminophen as a potent scavenger of peroxynitrite. Electron spin resonance spectrometry in combination with 5,5-dimethyl-1-pyrroline N-oxide (DMPO)-spin-trapping supported the ability of acetaminophen at high concentrations to diminish the production of a free radical species (likely hydroxyl radical) from SIN-1. Fenton chemistry-based DMPO-spin trapping further demonstrated the hydroxyl radical-scavenging capacity of acetaminophen. Collectively, the results of this study for the first time revealed the potential of acetaminophen to protect against peroxynitrite toxicity, which may have important implications in neuroprotection associated with the use of this popular analgesic and antipyretic drug.

KEYWORDS | Acetaminophen; Chemiluminometry; DNA damage; Electron paramagnetic resonance; Peroxynitrite; SIN-1; Spin-trapping

ABBREVIATIONS | CL, chemiluminescence; DMPO, 5,5-dimethylpyrroline N-oxide; EPR, electron paramagnetic resonance; PBS, phosphate-buffered saline; SIN-1, 3-morpholinosydnonimine
1. INTRODUCTION

Acetaminophen, the active ingredient of Tylenol, is among the most widely used over-the-counter analgesic drugs. On the other hand, the drug is also well known for its ability to cause liver injury upon overdose. Recent studies suggest that acetaminophen at therapeutic doses may protect against dopaminergic neuron degeneration and neuroinflammation in animal models [1–3]. In this context, peroxynitrite, a reactive species formed from the bi-radical reaction between superoxide and nitric oxide, plays an important role in neurocytotoxicity and inflammatory injury via, at least partially, inducing DNA strand breakage and the subsequent activation of poly ADP ribose polymerase [4–6]. Accordingly, this study investigated the potential effects of acetaminophen at pharmacologically relevant concentrations on peroxynitrite-induced DNA damage and the underlying mechanisms. Our results for the first time demonstrated that acetaminophen inhibited peroxynitrite-induced DNA damage which appeared to result from its scavenging of peroxynitrite and/or peroxynitrite-derived secondary radical species.

2. MATERIALS AND METHODS

2.1. Materials

φX-174 RF I plasmid DNA was from New England Biolabs (Beverley, MA, USA). Authentic peroxynitrite was from Calbiochem (San Diego, CA, USA). 3-Morpholinosydnonimine (SIN-1), acetaminophen, and other chemicals were from Sigma–Aldrich (St. Louis, MO, USA). SIN-1 was dissolved in cold phosphate-buffer saline, pH 5.5, and stored at −80°C. The concentration of authentic peroxynitrite was determined spectrophotometrically at 302 nm (extinction coefficient = 1670 M⁻¹ cm⁻¹). The peroxynitrite stock solution was aliquot and stored at −80°C under nitrogen and used within 3 months.

2.2. DNA Strand Cleavage Assay

DNA strand breaks were measured by the determining the conversion of supercoiled φX-174 RF I double-stranded DNA to open circular and linear forms, according to the method described before [7]. In brief, 0.2 µg DNA was incubated with SIN-1 in the presence or absence of acetaminophen in phosphate-buffered saline (PBS, pH 7.4) in a final volume of 24 µl at 37°C for 60 min. Following the incubation, the samples were immediately loaded in a 1% agarose gel containing 40 mM Tris, 20 mM sodium acetate and 2 mM ethylenediaminetetraacetic acid (EDTA), and electrophoresed in a horizontal slab gel apparatus in Tris/acetate/EDTA gel buffer. After electrophoresis, the gels were stained with a 0.5 µg/ml solution of ethidium bromide for 30 min, followed by another 30 min destaining in water. The gels were then photographed under ultraviolet illumination and quantified using an Alpha Innotech Imaging system (San Leandro, CA, USA).
2.3. Chemiluminometry

SIN-1 autoxidizes at a physiologically relevant pH to give rise to both superoxide (O$_2^−$) and nitric oxide (’NO). Reaction between superoxide and NO forms peroxynitrite.

2.4. Oxygen Polarography

Oxygen consumption caused by SIN-1 autoxidation was monitored with a Clark oxygen electrode (YSI 5300, Yellow Springs, OH, USA) upon mixing SIN-1 in 2.5 ml air-saturated PBS at 37°C in the presence or absence of acetylsalicylic acid. The oxygen consumption caused by SIN-1 was expressed as percentage of saturation oxygen [9].

2.5. EPR SPIN-Trapping

The spin trap 5,5-dimethylpyrrrole N-oxide (DMPO) was used to measure hydroxyl radicals derived from the decomposition of peroxynitrite or the Fenton reaction (H$_2$O$_2$/Fe$^{2+}$) in the presence or absence of acetylsalicylic acid. The electron paramagnetic resonance (EPR) spectra were recorded at room temperature with a spectrometer (Bruker D-200 ER, IBM–Bruker), operating at X-band with a TM cavity and capillary cell, as described by us before [9]. The EPR spectrometer settings were as following: modulation frequency, 86 kHz; microwave frequency, 9.845 GHz; microwave power, 29.8 mW; modulation amplitude, 2.17 G; and scan time, 400 s. The reactants were mixed in test tubes to a final volume of 0.2 ml and then transferred to a capillary cell for EPR spectral acquisition at room temperature under conditions described above.

2.6. Statistical Analysis

All data are expressed as mean ± SD from at least three separate experiments unless otherwise indicated. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. Statistical significance between the values was considered at p < 0.05.

3. RESULTS AND DISCUSSION

3.1. Induction of DNA Strand Cleavage by SIN-1

Induction of single-strand and double-strand breaks of supercoiled φX-174 RF I plasmid DNA led to the formation of open circular and linear forms, respectively. Previous studies showed that autoxidation of SIN-1 at a physiological pH led to the formation of peroxynitrite, which caused DNA cleavage [9]. To set the stage for determining the effects of acetylsalicylic acid on peroxynitrite-induced DNA damage, we first characterized the induction of DNA strand cleavage by SIN-1 at various concentrations. As shown in Figure 2, incubation of φX-174 RF I plasmid DNA with SIN-1 at 25–500 µM for 60 min resulted in the increased conversion of supercoiled DNA (SC) to both open circular (OC) and linear (L) forms in a concentration-dependent manner. Thus,
under the present experimental conditions, SIN-1 autoxidized to form the DNA-cleaving peroxynitrite.

3.2. Inhibition of SIN-1-Mediated DNA Cleavage by Acetaminophen

A high concentration of SIN-1 (250 µM) was chosen to determine the effects of acetaminophen on SIN-1-mediated DNA cleavage. As shown in Figure 3, the presence of acetaminophen significantly inhibited SIN-1-mediated DNA strand cleavage in a concentration-dependent manner. Notably, the ratio of linear form to supercoiled form (L/SC) was significantly reduced by acetaminophen even at 25 µM. As 250 µM SIN-1 caused drastic DNA cleavage, which might be too severe to be pathophysiologically relevant. As such, a lower concentration of SIN-1 (25 µM) was used to cause mild DNA damage. As shown in Figure 4, the presence of acetaminophen at 400 µM nearly completely prevented SIN-1-induced DNA cleavage. The above results thus suggested that acetaminophen could markedly inhibit peroxynitrite-induced DNA damage. Plasma concentrations of acetaminophen can reach up to 100–200 µM after therapeautic dosages [10–12]. Hence, the concentrations
3.3. Inhibition of Peroxynitrite-Dependent CL by Acetaminophen

To investigate the mechanisms underlying acetaminophen-mediated inhibition of SIN-1-induced DNA damage, we next determined the effects of acetaminophen on peroxynitrite-dependent CL. Bicarbonate-enhanced luminal-derived CL is indicative of peroxynitrite formation [8]. As shown in Figure 5, acetaminophen at 25–400 µM nearly completed inhibited the CL response. The nearly complete abolishment of bicarbonate-enhanced CL response from SIN-1 autoxidation by acetaminophen at a concentration as low as 25 µM suggested that this drug might act as a potent scavenger of peroxynitrite. The minimal effects of acetaminophen at 100 and 400 µM on SIN-1-mediated oxygen consumption (Figure 6) indicated that the inhibitory effect of acetaminophen on peroxynitrite-dependent CL was unlikely due to its inhibition of SIN-1 autoxidation. As depicted in Figure 1, consumption of oxygen is a critical step leading to the formation of peroxynitrite by SIN-1.

3.4. Effects of Acetaminophen on Peroxynitrite-Derived Free Radical Production

It has been suggested that decomposition of peroxynitrite may lead to the formation of hydroxyl radicals [13], and this radical formation may be responsible, at least partly, for peroxynitrite-induced DNA cleav-
Hence, we used the DMPO-spin trapping technique to determine the effect of acetaminophen on the above radical formation from peroxynitrite. As shown in Figure 7, incubation of DMPO with authentic peroxynitrite led to the formation of a DMPO-spin adduct characteristic of a hydroxyl radical adduct. Interestingly, the presence of acetaminophen as 100 µM significantly increased the formation of this spin adduct, whereas 400 µM acetaminophen diminished this radical production. It remains unknown how acetaminophen caused this biphasic response. To further investigate the effect of acetaminophen on hydroxyl radicals, we employed the Fenton chemistry to generate hydroxyl radicals. As shown in Figure 8, incubation of DMPO with hydrogen peroxide and Fe²⁺ led to the detection of a strong EPR spectrum characteristic of DMPO-hydroxyl radical adduct. It is well-established that the Fenton reaction between hydrogen peroxide and Fe²⁺ generates hydroxyl radicals. Addition of acetaminophen at 100 and 400 µM significantly inhibited the formation of the hydroxyl radicals, suggesting that acetaminophen might be a potential hydroxyl radical scavenger.

3.5. Conclusion

Taken together, this study demonstrated that acetaminophen at pharmacologically relevant concentra-
tions is able to inhibit peroxynitrite-induced DNA damage in vitro. The results of both chemilumino-
metric and EPR spectrometric experiments also in-
dicated that acetaminophen might directly scavenge
peroxynitrite or the secondary hydroxyl radical gen-
eration, especially at high concentrations. While the
detailed chemical mechanisms of acetaminophen as
an inhibitor of peroxynitrite toxicity remain to be eluci-
dated, the findings of the present study might have
important implications in neuroprotection as well as
other beneficial effects associated with the use of aceta-
maminophen at therapeutic dosage. Further
studies are also warranted to determine if acetami-
nophen protects against peroxynitrite toxicity in cul-
tured cells as well as in vivo animal models.

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