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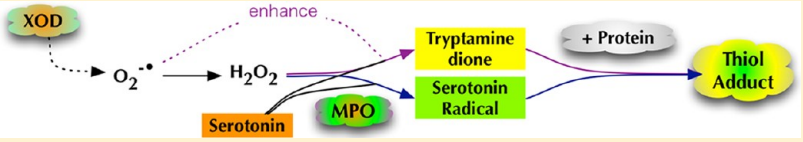
Myeloperoxidase Catalyzes the Conjugation of Serotonin to Thiols via Free Radicals and Tryptamine-4,5-dione

Yoji Kato,*[,†] Alexander V. Peskin,[‡] Nina Dickerhof,[‡] D. Tim Harwood,[‡] and Anthony J. Kettle[‡]

[†]School of Human Science and Environment, University of Hyogo, Himeji 670-0092, Japan

[‡]Centre for Free Radical Research, Department of Pathology, University of Otago Christchurch, Christchurch, New Zealand



ABSTRACT: Serotonin (5-hydroxytryptamine; 5HT) is a favorable substrate for myeloperoxidase and is likely to be oxidized by this heme enzyme during inflammation. In this study, we have investigated how serotonin becomes conjugated to amino acid residues and proteins when it is oxidized by myeloperoxidase. 5HT formed three adducts with *N*-acetylcysteine (NAC) when it was incubated with myeloperoxidase, xanthine oxidase, and acetaldehyde. One of the adducts was identified as 5HT-NAC, and the others were conjugates of NAC and tryptamine-4,5-dione (TD). There was no evidence for coupling of oxidized serotonin to amine residues. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was exposed to 5HT with the enzymatic system or synthetic TD. Both caused a loss of thiols on GAPDH and covalent attachment of quinones derived from TD to the protein. Biotin-labeled 5HT was used instead of 5HT to confirm the conjugation of 5HT to GAPDH. It was incorporated into the GAPDH when oxidized by myeloperoxidase. Analysis of tryptic peptides of human GAPDH by liquid chromatography with mass spectrometry revealed that an adduct of TD was formed with the peptide containing Cys^{152} and Cys^{156}. Our results indicate that myeloperoxidase can oxidize serotonin to species that form adducts with low molecular weight thiols and cysteine residues in proteins. Low molecular weight conjugates will redox cycle and fuel oxidative stress. Conjugation of serotonin to proteins will affect their function and may provide useful biomarkers of serotonin oxidation during inflammatory events.

INTRODUCTION

Serotonin (5-hydroxytryptamine; 5HT) is secreted into the blood from enterochromaffin cells in the gut, where it is avidly taken up and stored by platelets.[1] It is released from platelets in response to numerous signals including contact with damaged endothelium.[2] 5HT aids in hemostasis by exerting a direct vasoconstrictor effect.[3] It also causes endothelial cells to release nitric oxide. The interplay of 5HT and nitric oxide on smooth muscle cells results in either vasoconstriction or vasodilation.[2] These effects on vascular tone will depend on the local concentration of 5HT. Perturbation of the normal physiological concentration of 5HT can result in cardiovascular dysfunction.[3,4] This may arise from oxidation of 5HT by myeloperoxidase (MPO), which is attached to the endothelium.[5] Platelets have been considered to be an extension of neutrophils because they bind to these phagocytic cells, leading to the transcellular synthesis of numerous inflammatory molecules.[6] Oxidation products of 5HT may be included in this group because 5HT as well as MPO and hydrogen peroxide will be released from these cells when they interact at sites of inflammation and during thrombosis.[7] 5HT is a preferred substrate for this heme enzyme and under suitable physiological conditions should be oxidized by MPO.[8,9]

Although chloride is the physiological substrate for MPO, 5HT is oxidized because it reacts rapidly with the redox intermediates of the enzyme.[8] The initial oxidation product is the free radical of 5HT, which is eventually transformed into tryptamine-4,5-dione (TD) via superoxide-dependent and -independent pathways.[9] Thus, oxidation of 5HT by MPO has the potential to contribute to the sequel of inflammation. Their interactions may explain the associations of both MPO and 5HT with adverse outcomes in cardiovascular disease.[10−12] Reactive quinones similar to those derived from 5HT have been implicated in neurodegenerative diseases and are likely to be associated with the oxidative stress that is commonly linked to these pathologies.[13] Indeed, TD is thought to be a potent neurotoxin.[14,15]

When incubated with isolated neutrophils, 5HT formed adducts with their proteins in a reaction that was partially dependent on MPO and hydrogen peroxide.[16] Thus, at sites of inflammation where neutrophils and platelets interact, MPO may modulate the function of proteins by promoting the attachment of 5HT. Such adducts may also prove useful as biomarkers of oxidative stress and inflammation. To date, however, the mechanism by which MPO catalyzes attachment of 5HT to residues on proteins and the chemical nature of these adducts is unknown. It has been shown that TD forms adducts with thiols, including cysteine residues on proteins.[15,17] Other possibilities include the attachment of 5HT radicals to thiol and/or amine groups. Therefore, we have determined

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whether MPO catalyzes the attachment of 5HT to amino acid residues and identified the adducts that are formed.

EXPERIMENTAL PROCEDURES

Materials. Human MPO was obtained from Planta Natural Products (Vienna, Austria). Xanthine oxidase (XOD, type X4500), *N*-acetylcysteine (NAC), *Nα*-acetyl-lysine, *Nα*-acetyl-histidine, 5HT (HCl form), catalase, allopurinol, nitroblue tetrazolium (NBT), sodium dodecyl sulfate (SDS), potassium nitrosodisulfonate, diethylenediaminetetraacetic acid (DTPA), streptavidin-peroxidase, and Cu/Zn-superoxide dismutase (SOD) were purchased from Sigma. Glyceraldehyde-3-phosphate dehyderogenase (GAPDH) from rabbit muscle was obtained from ICN and Sigma. Iodoacetamide-fluorescein (IAF) was purchased from Invitrogen. Sulfosuccinimidyl-6-(biotinamido)hexanoate (sulfo-NHS-LC-biotin) and *N*-iodoacetyl-*N*-biotinylhexylenediamine (IAB) were purchased from Pierce. Acetaldehyde (ALD) was obtained from BDH laboratory supplies.

Synthesis of Biotinylated 5HT. 5HT (5 mg) was reacted with 25 mg of sulfo-NHS-LC-biotin in 0.1 M borate buffer (pH 9.0) for 24 h. The reaction mixture was injected into a high-performance liquid chromatography (HPLC, Shimadzu Prominence) using a Combi-RP column (Nomura Chemical Co., 20 mm × 100 mm), which was equilibrated with 0.1% acetic acid/CH_3CN (3/1) at a flow rate of 5 mL/min. A major peak was collected and concentrated. Structural identification was carried out by infusing the material into a mass spectrometer (AB Sciex API3000), which gave m/z ratios of 516 $[M + H]^+$, 498 $[M + H - H_2O]^+$, 340.4 $[M + H - 5HT]^+$, and 227 [Biotin-LC]$^+$. The concentration of 5HT−biotin conjugate was calculated from UV 290 nm intensity by comparison with standard 5HT.

Synthesis of Tryptamine Dione. TD was synthesized daily according to the published method with some modifications.[18,19] 5HT·HCl (1 mg) was dissolved in 500 μL of water, and then, 6.3 mg of potassium nitrosodisulfonate was added and mixed. After 25 min of incubation at room temperature, the purple reaction mixture was applied to a solid-phase extraction (SPE) column (C18, Alltech, 3 mL). The column was washed with 2 mL of water, and the purple compound was then eluted with 1 mL of 1% formic acid/75% methanol. The purity of TD was confirmed as >99% by reversed phase HPLC as described below. The concentration of TD was calculated by measuring its absorbance at 540 nm (ε_{540} = 880 M^{-1} cm^{-1}).[15,18] The TD was kept at 4 °C before use. Synthetic biotinylated 5HT was also treated with potassium nitrodisulfonate similarly and then purified by SPE using 1% formic acid/75% CH_3CN to eluate the bound material. The synthetic TD-biotin had m/z ratios 530 $[M + H]^+$, 340 $[M + H - 5HT]^+$, and 227 [Biotin-LC]$^+$. The concentration of TD-biotin was calculated by measuring its absorbance at 540 nm using the absorption coefficient for TD.

Reaction Conditions for NAC with MPO/XOD/ALD/5HT. The continuous system contained MPO (50 nM), XOD (1/1000), 5HT (0.1 mM), and NAC (1 mM) in 50 mM phosphate buffer (pH 7.4), and the reactions were started by adding 10 mM ALD. The generation of superoxide by XOD in the system was 3.8 μM/min as measured by the cytochrome *c* assay.[20] The reaction was terminated by the addition of allopurinol (0.1 mM), and then, the enzymes were removed by filtration using an Ultrafree MC30000 (Millipore, 5000*g*, 12 min). It is noteworthy that the removal of enzymes was a critical step for preventing an undesirable further reaction.

The discontinuous system contained MPO (50 nM), XOD (1/1000), and 5HT (0.1 mM) in 50 mM phosphate buffer (pH 7.4), and reactions were started by adding ALD (10 mM) (volume, 100 μL). After 10 min, the reaction was filtered using the Ultrafree MC30000 (5000*g*, 12 min). Twenty microliters of 10 mM of NAC aqueous solution, or water as a blank, was added to the receiver of the filtration apparatus to determine whether reactive species were present in the filtrate.

Reaction Conditions for GAPDH with MPO/XOD/5HT. The continuous system contained MPO (50 nM), XOD (1/1000), 5HT or 5HT-biotin (0.1 mM), and GAPDH (0.7 mg/mL) in 50 mM phosphate buffer containing 1 mM DTPA (pH 7.4), and reactions were started by adding ALD (10 mM). After 10 min, the reaction was terminated by the addition of allopurinol (0.1 mM).

The discontinuous system contained MPO (50 nM), XOD (1/1000), and 5HT or 5HT-biotin (1 mM) in 50 mM phosphate buffer (pH 7.4), and reactions were started by adding and ALD (10 mM) (volume 100 μL). After 5 min, the solution was filtrated by Ultrafree MC30000 (5000*g*, 12 min). Before the centrifugation, 20 μL of a solution containing GAPDH was added to the receiver of the Ultrafree MC apparatus to determine whether reactive species were present in the filtrate.

HPLC Analysis of 5HT Oxidation Products. Samples were analyzed using an HPLC (Waters 2690 alliance separation module) with a photodiode array detector (Waters 996 PDA). Analytes were separated using a Luna C18 column (4.6 mm × 250 mm) with a flow rate of 0.8 mL/min. The eluant consisted of solvent A (5 mM ammonium acetate/1% acetic acid) and solvent B (100% CH_3CN), which were mixed according to the following gradient: 0−5 min (100% A), 5−25 min (100−85% A), 25−30 min (85−50% A), 30−35 min (50% A), and 36−50 min (100% A).

Structural Analyses of 5HT Oxidation Products and Adducts by Liquid Chromatography/Mass Spectrometry (LC/MS). The mass spectrometric analyses of products were performed with an ion-trap mass spectrometer (LCQ-DECA) with reversed-phase HPLC (LC/MS). Analytes were separated using a Luna C18 column (2 mm × 150 mm) with a flow rate of 0.2 mL/min. The solvents and gradient program were the same as described above for the HPLC-PDA.

Thiol Estimations. Visualization of thiols in polyacrylamide gels was performed as described previously.[21] Briefly, protein samples were incubated with 0.2 mM IAF for 10 min in the dark and then mixed with loading buffer. The sample was applied to nonreducing 12% SDS−polyacrylamide gel electrophoresis (PAGE). The gels were scanned using a Bio-Rad Molecular Imager FX (Bio-Rad Laboratories) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The protein in the gel was then stained with Coomassie brilliant blue.

To detect thiols on a blotted membrane, protein was incubated with 0.25 mM IAB for 30 min in the dark and then applied to a microbiospin column (Bio-Rad). The biotin-labeled protein sample was then applied to two gels. One gel was blotted on to a polyvinylidene difluoride (PVDF) membrane as described below. The other gel was used for protein staining using a silver stain kit (Kanto Chemical Co.).

Quinone Staining. The proteins in gels were blotted onto a PVDF membrane, and quinones on the membrane were stained using NBT (0.24 mM) in 2 M glycine-NaOH (pH 10) as described previously.[22] Detection was carried out using a Fluor-S (Bio-Rad Laboratories).

Blotting and Detection of Biotin-Incorporated Protein. The modification of GAPDH by the continuous system was done as described previously. Synthetic TD-biotin (0−50 μM) was reacted with GAPDH (0.7 mg/mL) in the 50 mM phosphate buffer. After treatment of a spin column, the proteins were mixed with a loading buffer and applied to the PAGE using two gels. The biotinylated proteins in one gel were blotted onto a PVDF membrane. The membrane was incubated with 1% Block Ace (DS Pharma Biomedical Co.) for 30 min and then reacted with streptavidin-peroxidase polymer (1/100000) at room temperature for 30 min. The chemiluminescent detection was done by ECL plus (GE Healthcare Bioscience) or Dura (Pierce) as according to the methods by manufacturer's recommendation. The other gel was stained by Sypro-Orange (Invitrogen) for protein staining.

Estimation of Stability of Biotinylated 5HT-GAPDH Adducts. GAPDH was modified by MPO/XOD/ALD in the presence of 5HT-biotin (continuous system) and then applied to a microspin column. The protein fraction was diluted with phosphate-buffered saline (PBS) at a concentration of 0.01 mg/mL. The sample was incubated at 4 or 37 °C for 0−8 h. After the various incubations, an aliquot of the sample was taken and frozen at −20 °C until the following analysis. The remaining/binding of biotin probe on the GAPDH was confirmed as follows. The sample (50 μL) was coated onto a microplate at 4 °C overnight. The plate was washed with 0.01% Tween containing PBS

and then reacted with the streptavidin-peroxidase in 1% bovine serum albumin-PBS for 30 min at room temperature. The plate was washed, and 3,3′-5,5′-tetramethylbenzidine reagent (Kirkegaard & Perry Laboratories, Inc.) was then added. Color development was terminated by the addition of 1 N phospholic acid and measured at 450 nm by a microplate reader.

Reaction of Human GAPDH with TD and Its Analysis by LC/MS. Human recombinant GAPDH (Creative BioMart, Shirley, NY) was reduced for 30 min with 15 mM DTT. Excess DTT was removed using a Micro Bio-Spin 6 column (Bio Rad), which was pre-equilibrated with 100 mM phosphate buffer, pH 7.5. GAPDH (17 μM) was incubated with TD (0.7 mM) for 30 min and then with 20 mM *N*-ethylmaleimide (NEM) for 15 min to alkylate unreacted thiols. Samples were either used directly for LC/MS of the whole protein, or excess reagents were removed using a Micro Bio-Spin 6 column pre-equilibrated with 25 mM bicarbonate buffer, pH, before digesting with trypsin.

To determine modifications of undigested GAPDH, 5 μg of protein was loaded onto a Jupiter C-18 HPLC column (150 mm × 2 mm, 5 μm, 100 Å, Phenomenex, Torrance, CA) for HPLC-MS analysis using a Surveyor HPLC system (Thermo Scientific, San Jose, CA). An acetonitrile gradient from 90% solvent A (0.1% formic acid in water) to 50% solvent B (0.1% formic acid in acetonitrile) was run over 8 min at a flow rate of 200 μL/min. After each gradient, solvent B was held at 50% for 8 min followed by column equilibration over 8 min with 90% solvent A. Voltage was 5 kV, and nitrogen gas flow was 20. The HPLC was coupled inline to an electrospray ionization source of a Velos Pro mass spectrometer (Thermo Scientific). Mass spectral data were acquired from 8 to 16 min of each chromatographic separation scanning between 410 and 2000 m/z in positive ion mode at a normal scan rate. Mass spectra were averaged over the full length of each protein peak and deconvoluted to yield the molecular mass using ProMass for Xcalibur (Version 2.8, Novatia LLC, Monmouth Junction, NJ).

For analysis of tryptic peptides, trypsin (Promega) was added to treated GAPDH at a ratio of 1/20 w/w in 25 mM bicarbonate buffer, pH 8, and then incubated for 4 h at 37 °C. Samples were adjusted to a volume of 60 μL with water containing 0.1% FA. Fifty microliters of sample was loaded onto a Jupiter 4 μm C-12 HPLC column (150 mm × 2 mm, 100 Å, Phenomenex) for HPLC-MS using the LC-MS system described above. An acetonitrile gradient from 98% solvent A (0.1% formic acid in water) to 98% solvent B (0.1% formic acid in acetonitrile) was run over 55 min at a flow rate of 200 μL/min. After each gradient, solvent B was held at 98% for 5 min followed by column equilibration over 10 min with 98% solvent A. CID-MS/MS spectra were acquired from 200 to 2000 m/z in positive ion mode for selected precursor ions from 2 to 70 min of each chromatographic separation. The precursor ions represented the doubly or triply charged species of the cysteine containing GAPDH tryptic peptides with varying numbers of added NEM or dione groups as well as one reference peptide containing no oxidizable groups. The window for precursor selection was 3 Da on either side of the m/z value. The normalized collision energy was 35 eV, and the activation time was 10 ms. In the same run, multiple reaction monitoring data for quantification were acquired for the unreacted cysteine containing peptides as well as the control peptide.

RESULTS

MPO-Dependent Formation of Adducts between 5HT and Amino Acid Residues. Initially, we investigated what products were formed when MPO oxidized 5HT in the presence of NAC. We chose to use NAC as a model of cysteine residues on proteins because it has a single thiol group only that can react with oxidized products of 5HT. Thus, the products formed should involve the nucleophilic sulfur and not be potentially confounded by the amine groups that are present in either cysteine or glutathione. XOD was used as a source of hydrogen peroxide and superoxide because it is a good mimic of the NADPH-oxidase, which normally provides MPO with these substrates. As we have previously demonstrated,[9] MPO and the XOD system readily oxidized 5HT and converted it to its dimer and TD, which were identified by HPLC (Figure 1A). The dimer, which eluted at 4 min, was identified by its characteristic UV absorbance peaks at 278 and 304 nm and its m/z ratio of 351.[23] The peak at 17 min coeluted with synthetic TD and had the same absorbance maxima at 354 and 540 nm as well as an identical m/z ratio of 191.1 for its $[M + H]^+$ parent ion.

In the presence of NAC, very little dimer or TD was detected. However, three new products were detected by HPLC (Figure 1B). These were identified by mass spectrometry as a conjugate of 5HT and NAC (peak A; retention time, 20 min; $[M + H]^+$ 338 m/z) and adducts with TD containing one (peak B; retention time, 23 min; $[M + H]^+$ 352 m/z) and two molecules of NAC (peak C; retention time, 24 min; $[M + H]^+$ 513 m/z). These products have been identified before when 5HT underwent oxidation in the presence of NAC.[24] Further confirmation that the products in peaks B and C were adducts formed between TD and NAC was that they were also generated by incubation of synthetic TD with NAC (data not shown). Prolonged incubation of the complete reaction system caused the loss of TD-NAC and increased formation of TD-2NAC (Figure 1C) but minimal change in 5HT-NAC. The addition of *N*-α-acetyl-lysine or *N*-α-acetyl-histidine (1 mM) instead of NAC to the reaction system had no effect on production of the 5HT dimer or TD (data not shown). This result demonstrates that the oxidation products of 5HT react most avidly with thiol groups and show relatively little reactivity with amines.

Each component was individually excluded from the reaction system to confirm that they were all required for oxidation of 5HT and production of dimer and 5HT-NAC (Figure 2A). The complete system caused the loss of approximately 90% of 5HT and the formation of dimer and 5HT-NAC. Exclusion of MPO, 5HT, XOD, or ALD resulted in minimal loss of 5HT and formation of products. Exclusion of NAC resulted in the loss of 5HT but formation of dimer only. Thus, all components of the reaction system were required to enable 5HT to form adducts with NAC.

To clarify the mechanism of oxidation of 5HT and formation of 5HT-NAC, various scavengers and inhibitors of reactive oxygen species and enzymes were added to the reaction system (Figure 2B). The reaction was inhibited by catalase, which converts hydrogen peroxide to oxygen and water. It was also inhibited by allopurinol and azide, which poison XOD and heme proteins, respectively. These results establish that hydrogen peroxide generated by xanthine oxidation is required by MPO to promote the conversion of 5HT to adducts with NAC. The addition of SOD enhanced the loss of 5HT and generation of 5HT-NAC. These results indicate that superoxide can modulate the loss of 5HT and formation of its oxidation products.

We also determined the effect of reagent hydrogen peroxide on the formation of the dimer and adduct by MPO (data not shown). There was a similar loss of 5HT and formation of products as observed with XOD in the presence of SOD (Figure 2B). In the absence of NAC, there was almost complete loss of 5HT and a yield of dimer similar to that observed with the XOD system without NAC (Figure 2A). No products were formed by MPO in the absence of hydrogen peroxide. Thus, we conclude that hydrogen peroxide is required for the generation

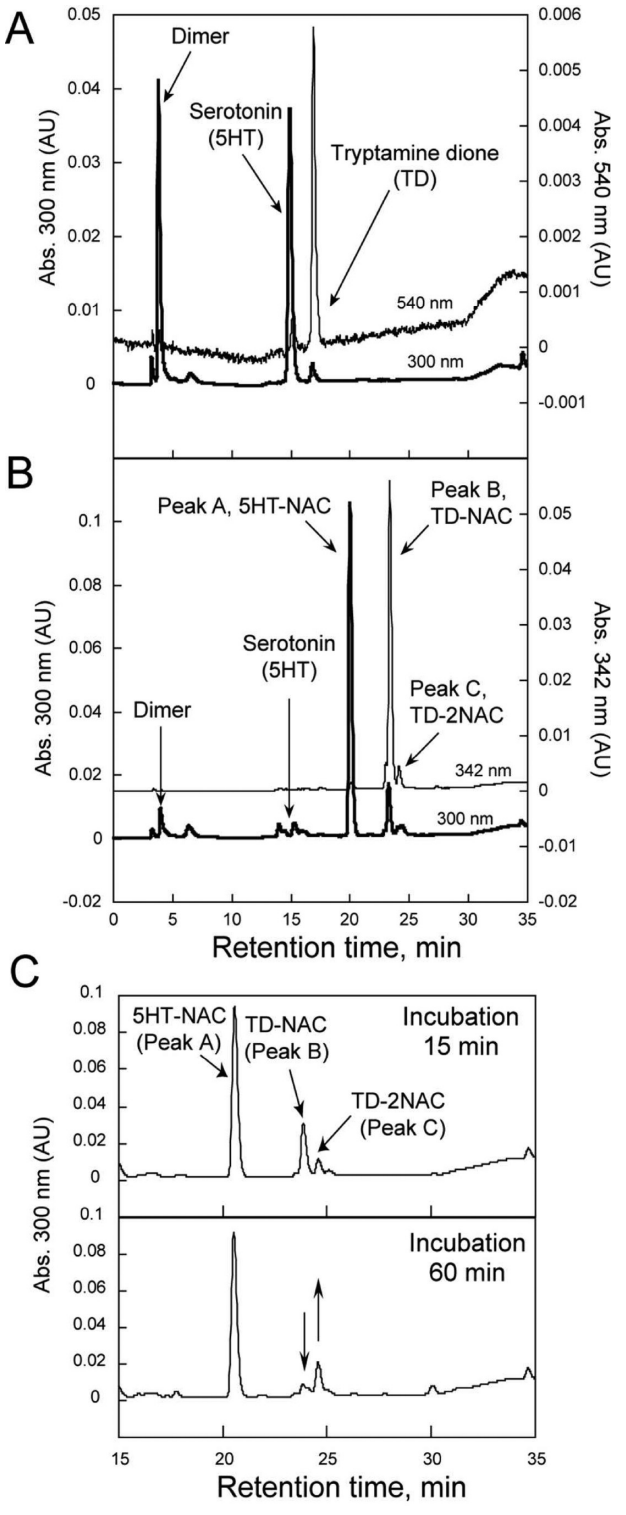

Figure 1. Detection of oxidation products of 5HT. (A) 5HT (100 μM) was incubated in 50 mM phosphate buffer, pH 7.4, containing MPO (50 nM), XOD, and 100 μM DTPA. Reactions were started by the addition of 10 mM ALD. The rate of superoxide production was 3.8 μM/min. After 10 min, the reaction mixture was injected into the HPLC, and elution of 5HT and its oxidation products was monitored at 300 and 540 nm using a diode array detector. (B) Oxidation of 5HT was carried out as in panel A except that 1 mM NAC was included in the buffer and oxidation products detected at 300 and 342 nm. (C) The reaction system with NAC was also analyzed at 15 (upper trace) and 60 min (lower trace) with monitoring at 300 nm.

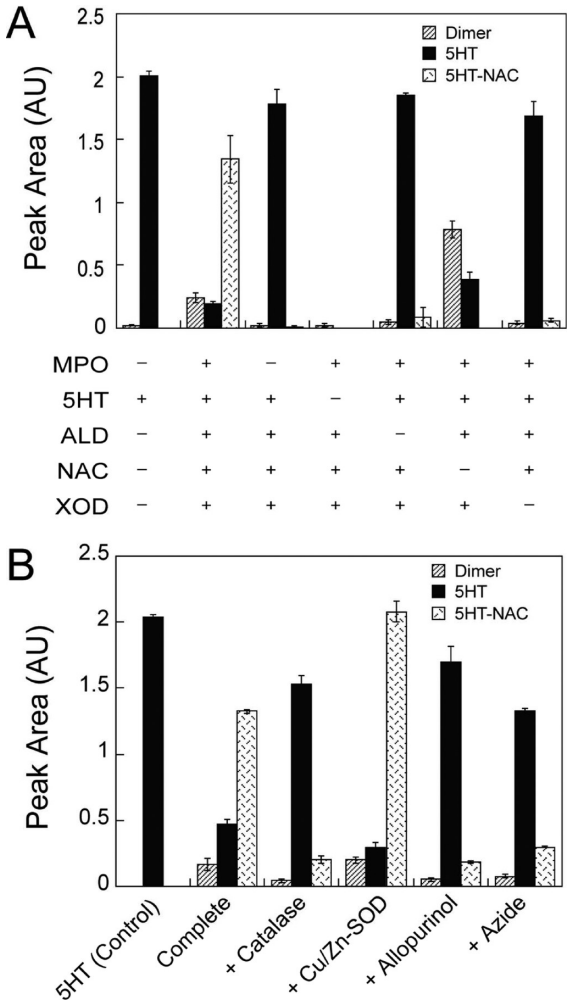

Figure 2. Analysis of essential factors for oxidation of 5HT by MPO in the presence of NAC. (A) Reactions were carried out under the conditions described in Figure 1B, and oxidation of 5HT (black bar) and formation of dimer (hatch bar) or 5HT-NAC (speckled bar) were determined for reactions containing all components or without a single component as indicated. (B) As in panel A but reactions were also carried out in the presence of 100 μg/mL catalase, 10 μg/mL SOD (Cu/Zn-SOD), 100 μM allopurinol, or 1 mM azide. Data are means and SDs of three independent experiments.

of these products, but the flux of hydrogen peroxide does not influence the relative yield of products.

The time course was followed for loss of 5HT and formation of dimer and 5HT-NAC when 5HT was oxidized by MPO, XOD, and ALD in the presence of NAC (Figure 3A). 5HT was completely lost from the reaction system within 10 min. Its loss was mirrored by the formation 5HT-NAC, which ceased at 10 min. The formation of the dimer reached a maximum within a minute, but after 10 min, it was lost from the system. This latter result indicates that production and loss of the dimer must reach a steady state, while 5HT is still present. However, once all of the 5HT is gone, further oxidation of the dimer also results in its elimination from the system.

Increasing the concentration of NAC in the reaction system enhanced the loss of 5HT and formation of 5HT-NAC (Figure 3B). It also decreased the formation of dimer. These results suggest that the 5HT radical is a common intermediate for both the dimer and the 5HT-NAC. By reacting with this radical, NAC would prevent dimer formation while enhancing the

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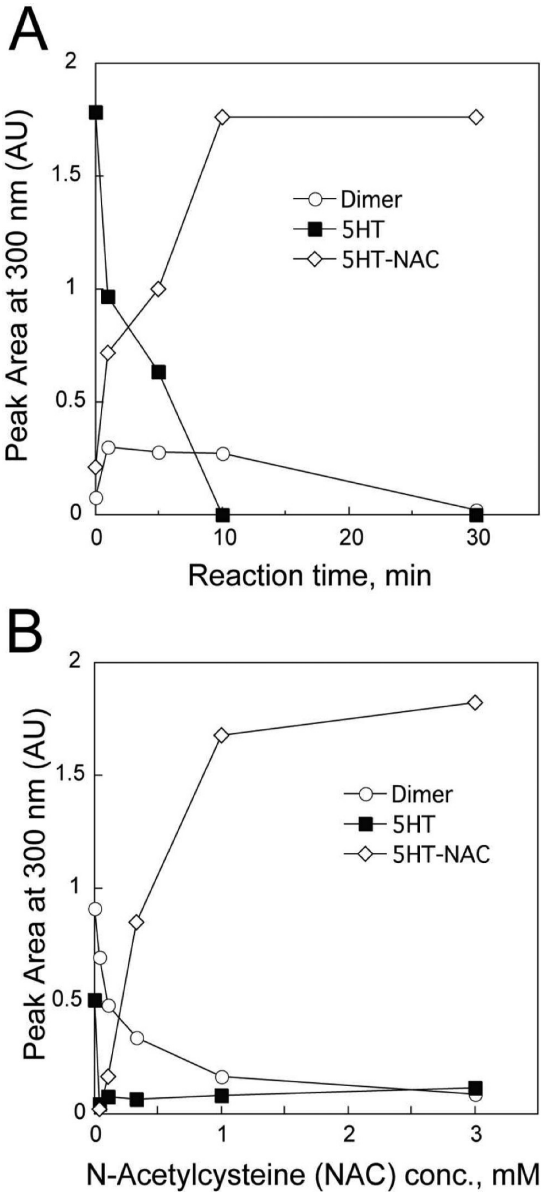

Figure 3. Kinetics and dependence of 5HT oxidation by MPO on the concentration of NAC. (A) The time courses for formation of dimer and 5HT-NAC as well as the loss of 5HT were followed by HPLC. Reaction conditions were as described in Figure 1 except reactions were stopped by adding 100 μM allopurinol to inhibit XOD and then filtered to remove proteins. (B) Reactions were run under the conditions described in Figure 1 except that the concentration of NAC was varied. They were stopped after 10 min and analyzed by HPLC.

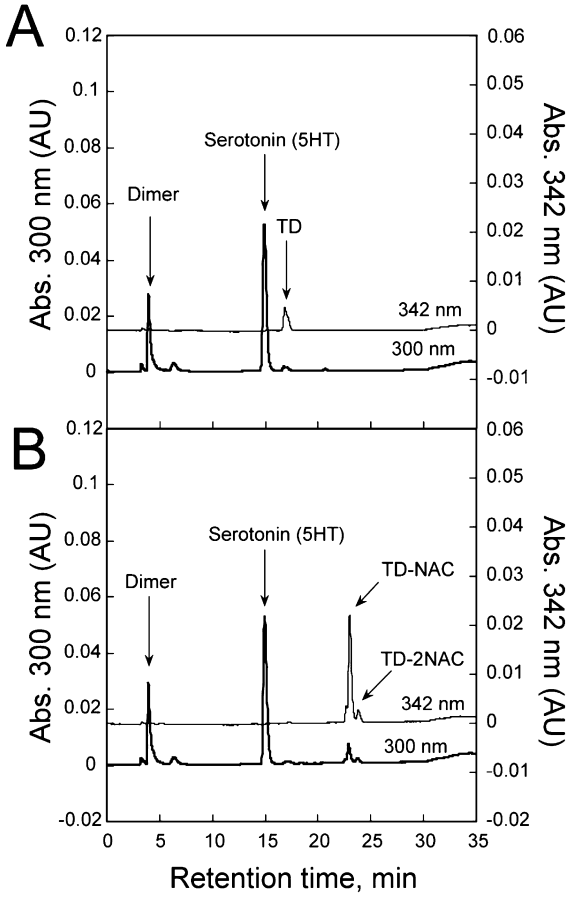

Figure 4. Reaction of NAC with stable products formed from MPO-dependent oxidation of 5HT. 5HT was oxidized in the absence of NAC under the conditions described in Figure 1A. After 10 min, the reaction mixture was filtered and collected into a solution without (A) or with (B) 1 mM NAC. The filtered material was then analyzed by HPLC.

production of 5HT-NAC. Its enhancement of 5HT oxidation also suggests that 5HT radical normally disproportionate to regenerate 5HT and form a quinine imine.

A discontinuous reaction system was also used to further elucidate the reaction mechanism. Initially, MPO, XOD, and ALD were used to oxidize 5HT. After oxidation of 5HT, the solution was ultrafiltered to remove the enzymes. The filtrate was collected into an aqueous solution with or without NAC. In the absence of NAC, dimer, 5HT, and TD were detected (Figure 4A). In contrast, with NAC present in the collection buffer, both TD-NAC and TD-2NAC were detected (Figure 4B). However, no 5HT-NAC was detected. These results indicate that TD-NAC and TD-2NAC are produced by reaction of NAC with TD, in a reaction that is independent of the enzymes. In contrast, 5HT-NAC was produced only with coincubation of NAC with the continuous reaction system (Figure 1B), suggesting that this adduct is derived from a shorted-lived precursor such as the 5HT radical.

MPO-Dependent Formation of Adducts between 5HT and Protein Thiols. We also determined whether MPO could promote the formation of adducts between 5HT and cysteine residues on proteins. We chose to investigate its reactivity with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which contains four thiols. When GAPDH was incubated with a complete continuous reaction system containing 5HT, MPO, XOD, and ALD, there was substantial loss in its thiol residues as detected by labeling cysteine residues with iodoacetamide-FITC (Figure 5A, lane a). Loss of thiols was negligible when MPO, XOD, ALD, or 5HT was excluded from the reaction system. The loss in thiols was accompanied by an increase in staining for quinone adducts, which was maximal in the complete reaction system (Figure 5A, lane b). The amount of protein in the reaction system was not affected by any of the reagents used to oxidize 5HT (Figure 5A, lane c). These results suggest that oxidation of 5HT by MPO in the presence of GAPDH results in oxidation of its cysteine residues and attachment of a quinone to the protein.

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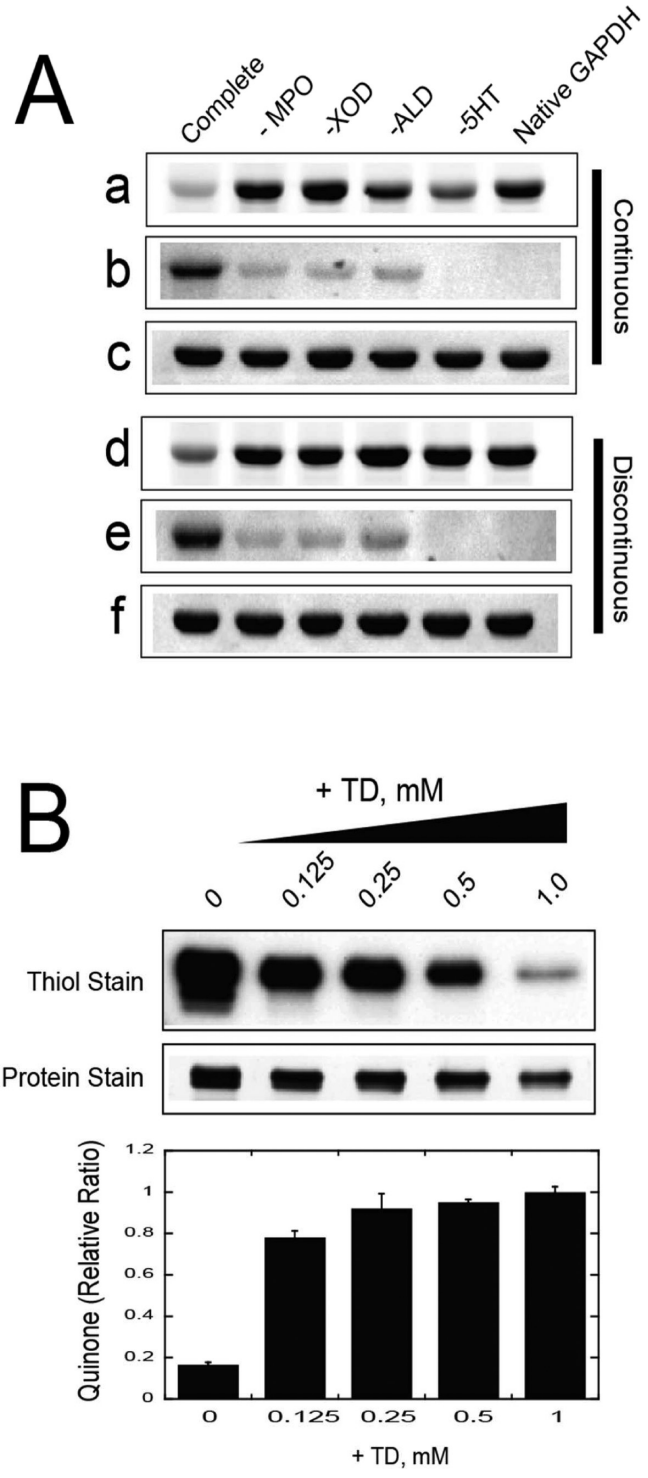

Figure 5. Oxidation of protein thiols and identification of quinone reactivity on GAPDH after incubation with MPO and 5HT. (A) For the continuous reaction (a–c) of GAPDH with oxidation products of 5HT, reactions were carried out in 50 mM phosphate buffer, pH 7.4, containing 5HT (5HT, 100 μM), GAPDH (0.7 mg/mL), MPO (50 nM), XOD, and 100 μM DTPA. Reactions were started by the addition of 10 mM ALD. The rate of superoxide production was 3.8 μM/min. Allopurinol (100 μM) was added after 10 min to stop the reaction. Alternatively, reactions were run in the absence of GAPDH and after 5 min filtered into a solution containing GAPDH (d–f). An iodoacetamide-FITC reagent was added to samples and reacted for 10 min in the dark. The protein was separated by SDS-PAGE using two gels. One gel was scanned to measure FITC as an estimate of residual proteins thiols on GAPDH (a and d), and the other (b and e) was blotted onto a PDVF membrane and stained by NBT reduction for quinone reactivity on GAPDH. The gel in (a or d) was further stained with Coomassie Brilliant Blue and then scanned to for protein content (c and f). (B) GAPDH (0.7 mg/mL) was reacted with increasing concentrations of synthetic TD for 30 min, and residual thiols were determined by reacting them with iodoacetyl-LC-biotin and visualized using streptavidin-peroxidase as described in the Experimental Procedures. The protein content was determined as described in panel A. The proteins were also blotted onto the PVDF membrane using a slot-blot apparatus and then stained by NBT reduction. The bands were scanned and analyzed the intensity using Image-J software (version 1.41).

We also investigated the reaction of oxidized 5HT with GAPDH in the discontinuous system as outlined above for NAC (Figure 4). 5HT was oxidized by MPO, XOD, and ALD in the absence of GAPDH, enzymes removed by ultrafiltration, and the filtrate collected in a solution containing GAPDH. Under these conditions, the thiol residues of GAPDH were still lost (Figure 5A, lane d), and quinone adducts formed (Figure 5A, lane e). With this discontinuous system, the 5HT radical would not be available to react with thiol residues on GAPDH because it is too short-lived. Thus, the adducts are most likely derived from reactive quinone, TD. To support this proposal, we found that increasing concentrations of synthetic TD caused progressive loss of thiols on GAPDH and an increase in quinone staining on the protein (Figure 5B).

To demonstrate that an oxidation product of 5HT becomes conjugated to GAPDH, 5HT was biotinylated on its terminal amine functionality and used as a substrate for MPO (Figure 6A). Biotinylated 5HT (5HT-biotin) was oxidized by the enzyme in the presence of GAPDH. Subsequently, GAPDH was probed with streptavidin, and biotin was detected abundantly on the protein when the complete reaction system was used (Figure 6B). Elimination of 5HT-biotin, MPO, XOD, or ALD from the reaction system resulted in a substantial decrease in the biotin signal. XOD could be replaced by hydrogen peroxide (Figure 6C, lane 5). Biotin either alone or in combination with 5HT did not cause the formation of a biotin adduct on GAPDH (Figure 6C, lanes 2 and 3). Biotin was also chemically conjugated to TD on its terminal amine. This conjugate also formed adducts with GAPDH, which were detectable at low micromolar levels (Figure 6D). To confirm the stability of the adducts using the biotin probe, modified GAPDH was separated from the oxidation system and further incubated in a neutral buffer. The conjugated biotin moiety was liberated from the modified protein after several hours of incubation at 37 °C but not 4 °C (Figure 6E). This indicates that the adducts are moderately stable under physiological conditions.

Reaction of TD with Human GAPDH. Next, mass spectrometry was used to confirm that TD forms adducts with cysteine residues on GAPDH. We chose to use human GAPDH in these experiments because of the potential relevance of the toxicity of TD in inflammatory pathologies. Synthetic TD was incubated with GAPDH for 30 min, and then, unreacted thiols were blocked with *N*-ethylmaleimide (NEM). The total mass of the treated protein was compared with GADPH that had been reacted with NEM only (Table 1). The dominant species in each deconvoluted mass spectrum was attributed to GAPDH (35922 Da) plus three NEMs (375 Da).

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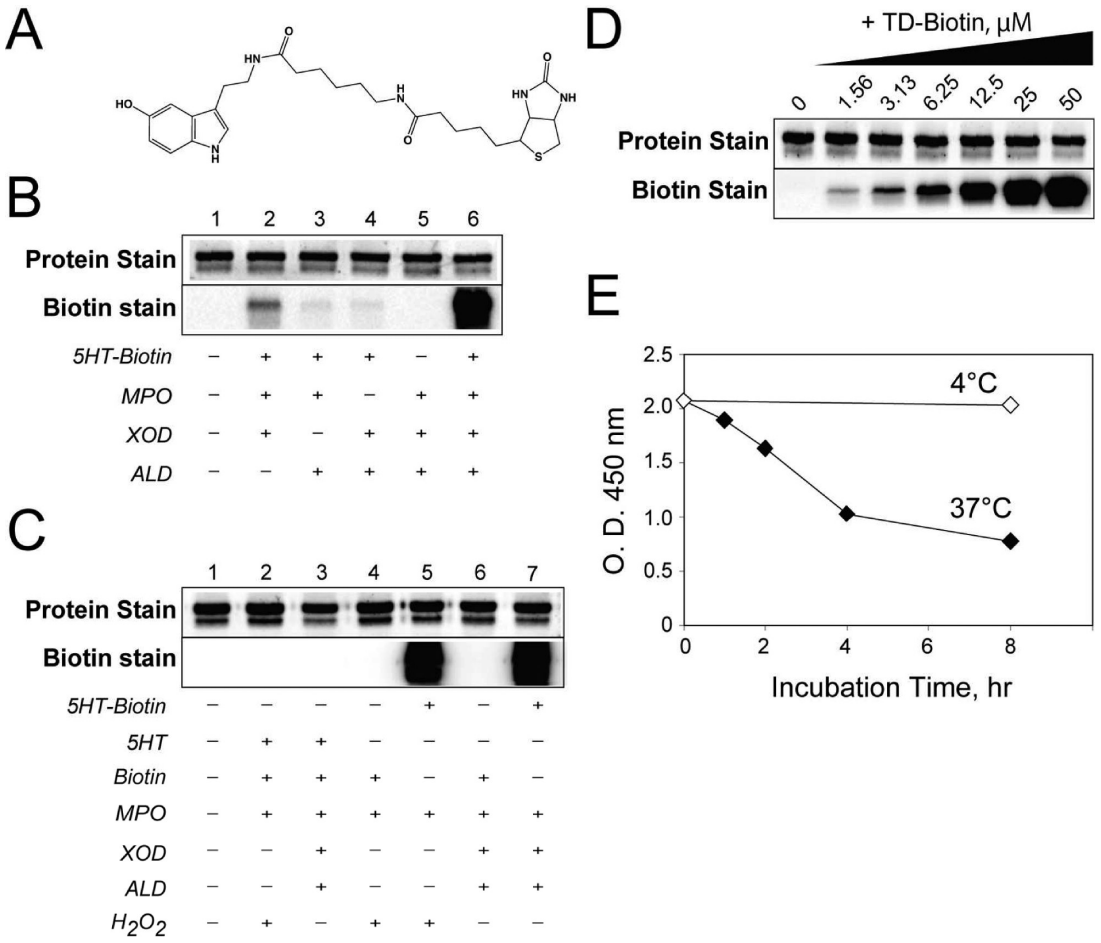

Figure 6. Identification of 5HT adducts on GAPDH. (A) The structure of the conjugate of 5HT and biotin (5HT-biotin). (B) Incorporation of 5HT-biotin into GAPDH. GAPDH was exposed to the MPO, XOD, ALD system as described in Figure 5 except that 5HT was replaced with 100 μM 5HT-biotin. After reactions were stopped, the proteins were separated by SDS-PAGE, gels were either stained for protein or blotted, and the biotin moiety on GAPDH was estimated using streptavidin-peroxidase visualization. Each component of the reaction mixture was excluded to confirm its requirement in the formation of the conjugate. (C) The effect of free biotin and hydrogen peroxide on formation of 5HT-biotin conjugates with GAPDH. 5HT-biotin was replaced with free biotin (100 μM) in the presence or absence of 5HT (100 μM) to assess whether the biotin tag became bound to GAPDH independently of 5HT. Also, XOD and ALD were replaced with hydrogen peroxide (100 μM) to confirm its requirement in the reaction. Other conditions were as described in panel B. (D) The reaction of synthetic TD-biotin with GAPDH. TD-biotin was reacted with GAPDH (0.7 mg/mL), and the biotin moiety on the membrane was estimated by streptavidin-peroxidase as described above. (E) Stability of adducts on GADPH. GAPDH was modified with 5HT-biotin as described in panel B, and the stability of the resulting adducts was determined at either 4 or 37 °C for 0−8 h. The conjugated moiety was evaluated by measuring its binding to streptavidin-peroxidase using a microplate assay as described in the Experimental Procedures.

There were two changes in the treated protein that can be assigned with confidence. The decrease of 60 Da is consistent with the loss of two NEMs (−250 Da) from the dominant species plus the addition of one TD (+190 Da). The increase of 47 Da is consistent with the addition of three oxygens to a cysteine residue to give cysteic acid. An additional potential modification to explain the increase in mass of 130 Da is the replacement of two NEMs (−250 Da) with two TDs (+380 Da). However, this species could also be GAPDH with four NEMs as indicated in the control protein. From these data, we can conclude that TD forms adducts with human GAPDH, and these prevent subsequent reactions of NEM with cysteine residues.

We then analyzed the tryptic digest of GAPDH to identify peptides that TD had formed adducts with. Human GAPDH has three free cysteine residues in the two tryptic peptides IISNASC^{152}TTNC^{156}LAPLAK and VPTANVSVVDLTC^{247}R.[21] These peptides were initially identified based on their mass and fragmentation patterns using LC/MS/MS. Once identified, they were quantified by multiple reaction monitoring of the ions shown in Table 2. The peptides that were monitored included all possible permutations for the alkylated forms. The relative signals for their ions were compared to that for a control tryptic peptide that should not react with TD. The peptide containing Cys^{152} and Cys^{156} underwent a substantial loss when GAPDH was reacted with TD. In contrast, there were no appreciable losses of the peptide containing Cys^{247}. These results suggest that TD reacts predominantly with either or both Cys^{152} and Cys^{156}. Interestingly, the unalkylated form of the peptide with Cys^{247}, which is likely to be a minor species in the unreacted protein, increased substantially upon treatment of GAPDH with TD. This somewhat counterintuitive result is most likely explained by the fact that adducts of TD on GAPDH may prevent alkylation of this thiol residue.

To identify adducts of TD with the cysteine-containing peptides, we predicted m/z values for the doubly and triply

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Table 1. Deconvoluted Masses of GAPDH after Treatment with TD and or *N*-Ethylmaleimide[a]

sample (peak no.)	mass (Da)	relative intensity	Δmass vs peak 1 (Da)	possible modification
control GAPDH + NEM				
1	36311 ± 15	1.38×10^6		+3 NEM
2	36188 ± 15	0.98×10^6	−123	+2 NEM
3	36433 ± 15	0.7×10^5	+122	+4 NEM
4	36484 ± 15	0.5×10^5	+173	unknown
5	36372 ± 11	0.4×10^6	+61	unknown
treated GAPDH + TD + NEM				
1	36306 ± 15	1.2×10^6		+3 NEM
2	**36246 ± 17**	0.95×10^6	**−60**	**+TD + NEM**
3	**36353 ± 15**	0.9×10^6	**+47**	**cysteic acid**
4	**36436 ± 11**	0.9×10^6	**+130**	**+2 TD + NEM** or 4 NEM
5	**36093 ± 11**	0.78×10^6	−213	unknown
6	36185 ± 13	0.7×10^6	−121	+2 NEM

[a]GAPDH (17 μM) was reacted with TD (700 μM) in 100 mM phosphate buffer, pH 7.5, for 30 min and then 20 mM NEM for 15 min. The control was GAPDH reacted with NEM only. The whole protein species present in reaction mixtures were analyzed by mass spectrometry. The major deconvoluted masses obtained from the ion spectra are shown. Errors represent half the peak width at half the peak height. The theoretical mass of human GAPDH is 35922 Da, and those for NEM and TD are 125 and 190 Da, respectively. New masses and modifications that appear in the treated sample are shown in bold.

charged species for all of the possible combinations of these peptides containing TD and NEM. Ions with these m/z values were selected for MS^2 fragmentation with a window of ±3 Da. This window was chosen to detect addition of TD to a single cysteine residue to give a diol (+190 Da), its dione (+188 Da), or a dione bonded to both cysteines (+186 Da). Doubly and triply charged ions for the peptide containing Cys^{152} and Cys^{156} with one addition of TD but no NEM were detected in the treated protein but not the control (Figure 7A,B). These ions coeluted, which suggests that they originated from the same peptide. The doubly charged ion of this peptide ($[M + 2H]^{2+}$ = 954.5 m/z) was concurrently fragmented, and its mass spectrum was compared with that for the unreacted and unalkylated parent peptide ($[M + 2H]^{2+}$ = 860.1 m/z). Fragmentation of the parent peptide gave good sequence coverage (Figure 7C). All of the y ions above an m/z value of 1100 had m/z values two mass units less than expected for this peptide ($[M + H]^+$ = 1719.9 m/z), which indicates that the cysteine were present as a disulfide. Fragmentation of the suspected peptide containing TD gave the same y_4, y_5, and y_6 ions as the parent peptide (Figure 7D). The major ion in the spectrum had a m/z value of 944.3. This is likely to be a doubly charged ion of the molecular ion after the loss of water. Thus, the singly charged molecular ion must have an m/z value of 1905.6. This value indicates that when TD reacts with the peptide containing Cys^{152} and Cys^{156}, there is an increase in mass of 185.7 Da. Given the m/z value of the molecular ion, it was possible to identify the y_{12} and y_{13} ions. Collectively, these data indicate that TD reacts with the peptide containing Cys^{152} and Cys^{156} to form a covalent adduct with both thiols.

DISCUSSION

In this investigation, we have shown that MPO oxidizes 5HT to products that become conjugated to thiols. We could not detect analogous adducts with amines. The conjugates with thiols can result from either their reaction with 5HT radicals or via coupling to TD. Consequently, oxidation of 5HT by MPO at sites of inflammation will not only lower the concentration of this important vasoactive mediator but also promote modifications of reactive thiols. The formation of adducts with glutathione and cysteine are known to be toxic,[24] whereas conjugation to cysteine residues on proteins may affect their function.[17,19] These protein adducts will be toxic if quinone adducts are able to redox cycle.[25] The adducts between thiols and 5HT oxidation products also have the potential to be exploited as biomarkers to demonstrate the contribution that MPO and 5HT make to inflammation.

5HT has previously been shown to be oxidized by endogenous oxidants, such as superoxide, nitric oxide, and peroxynitrite, to produce its radical and TD.[14,15] We contend that under physiological conditions, oxidation of 5HT by MPO is likely to be more relevant than via these transient species. This is because these short-lived oxidants will to be scavenged by other nucleophiles, which collectively will be more concentrated in cells and extracellular fluid than 5HT. MPO promotes oxidation by initially reacting with hydrogen peroxide to form compound I. The dominant substrates for compound I are chloride and thiocyanate.[26] 5HT would be expected to compete poorly with these substrates in vivo unless its local concentration approaches 100 μM. However, numerous other endogenous substrates, including urate, tyrosine, and ascorbate, reduce compound I to compound II.[27] Turnover of compound II will limit the rate at which MPO generates oxidants at sites of inflammation. The rate constant for reaction of 5HT with compound II is 100-fold greater than those for the other endogenous substrates. [8] Hence, only low micromolar levels of 5HT are required for it to be effectively oxidized by compound II in vivo. Alternatively, when neutrophils generate superoxide,

Table 2. Multiple Reaction Monitoring for the Quantification of Cysteine Containing Tryptic Peptides of GAPDH[a]

residues	Cys	peptide sequence	MS1/MS2	area control	area treated	dione-treated/ control
67−80		K.LVINGNPITIFQER.D	807.5/1003.55	1000	1000	1.00
146−162	152, 156	K.IISNAScTTNcLAPLAK.V	986/1156.6	3.1 ± 0.2	0.1 ± 0.05	0.03
146−162	152, 156	K.IISNAScTTNCLAPLAK.V or K.IISNASCTTNcLAPLAK.V	922.5/1260.59	9.9 ± 3.0	0.07 ± 0.01	0.01
146−162	152, 156	K.IISNASCTTNCLAPLAK.V	860/1292.63	47.0 ± 2.0	26.4 ± 2.0	0.56
235−248	247	R.VPTANVSVVDLTcR.L	800.1/1017.62	93.7 ± 82	56.9 ± 6.0	0.61
235−248	247	R.VPTANVSVVDLTCR.L	737.5/483.25	53.0 ± 4.0	1750 ± 1000	33.03

[a]GAPDH was reacted with TD as described in Table 1 and digested with trypsin. The peptides for treated and untreated GAPDH that are listed were quantified using multiple reaction monitoring with the respective transitions (MS1/MS2). The peak areas for each species were determined relative to the control peptide LVINGNPITIFQER and normalized. Data are from two independent experiments. C = cysteine, and c = cysteine-NEM.

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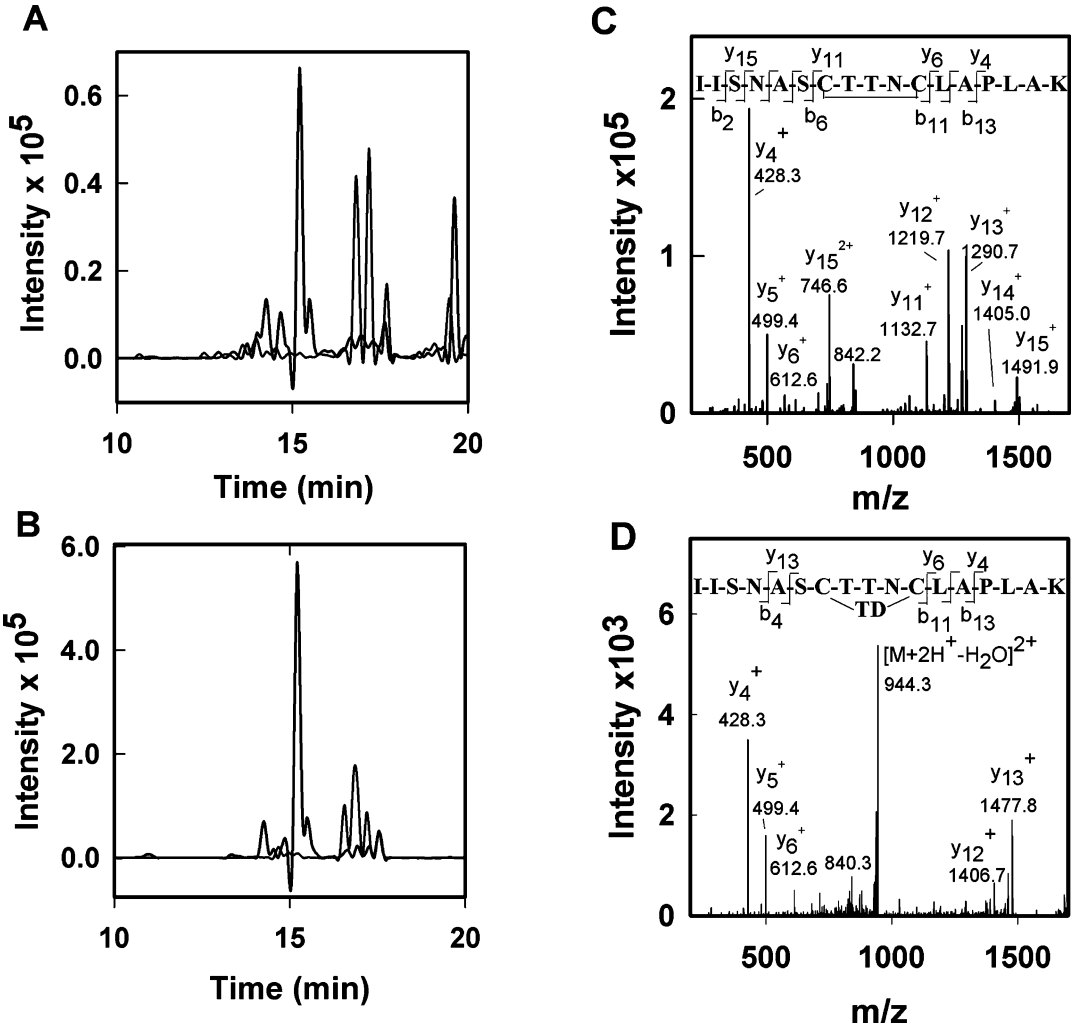

Figure 7. Analysis of tryptic peptides from GAPDH modified by reaction with TD. GAPDH was reacted with TD as described in Table 1 and then digested with trypsin. LC with selected ion monitoring was used to detect TD conjugated to the peptide IISNASC[152]TTNC[156]LAPLAK as either a doubly charged ion (A) $[M + 2H]^{2+} = 954.5 \pm 3$ *m/z* or a triply charged ion (B) $[M + 3H]^{3+} = 636.7 \pm 3$ *m/z*. The black and gray traces in A and B are for the protein treated with and without TD, respectively. (C) The MS^2 spectrum of the precursor $[M + 2H]^{2+} = 860.1$ *m/z*. (D) The MS^2 spectrum of the precursor $[M + 2H]^{2+} = 954.5$ *m/z* of the tryptic peptide from protein treated with TD as shown in panel A. Data are representative of two independent experiments.

MPO is converted to oxy-MPO or compound III.[28] Only 5HT and superoxide react appreciably with this redox intermediate.[9,29] Therefore, in the presence of stimulated neutrophils, 5HT will have little competition for oxidation by compound III and be readily oxidized to its radical.

Our results demonstrate that when MPO oxidizes 5HT to radicals, these transient species have several fates (see the proposed reaction, Scheme 1). They can react with themselves to form dimers or disproportionate and ultimately form TD.[9,15,30] They may also react with superoxide to form TD via a transient hydroperoxide. [9] In line with the early work of Dryhurst and co-workers, 5HT radicals produced by MPO reacted with thiols to produce conjugates.[24] At sufficiently high concentrations of NAC, we found that the conjugation reaction predominated, and there was little formation of dimer of 5HT or TD. Furthermore, the conjugate between 5HT and NAC did not form if the thiol was added after MPO had oxidized 5HT. These results support the existence of a transient reactive radical that unless captured by thiols undergoes radical−radical reactions.

In accord with previous investigations by the Dryhurst group, we found that TD produced by MPO also reacted with NAC.[24] Hence, at sites of inflammation, low molecular weight thiols will be a target for this reactive quinone. The conjugates that are formed undergo redox cycling to produce superoxide and hydrogen peroxide, which would provide MPO with further fuel to catalyze its array of damaging oxidants. The reaction of TD and 5HT radicals with thiols appears to be selective because we found no evidence for their reaction with biological amines.

Our results show that TD became conjugated to GAPDH. There was no evidence to support the reaction of 5HT radicals with protein thiols. Presumably, the steric constraints might limit the interaction of radicals with protein thiols. Conjugation of TD to a thiol in GAPDH was supported by the observed loss in thiols and concomitant increase in quinone reactivity on the protein when it was treated with 5HT, MPO, and XOD/ALD (Figure 5). This occurred regardless of whether GAPDH was added before or after the oxidative event. With biotin-labeled 5HT, we observed increased biotinylation of GAPDH, which was also reliant on the complete MPO system. TD either alone

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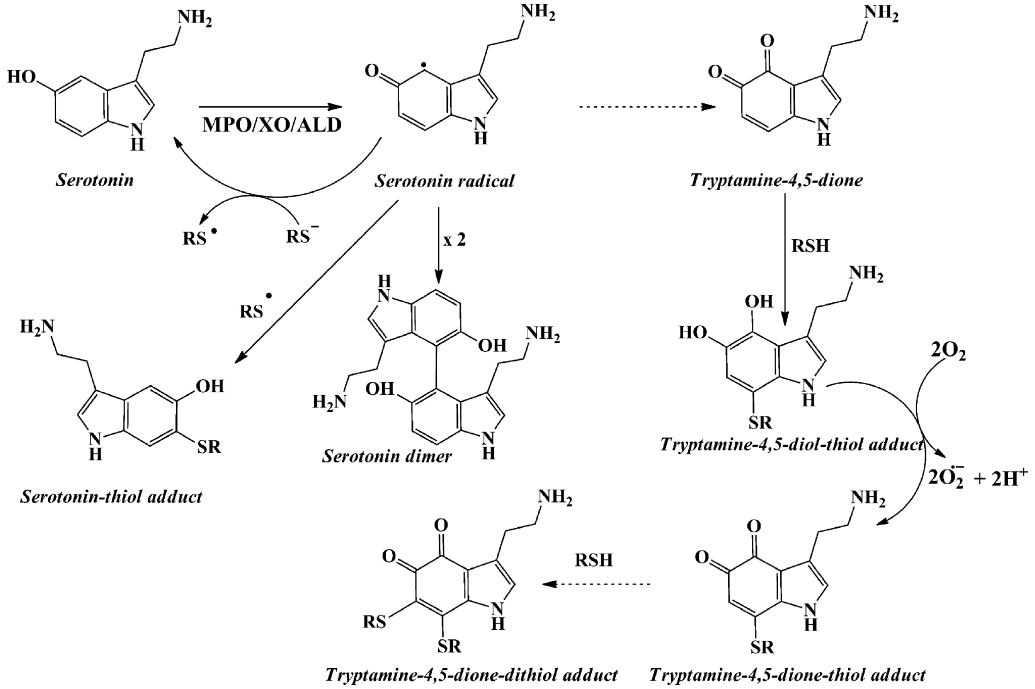
Scheme 1. Probable Reaction Pathways for the Generation of 5HT Oxidation Products and Adducts with Thiols (RSH) Such as NAC and Protein Cysteine Residues[a]



[a]It is also conceivable that the dimer and the 5HT−thiol adduct are formed via nonradical pathways in which the quinone imine of 5HT reacts with 5HT or a thiol, respectively.

or conjugated to biotin reacted with thiols on GAPDH. Finally, we identified a new tryptic peptide after human GAPDH reacted with TD, and this corresponded to covalent attachment of TD to the peptide IISNASC[152]TTNC[156]LAPLAK. Interestingly, our data indicated that TD had reacted with both cysteine residues to form a doubly cross-linked dione species. GAPDH is a tetrameric enzyme (a dimer of dimers) consisting of four 35 kDa subunits. Each subunit in human GAPDH has three cysteines (Cys[152], Cys[156], and Cys[247]) with Cys[152] being typically considered as the most nucleophilic.[21] Thus, it is likely that once Cys[152] reacts with TD, the resulting diol is oxidized back to a dione that subsequently reacts with Cys[156]. The presence of this double cross-link is supported by our data that TD was able to become conjugated to two molecules of NAC.

Considerable evidence has accumulated indicating that oxidation products of dopamine are neurotoxic and become covalently coupled to proteins.[31−33] For example, its attachment to parkin via dopaquinone was suggested to cause progressive loss of parkin function in dopaminergic neurons during aging and Parkinson's disease.[34] Our findings demonstrate that 5HT may have similar toxicity by analogues routes when it is oxidized by MPO. Its subsequent conjugation to low molecular weight thiols will exacerbate inflammation by redox cycling, while coupling to protein thiols may inactivate critical enzymes and disrupt normal protein function. Our findings are relevant to neurological diseases, such as Alzheimer's disease, where MPO is associated with amyloid plaques and is present in neuronal cells that contain 5HT.[34] They are also pertinent to the interaction of neutrophils and platelets in cardiovascular disease, which results in the release of MPO and 5HT, respectively, from these inflammatory cells.[2] In the inflamed gut, MPO has the potential to oxidize 5HT that is locally produced by enterochromaffin cells[1] and modify thiols. Development of specific assays using either immunological techniques or mass spectrometry will be crucial for determining what role the chemistry elaborated in this study plays during the inflammation.

AUTHOR INFORMATION

Corresponding Author

*Tel: 81-79-292-9413. Fax: 81-79-293-5710. E-mail: yojikato@shse.u-hyogo.ac.jp.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

5HT, serotonin; TD, tryptamine-4,5-dione; MPO, myeloperoxidase; XOD, xanthine oxidase; NAC, *N*-acetylcysteine; NBT, nitroblue tetrazolium; SDS, sodium dodecyl sulfate; DTPA, diethylenediaminetetraacetic acid; SOD, superoxide dismutase; GAPDH, glyceraldehyde-3-phosphate dehyderogenase; IAF, iodoacetamide-fluorescein; sulfo-NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamido)hexanoate; IAB, *N*-iodoacetyl-*N*-biotinylhexylenediamine; ALD, acetaldehyde; HPLC, high-performance liquid chromatography; SPE, solid-phase extraction; PDA, photodiode array detector; LC/MS, liquid chromatography/mass spectrometry; PAGE, polyacrylamide gel electrophoresis;

PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline

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