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Comparison of Spectrophotometric, HPLC and Chemiluminescence Methods for 3-Nitrotyrosine and Peroxynitrite Interaction

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We have studied the interaction of 3-nitrotyrosine with peroxynitrite using three different methods; chemiluminescence, spectrophotometry and HPLC. Peroxynitrite-induced luminol or lucigenin chemiluminescence were significantly decreased by 3-nitrotyrosine, in concentration-dependent manners. The intensity of the peroxynitrite spectrum was also markedly reduced in the presence of 3-nitrotyrosine in the spectrophometric assay. However, there was no attenuation of the 3-nitrotyrosine signal in the HPLC assay after mixing with peroxynitrite. The interaction of 3-nitrotyrosine and hypochlorous acid (HOCl) was also studied *via* the chemiluminescence assay, where the HOCl-induced responses were markedly inhibited by 3-nitrotyrosine. These results suggest that caution should be taken when studying the levels or interactions of 3-nitrotyrosine.

Key words: 3-Nitrotyrosine, Peroxynitrite, Spectrophometric assay, Chemiluminescence assay

INTRODUCTION

The formation of reactive oxygen species and their metabolites appear to play a significant role in many pathological states, including inflammation, diabetes, ischemia-reperfusion injury and endotoxemia. Stimulated inflammatory cells represent a major source of oxygen radicals and metabolites (Reilly, 1991). It has been shown that superoxide radicals can react with nitric oxide to form the potent oxidant, peroxynitrite (Beckman, 1990). Peroxynitrite adds a nitro group to the ortho position of the phenolic ring of free and protein-associated tyrosines, to form 3-Nitrotyrosine (3-NT), leaving a detectable marker *in vivo*. This reaction occurs spontaneously, but can also be catalyzed by low molecular mass transition metals, superoxide dismutase or carbon dioxide (Ischiropoulos, 1992; Gow, 1996). Increased levels of 3-NT have been detected in various pathological conditions (Ischiropoulos, 1998; Radi, 2001). 3-NT is a stable product, which can be directly assessed by various methods, including spectrophotometric analysis, HPLC or gas chromatography (GC) and mass spectrometry (MS), depending on the required detection limits, equipment availability and expertise of the operator. ELISA or immunohistochemistry have been widely used to detect 3-NT from tissues (Radi, 2001).

Under conditions where there is concomitant reactive oxygen and nitrogen species formation, such as inflammatory conditions, it is pertinent to determine the direct interaction of these species with 3-NT. In the present study, chemiluminescence (CL), spectrophotometric as well as HPLC methods were used for the assessment of the interaction of 3-NT with peroxynitrite in cell-free systems. Since there is a reported interaction between HOCl and 3-NT in an HPLC assay (Whiteman, 1999), this interaction was also examined by a CL assay in the present study.

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MATERIALS AND METHODS

Reagents

The 3-nitrotyrosine, luminol sodium, lucigenin, catalase (from bovine liver) and sodium hypochlorite were obtained from Sigma Chemical Company (St. Louis, MO, USA). The peroxynitrite was prepared using a quenched flow reaction, as described previously (Beckman, 1994). Briefly,

an aqueous solution of 0.6 M sodium nitrite was rapidly mixed with an equal volume of 0.6 M H_2O_2, containing 0.7 M HCl, and immediately quenched with the same volume of 1.2 M NaOH. All reactions were performed on ice. Excess H_2O_2 was removed by the addition of manganese dioxide (MnO_2) powder to the peroxynitrite solution. The mixture was shaken for 5 min and the MnO_2 then removed by passage over a cellulose acetate disposable filter. The solution was used fresh, or frozen at -20°C for as long as a week. The final concentration of peroxynitrite was determined spectrophotometrically in 1.2 M NaOH (ε_{302}= 1670 M^{-1} cm^{-1}). Dilutions of this peroxynitrite stock solution were made in 1.2 M NaOH, with a final dilution in 0.1 M NaOH, prior to use.

Apparatus

The CL was measured at 37°C using a chemiluminometer (Bio-Orbit 1250 Luminometer, Turku, Finland). The CL produced was measured continuously, and recorded on a computer by the Luminometer 1250 programme (version 1.12, BioOrbit). All UV-visible absorption spectra were recorded using a spectrophotometer (Beckman DU-650, USA) equipped with 1-cm quartz cells. Samples were applied to HPLC (5 μm, C18 reversed phase column 250×4 mm, LiChrospher 100, HP, USA); with a C18 cartridge (5 μm particle-size, LiChrospher ODS, HP, USA) and UV detection (set at 365 nm, HP 1050 series, USA).

Chemiluminescence measurements

Peroxynitrite-induced luminol- and lucigenin-enhanced CL were measured at 37°C, as described previously (Kahraman, 1997; Yıldız, 1998). Phosphate buffered saline (PBS, 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4) was mixed with luminol (250 μM, prepared daily in 2 M NaOH and diluted with PBS) or lucigenin (250 μM, prepared daily in PBS) in a cuvette. After adding catalase (50 U mL^{-1}) to the cuvette to remove the excess H_2O_2 following MnO_2 treatment, 0.1 μM or 0.1 mM peroxynitrite was injected for luminol and lucigenin CL, respectively. The CL produced was measured for 3 minutes (Kahraman, 1997; Yıldız, 1998).

Spectrophotometric measurements

The direct peroxynitrite scavenging activity of 3-NT was also studied with a spectrophotometric assay. Peroxynitrite at a concentration of 1 mM was mixed with PBS (pH=12), and absorbance measured at 302 nm in a spectrophotometer, as described previously (Mouithys-Mickalad, 1998). 3-NT, between 0.1 μM-10 mM, was mixed with PBS containing catalase (50 U mL^{-1}) before the addition of 1 mM peroxynitrite, and the absorbance measured again.

HPLC measurements

3-NT was measured using HPLC, with UV detection, as described previously (Van der Vliet, 1996). 2.5 μL dilutions of 3-NT were analyzed by HPLC, with a mobile phase consisting of 50 mM KH_2PO_4-H_3PO_4 (pH=3) and methanol (75:25, v/v), at a flow rate of 1 ml/min. The 3-NT peak was identified on the basis of the retention time of authentic 3-NT. The detection limit for 3-NT was 0.1 μM in this setting. The peak heights of 3-NT were measured, with the concentrations calculated from a standard curve.

HOCl-induced chemiluminescence

The HOCl was prepared as previously described by Vissers *et al.* (Vissers, 1994). NaOCl was diluted with PBS, and the pH readjusted to 7.4 immediately prior to addition to the CL cuvette. At this pH, the solution will contain approximately 1:1 HOCl plus OCl^-; subsequently referred to as HOCl (Vissers, 1994). HOCl (50 μM) was injected into the PBS and luminol (250 μM) mixture to induce CL, which was continuously measured for 3 min (Yıldız, 1998).

Experimental protocol and statistics

The effects of various concentrations of 3-NT were examined by its addition into the mixture prior to the stimulant. Duplicate assays were performed in all experiments. The results were calculated as the peak CL, a % of the peak CL or absorbance, and expressed as the mean ± SEM. n refers to the number of experiments. An analysis of variance (ANOVA) was used to assess the observed differences between concentrations. If significant differences were detected by ANOVA, individual means were compared, with control, using a Student-Newman-Keuls test. Differences were considered to be statistically significant when the P value was less than 0.05.

RESULTS

Chemiluminescence assay

Peroxynitrite at a concentration of 0.1 μM produced a 6615 ± 163 mV (n=15) signal in the presence of luminol. However, the lucigenin CL was less sensitive, and only a 1610 ± 134 mV (n=8) signal was measured with 0.1 mM peroxynitrite. 3-NT showed marked inhibition of both the peroxynitrite-induced luminol (99.9 ± 0.0%, n=8, 10^{-2} M) and lucigenin CL (98.8 ± 0.1%, n=6, 10^{-2} M). 3-NT started to inhibit the peroxynitrite signal at 10^{-5} M, and led to complete inhibition at 10^{-2} M, with IC_{50} values of 7.5×10^{-5} and 2.5×10^{-5} M for luminol and lucigenin, respectively (Fig. 1).

Spectrophotometric assay

The interaction of 3-NT with peroxynitrite has been

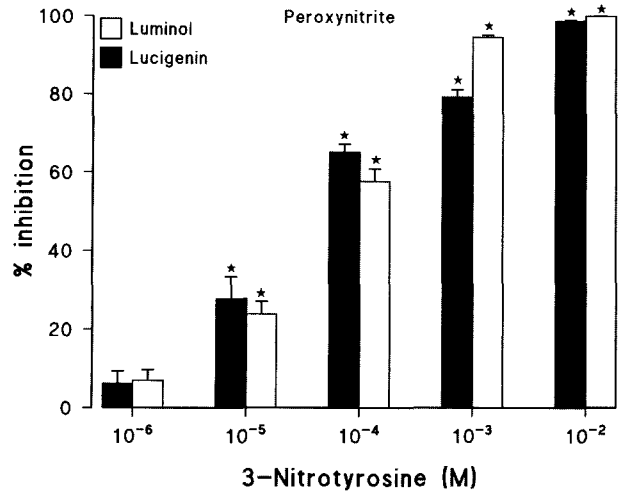

Fig. 1. Concentration-dependent effects of 3-NT on peroxynitrite (0.1 μM)-induced luminol (n=6-13) and lucigenin (0.1 mM peroxynitrite, n=5-12) CL. *P<0.05 compared with control values.

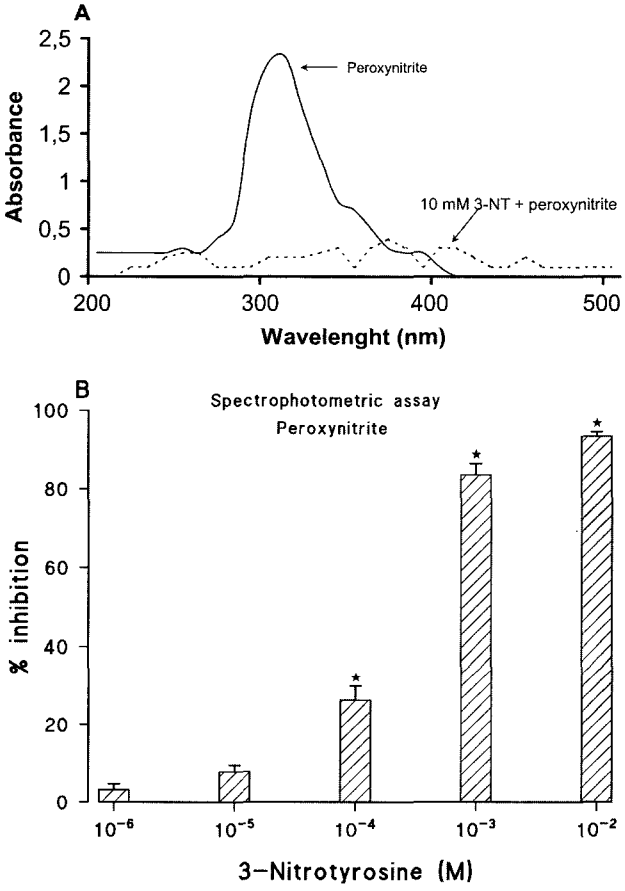

Fig. 2. Absorbance spectrum of peroxynitrite [1 mM) in alkaline solution (pH=12) and after reaction with 3-NT (10^{-2} M) (A). Concentration-dependent effects of 3-NT on absorbance are shown in B (n=6-8). *P<0.05 compared with control values.

examined by spectrophotometric and CL methods. Peroxynitrite at a concentration of 1 mM generated a

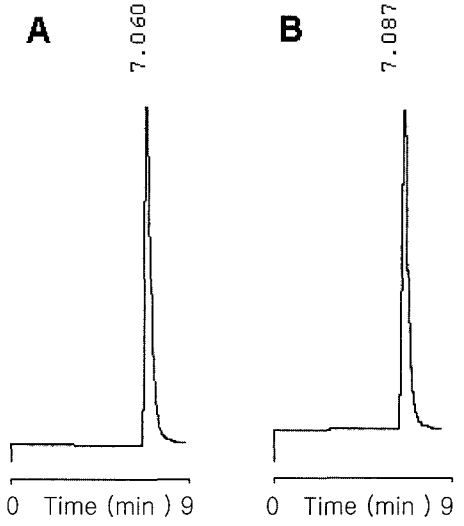

Fig. 3. Representative HPLC chromatogram of 3-NT (10^{-2} M) alone (A) and after mixing with 1 mM peroxynitrite (B).

peak at 302 nm in spectrophometric assay, with 3-NT producing concentration-dependent inhibition of the peroxynitrite-induced absorbance. A significant decrease was observed to start at a concentration of 10^{-4} M (26 ± 4%, n=6), with maximum inhibition obtained at the highest 3-NT concentration (10^{-2} M) used in this study (93 ± 1%, n=8) (Fig. 2A and B).

HPLC assay

Fig. 3 shows representative HPLC chromatograms of 3-NT alone and after mixing with peroxynitrite. There were no changes in the 3-NT (10^{-2} M) signals in the presence of 1 mM peroxynitrite (n=5), suggesting there is no interaction between 3-NT and peroxynitrite in this assay.

HOCl-induced chemiluminescence

Concentrations of 50 μM and 0.1 μM were selected for the HOCl and peroxynitrite-induced CL, respectively, and generated comparable luminol CL peaks (6820 ± 510 mV, n=7, HOCl, and 6615 ± 163 mV, n=15, peroxynitrite). Similar to peroxynitrite, only high concentrations of 3-NT caused a marked inhibition of the HOCl-induced luminol CL. Marked inhibitions of the CL signal were recorded at concentrations of 10^{-4} M and higher, with an IC_{50} value 1.5×10^{-4} M. Total inhibition (100 %, n=5) occurred at 10^{-2} M 3-NT (Fig. 4).

DISCUSSION

In the present study, in vitro experimental evidence was obtained of the 3-NT inhibition of the peroxynitrite CL signal in the presence of chemically different probes, i.e. luminol and lucigenin. Additionally, 3-NT also depressed

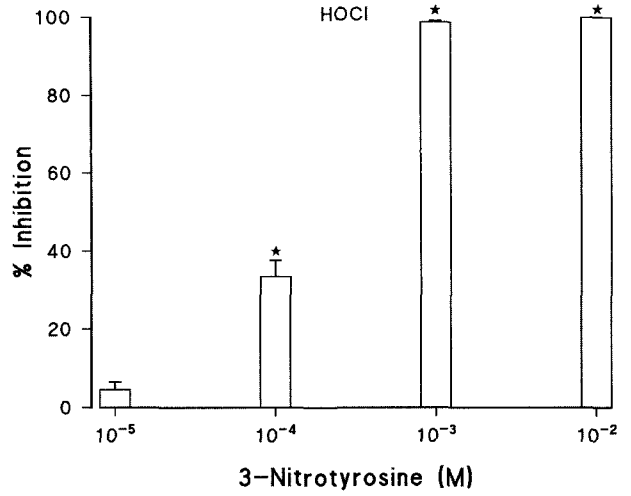

Fig. 4. Concentration-dependent effects of 3-NT on HOCl 50 μM)-induced luminol CL (n=5-7). *P<0.05 compared with control values.

the peroxynitrite absorbance in the spectrophotometric assay in the absence of any probe. However, peroxynitrite did not inhibit the 3-NT signal in the HPLC assay, and showed no interaction. Our HPLC results support the previous finding of Whiteman and Halliwell (Whiteman, 1999), who showed there was no loss of 3-NT in the presence of peroxynitrite, as studied by the HPLC method. However, marked concentration-dependent inhibitions were recorded in the CL and spectrophotometric assays in our study. The reasons for these differences are not known. These results suggest that one should be cautious when using CL and spectrophotometric assays to study the effects of 3-NT.

UV-visible spectra resulting from the reaction of peroxynitrite with free radical scavengers are commonly used in studies (Mouithys-Mickalad, 1998, 2000; Kohnen, 2003). Peroxynitrite-induced chemiluminescence is also used for screening the antioxidant activity of drugs (Valdez, 2004; Thiry, 2004). Additionally, peroxynitrite is believed to be one of the most efficient tyrosine-nitrating species of biological relevance so far identified. It is known that metal-containing compounds can also enhance the formation of nitrotyrosine through a bimolecular reaction with peroxynitrite. Moreover, if the metal is regenerated in the reaction, the compound is considered as a nitration catalyst, as the yield of tyrosine nitration can be enhanced several fold. Mn-superoxide dismutase, some cytochromes and several metalloporphyrins may serve as examples of peroxynitrite-dependent nitration catalysts (Pietraforte, 2003). Therefore, nitrotyrosine generation may interfere with the spectrophotometric and chemiluminescence methods. The results of our experiments suggest that such an interaction may possibly occur during *in vitro* experiments.

The detection and quantification of 3-NT have been employed in published studies using a variety of methods, including immunoassays, HPLC with a variety of detection methods, and gas or liquid chromatography coupled to mass spectrometry. HPLC is a powerful separation technique, but it must be combined with a detection method for quantitative analysis. UV (Crow, 1996), fluorescence (Kamaisaki, 1996), and electrochemical detection (Duncan, 2003) have been used to measure 3-NT. We also used HPLC with UV detection, and in this study compared the results with CL and spectrophotometric assays. A number of studies are available on 3-NT data in tissues and fluids from antibody-based methods. Immunohistochemical staining methods (Viera, 1999) have been used to demonstrate increased levels of 3-NT, such as in lung tissue from patients with cystic fibrosis (Morrissey, 2002). ELISA assays (ter Steege, 1998; Oldreive, 2001) and solid phase immunoradiochemical methods to measure 3-NT have also been developed (Banks, 1998). In all these studies, in the absence of rigorous assay validation, the differential appearance of immunoreactive proteins (i.e., nitrated proteins) in disease or treatment samples provides the investigators with some confidence in the selectivity of the assay. Another method involving the measurement of 3-NT in tissue and blood proteins was performed using gas chromatography with thermal energy analyzer (TEA) detection. Free and protein-bound tyrosine residues easily react with nitrating/nitrosating agents to yield 3-NT (Ohshima, 1990; Duncan, 2003). However, TEA detection does not offer the selectivity and sensitivity necessary to measure 3-NT levels in basal samples (Duncan 2003). Mass spectrometry is less readily accessible, and requires skilled operators and expensive hardware. However, this method has a unique benefit when applied to the analysis of 3-NT. Mass spectrometry is most frequently coupled with one of two commonly adopted chromatographic options, GC or HPLC, and GC-MS assays have been developed for 3-nitrotyrosine determinations (Crowley, 1998; Jiang, 1998; Frost, 2000; Gaut, 2002; Duncan 2003).

3-NT or its metabolites are not biologically inactive products, as systemic administration of 3-NT, which is also structurally similar to L-DOPA, selectively inhibits the subsequent hemodynamic responses to α1- and β-adrenoceptor agonists in anaesthetized rats (Kooy, 1996). In addition to the role of 3-NT in hemodynamic dysfunction, 3-NT levels are measured as a marker of endogenous peroxynitrite formation (Van der Vliet, 1996). It has been reported that high concentrations of peroxynitrite produce vascular and endothelium dysfunctions, lipid peroxidation and DNA damage (Beckman, 1994). Plasma 3-NT levels are not detectable in healthy volunteers, but high levels (about 120 μM) have been measured in renal patients

with septic shock (Fukuyama, 1997). The results of the present study may have implications when detecting 3-NT levels in disease conditions.

It has been demonstrated that 3-NT does not interact with H_2O_2, hydroxyl radical or superoxide (Whiteman, 1999). We also showed there was a marked inhibition of the HOCl signal in the presence of 3-NT in the CL assay. Our result with HOCl is in good agreement with those of previous experiments showing HOCl caused significant and rapid loss of free and protein bound 3-NT, as measured by an HPLC method (Whiteman, 1999).

In conclusion, these data strongly suggest a direct interaction between 3-NT and HOCl. However, caution should be taken when studying the effect of 3-NT, and conclusion should only be drawn after different assay methods have been studied. The direct interaction of 3-NT with HOCl may form other metabolites or reactive products, and will require further investigation.

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